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# 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 shutoff 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-1$^{SM}$, that was 100-fold more resistant to *in vitro* cleavage by Coxsackievirus 2A protease (2A$^{Pro}$) than wild-type eIF4G-1 (eIF4G-1$^{WT}$), 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-1$^{DM}$. eIF4G-1$^{DM}$ was 1,000–10,000-fold more resistant to cleavage *in vitro* than eIF4G-1$^{WT}$. Full functional activity of eIF4G-1$^{DM}$ was demonstrated *in vitro* by its ability to restore cap-dependent translation to a 2A$^{Pro}$-pretreated rabbit reticulocyte system. An isoform containing the binding site for poly(A)-binding protein, eIF4G-1e$^{DM}$, was more active in this assay than an isoform lacking it, eIF4G-1a$^{DM}$, but only with polyadenylated mRNA. Functional activity was also demonstrated *in vivo* with stably transfected HeLa cells expressing eIF4G-1$^{DM}$ from a tetracycline-regulated promoter. Cap-dependent green fluorescent protein synthesis was drastically inhibited by 2A$^{Pro}$ expression, but synthesis was almost fully restored by induction of either eIF4G-1a$^{DM}$ or eIF4G-1e$^{DM}$. By contrast, encephalomyocarditis virus internal ribosome entry site-dependent green fluorescent protein synthesis was stimulated by 2A$^{Pro}$; stimulation was suppressed by eIF4G-1e$^{DM}$ but not eIF4G-1a$^{DM}$.**

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 shutoff 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[1] 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 (2A$^{Pro}$) of Coxsackievirus B4 (CVB4) or human rhinovirus 2 (HRV2) into two functional domains, an N-terminal fragment ($cp_N$) that binds eIF4E and poly(A)-binding protein (PABP) and a C-terminal fragment ($cp_C$) 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 40 S 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, eIF-4γ, eIF4G-I, and eIF4GI)[2] 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-1a (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-1e (24). Recently, an even longer form, eIF4G-1f, was detected by mass spectrometric analysis (24). eIF4G-1e and eIF4G-1f contain the binding

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[1] The abbreviations used are: eIF4, eukaryotic initiation factor 4; aa, amino acid; nt, nucleotide; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; GFP, green fluorescent protein; EGFP, enhanced GFP; PABP, poly(A)-binding protein; MDL, messenger RNA-dependent lysate; HPLC, high pressure liquid chromatography; FAM, 6-carboxyfluorescein; TAMRA, tetramethylrhodamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRV2, human rhinovirus 2; CVB3, Coxsackievirus B3.

[2] The nomenclature system recommended by an *ad hoc* committee appointed by the IUBMB is used here (48).

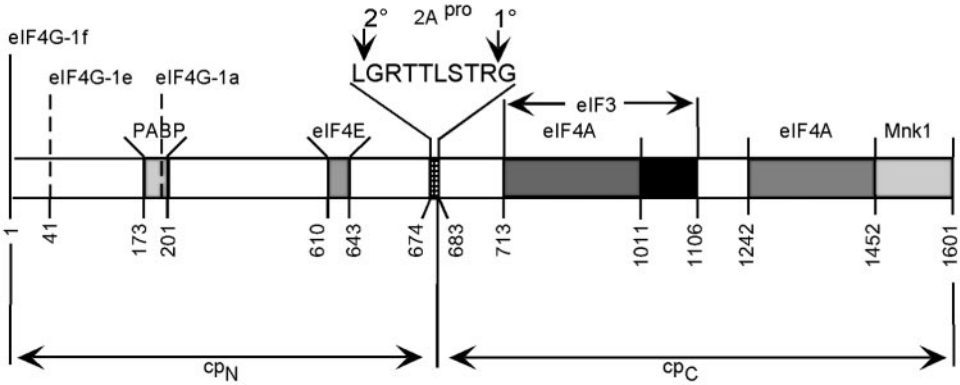


FIG. 1. **Schematic diagram of the primary structure of human eIF4G-1.** eIF4G-1a, eIF4G-1e, and eIF4G-1f are the 154- (21), 172- (25, 26), and 176-kDa (24) forms, respectively. N-terminal ($cp_N$) and C-terminal ($cp_C$) cleavage products are generated during infection of cells with picornaviruses of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

site for PABP, but eIF4G-1a does not (Fig. 1).

Cleavage of eIF4G-1 by $2A^{Pro}$ was initially thought to be the major mechanism utilized by entero- and rhinoviruses 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 shutoff 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 shutoff but not eIF4G cleavage (29). Expression of poliovirus $2A^{Pro}$ 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 shutoff (30). *In vivo* expression of $2A^{Pro}$ alone activates viral mRNA translation, independent of its role in the host shutoff (31, 32). Complete cleavage of eIF4G-1 in *Xenopus* oocytes (33) or HeLa cells (34) by introduction of exogenous $2A^{Pro}$ results in only a modest reduction of protein synthesis. Cleavage of eIF4G-2 appears to correlate better with host shutoff 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 most rapid period of viral protein synthesis, whereas complete 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 shutoff.

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

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

## EXPERIMENTAL PROCEDURES

*Materials*—The C4 column for reverse phase purification of $cp_C$ 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 pIRES2-EGFP, pEGFP-C1, and pTRE2pur 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-$2A^{Pro}$ domain of CVB3 from an encephalomyocarditis virus (EMCV) IRES was described previously (40) and is here referred to as pVP12A. Benzamidine, pepstatin, Pefabloc, and Expand™ High Fidelity PCR systems were purchased from Roche Molecular Biochemicals. T7 RNA polymerase, T4 polynucleotide kinase, RNase H, 5-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 from Promega (Madison, WI). [$^{35}$S]Met was obtained from ICN Biochemicals (Irvine, CA). Oligonucleotides used for site-directed mutagenesis and sequencing were acquired from Invitrogen. [5′-$^{32}$P]pCp was obtained from ICN Biochemicals (Irvine, CA). *Sgr*AI and T4 RNA ligase were purchased from New England Biolabs (Beverly, MA). The T7-1 primer 5′-AATACGACTCACTATAG-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 pSΔN1 and pSΔN1E were constructed by inserting the *Sma*I/*Xho*I fragments from $pAD4G^{WT}$ and $pAD4G^{G486E}$ (38),[3] respectively, into pET29A between the *Eco*RV and *Xho*I 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 $S\text{-}eIF4G\text{-}1(590\text{–}1601)^{WT}$ and $S\text{-}eIF4G\text{-}1(590\text{–}1601)^{SM}$, respectively.

Construction of the plasmid $pCITE4G\text{-}1a^{DM}$ (double mutant), which encodes eIF4G-1a with a second aa substitution (G675A), was achieved by site-directed mutagenesis of $pCITE4G^{G486E}$ (38). A mutated PCR product from nucleotide (nt) 1221–2033 was synthesized from $pCITE4G^{G486E}$ using the sense primer 5′-AGCCCCCTCGCCACCAG-3′ (nt 1221–1237) and the antisense primer 5′-GTTGTGCGCGCAAGGTTGGCAAAGGATGGAG-3′ (nt 2003–2033). A second mutated PCR product from nt 2016–2952 was produced from $pCITE4G^{G486E}$ using the sense primer 5′-CAACCTTGCGCGCACAACCCTTAGCACCCG-3′ (nt 2016–2045) and the antisense primer 5′-CACGTCCTGCAGCATAAAGCGG-3′ (nt 2931–2952). PCR was conducted with the Expand™ High Fidelity PCR system (Roche Molecular Biochemicals) for 20 cycles at 95 (1 min), 61 (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 *Bam*HI and *Bss*HII, and the other from the

[3] The names of plasmids $pCITE4G^{G486E}$ 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 *Bss*HII and *Nsi*I, were ligated into pCITE4G$^{G486E}$ that had been digested with *Bam*HI and *Nsi*I. The mutagenesis not only changed the encoded aa at position 675 from Gly to Ala but also created a restriction site for *Bss*HII at nt 2023–2028. Therefore, plasmids from colonies were initially screened by restriction digestion with *Bss*HII.

A plasmid expressing eIF4G-1e$^{DM}$, termed pCITE4G-1e$^{DM}$, was constructed by inserting the DNA sequence that encodes the 156-aa N-terminal extension of eIF4G-1e into pCITE4G-1a$^{DM}$. This was accomplished by digesting a plasmid containing this sequence in pCR2.1 (Ref. 26; kindly donated by Peter Sarnow) with *Xma*I and *Eco*RV, treating the reaction mixture with the Klenow fragment of DNA polymerase I (Promega), and then ligating the gel-purified 501-bp fragment into pCITE4G-1a$^{DM}$ that had been digested with *Eco*RV.

pTRE2pur4G-1a$^{DM}$ and pTRE2pur4G-1e$^{DM}$ were constructed as follows. First, the plasmid pFastBac4G-1a$^{DM}$ was constructed by digesting pCITE4G-1a$^{DM}$ with *Eco*RV and *Xho*I. The gel-purified insert was ligated into pFastBac HTb after cutting with *Stu*I and *Xho*I. pFastBac4G-1e$^{DM}$ was similarly constructed by digesting pCITE4G-1e$^{DM}$ with *Sgr*AI and *Xho*I. pTRE2pur4G-1a$^{DM}$ and pTRE2pur4G-1e$^{DM}$ were constructed by digesting pFastBac4G-1a$^{DM}$ and pFastBac4G-1e$^{DM}$, respectively, first with *Ssp*I and then partially with *Nhe*I. The gel-purified inserts, consisting of the entire eIF4G-1a$^{DM}$ or eIF4G-1e$^{DM}$ coding regions, were ligated into pTRE2pur that had been previously cut with *Pvu*II and *Nhe*I.

*Expression and Purification of the cp$_C$ Fragment*—One-liter cultures of the *Escherichia coli* expression strain BL21(DE3)pLysS bearing the plasmids pSΔN1 and pSΔN1E were grown in LB medium (41) containing 25 μg/ml kanamycin and 34 μg/ml chloramphenicol to an $A_{600}$ of 0.3. Expression of S-eIF4G-1(590–1601)$^{WT}$ or S-eIF4G-1(590–1601)$^{SM}$ 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, pelleted 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 (3–6 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 2A$^{Pro}$ 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 cp$_C$ 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 containing cp$_C$ were subjected to automated Edman degradation at the University of Kentucky Macromolecular Structure Analysis Facility.

*In Vitro Translation*—MDL was prepared from rabbit reticulocyte lysate by depletion of endogenous mRNA with micrococcal nuclease (42). In some cases, endogenous eIF4G was cleaved by pretreating MDL with recombinant CVB4 2A$^{Pro}$ at 50 μg/ml for 30 min at 30 °C. A two-phase cell-free translation protocol was used to test the function of eIF4G-1$^{DM}$. During phase I, *in vitro* translation was performed to synthesize the eIF4G-1 isoform of interest in the absence of radioactivity in CVB4 2A$^{Pro}$-pretreated MDL. During phase II, *in vitro* translation of globin mRNA was used to test whether the eIF4G-1 isoforms synthesized in phase I were functional for cap-dependent translation. Reaction mixtures of 25 μl in phase I contained 10 μl of either mock-treated or 2A$^{Pro}$-pretreated MDL and the following components: 150 mM potassium acetate, 1 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 40 μM of each aa with the exception of Met, 2 μM Met, 50 mM HEPES (pH 8.0), and 20 μg/ml eIF4G-1 mRNA. The latter was transcribed from pCITE4G-1a$^{DM}$ and pCITE4G-1e$^{DM}$ plasmids linearized by *Xho*I as described previously (43). Reactions were carried out at 30 °C for 1 h. Phase II reaction mixtures (25 μl) contained 12.5 μl of phase I reaction mixtures and 12.5 μl of fresh reaction mixtures consisting of 5 μl of either mock-treated or 2A$^{Pro}$-pretreated MDL, 160 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 40 μM of each aa with the exception of Met, 1 mCi/ml [$^{35}$S]Met, 50 mM HEPES (pH 8.0), and 40 μg/ml globin mRNA. The phase II reactions were incubated at 30 °C for 30 min, and aliquots were subjected to SDS-PAGE. eIF4G-1 synthesis was measured by immunoblotting and globin synthesis by incorporation of [$^{35}$S]Met into protein.

*Deadenylation of Globin mRNA*—Rabbit globin mRNA was labeled at 4 °C overnight in a 100-μl total reaction mixture containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 50 μg/ml bovine serum albumin, 1 unit/μl RNasin, 10% $Me_2SO$, 500 μCi/ml [5′-$^{32}$P]pCp, 200 μg/ml globin mRNA, and 100 units/ml T4 RNA ligase. After purification, an aliquot was mixed with nonradioactive globin mRNA (400 μg/ml) and hybridized to oligo(dT)$_{15}$ (20 μg/ml) by heating in a 50-μl reaction volume containing 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 30 μg/ml bovine serum albumin at 65 °C for 5 min and slowly cooling to 37 °C. RNase H (5 units) was added, and the reaction was incubated at 37 °C for 20 min. DNase (1 unit) was added, and the reaction was continued at 37 °C for 15 min. The RNA was isolated by phenol extraction and ethanol precipitation.

*Establishment of Stable Cell Lines Expressing Tetracycline-regulated eIF4G-1a$^{DM}$ and eIF4G-1e$^{DM}$*—The Tet-On HeLa cell line (Clontech) was transfected by electroporation with pTRE2pur4G-1a$^{DM}$ or pTRE2pur4G-1e$^{DM}$ linearized with *Xho*I. 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-1$^{DM}$ was detected by adding doxycycline to the culture medium (see figure legends). Cell lysates were treated with recombinant CVB4 2A$^{Pro}$ at 50 μg/ml for 2 h on ice. eIF4G-1 that was detected by immunoblotting with anti-eIF4G-1

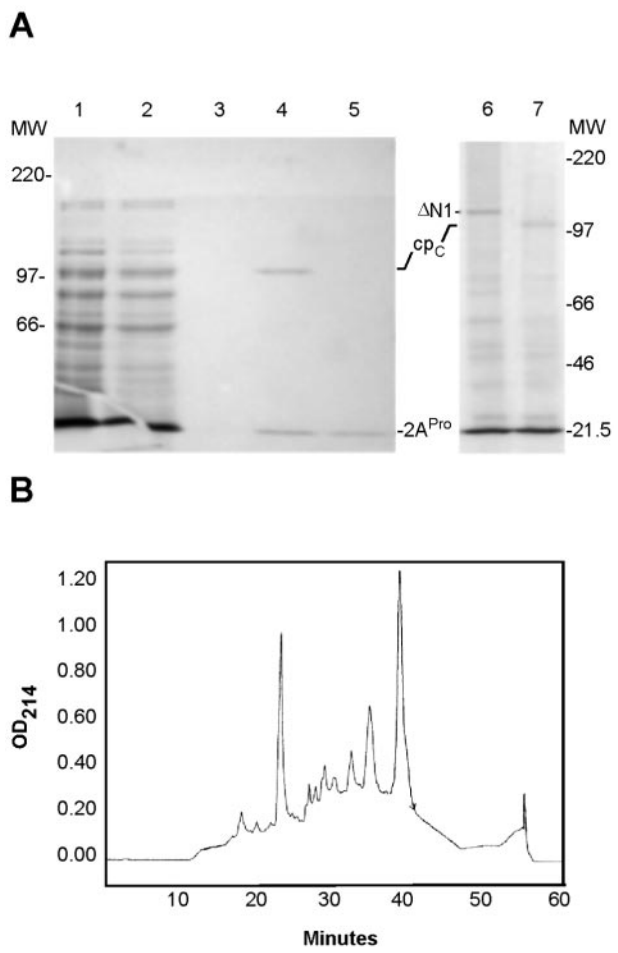


FIG. 2. **Purification of cp$_C$ from eIF4G-1$^{SM}$.** *A*, the truncated, S-peptide-tagged eIF4G fragment S-eIF4G-1(590–1601)$^{SM}$ was produced in *E. coli* and bound to S-Protein-agarose. The cp$_C$ fragment was cleaved from the resin by CVB4 2A$^{Pro}$ as described under "Experimental Procedures," and fractions were analyzed by SDS-PAGE on a 7.5% gel (*lanes 1–5*). In a separate experiment, S-Protein-agarose with bound S-eIF4G-1(590–1601)$^{SM}$ was eluted with SDS-PAGE loading buffer and analyzed on an 8% gel before (*lane 6*) and after (*lane 7*) incubation with CVB4 2A$^{Pro}$. Δ*N1* refers to uncleaved S-eIF4G-1(590–1601)$^{SM}$. *Lane 1*, bacterial extract; *lane 2*, fraction unbound to S-Protein-agarose; *lane 3*, last wash fraction; *lane 4*, eluate after CVB4 2A$^{Pro}$ treatment; *lane 5*, CVB4 2A$^{Pro}$ 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 2A$^{Pro}$ and cp$_C$, respectively (data not shown).

TABLE I
*In vitro cleavage sites for CVB4 2A$^{Pro}$ in human S-eIF4G-1(590–1601)$^{WT}$ and S-eIF4G-1(590–1601)$^{SM}$*

| Source of sequence | Sequence[a] |
|---|---|
| Protein sequence predicted from eIF4G-1$^{WT}$ cDNA[b] | $^{675}$GRTTLSTR ↓ GPPRGGPGGEL$^{693}$ |
| Edman degradation of cp$_C$ derived from S-eIF4G-1(590–1601)$^{WT}$ by CVB4 2A$^{Pro}$ digestion | *X*P*X*RGGPG*X*EL |
| Protein sequence predicted from eIF4G-1$^{SM}$ cDNA[c] | $^{671}$FANL ↓ GRTTLSTR EPPRGGPGGEL$^{693}$ |
| Edman degradation of cp$_C$ derived from S-eIF4G-1(590–1601)$^{SM}$ by CVB4 2A$^{Pro}$ digestion | *X*RTTLSTR EPPRG |

[a] *X* indicates that an unambiguous assignment could not be made. The gaps in some sequences are for alignment purposes only. Downward arrows indicate cleavage sites in the amino acid sequence that would explain the results of Edman sequencing.
[b] Ref. 21.
[c] Ref. 38.

antibodies but was resistant to 2A$^{Pro}$ digestion was scored as eIF4G-1$^{DM}$. Colonies were selected that had the lowest expression of eIF4G-1$^{DM}$ in the absence of doxycycline but highest expression in its presence.

*Expression and Detection of Green Fluorescent Protein (GFP) in Cell Lines Expressing eIF4G-1$^{DM}$*—The stably transfected HeLa cell lines expressing eIF4G-1a$^{DM}$ and eIF4G-1e$^{DM}$ were cultured in 6-well plates. Cells were either induced by doxycycline to express exogenous eIF4G-1$^{DM}$ for 24 h or mock-induced. Cells were then co-transfected with pVP12A and either pEGFP-C1 or pIRES2-EGFP using Polyfectin (Qiagen). Culture was continued for 24 h, and then cells were observed with a Nikon Eclipase 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 2A$^{Pro}$ in eIF4G-1a$^{SM}$ Is Different from the Site Previously Identified in eIF4G-1a$^{WT}$*—The fact that the eIF4G-1a$^{SM}$ variant is cleaved at sufficiently high CVB4 2A$^{Pro}$ 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-1$^{SM}$, *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-1$^{WT}$ and eIF4G-1$^{SM}$ containing the S-peptide of RNase A fused at the N terminus, designated S-eIF4G-1(590–1601)$^{WT}$ and S-eIF4G-1(590–1601)$^{SM}$, respectively. This allowed immobilization of the products on S-Protein-agarose. Digestion of the bound proteins with CVB4 2A$^{Pro}$ released soluble cp$_C$ (Fig. 2*A*, *lane 4*). The eluates were subjected to reverse phase HPLC to separate cp$_C$ from CVB4 2A$^{Pro}$ and other contaminating proteins (Fig. 2*B*). The cp$_C$ fragments from both S-eIF4G-1(590–1601)$^{WT}$ and S-eIF4G-1(590–1601)$^{SM}$ eluted at 40 min, similar to the elution position of natural rabbit cp$_C$ (10). N-terminal sequencing of cp$_C$ from S-eIF4G-1(590–1601)$^{WT}$ confirmed the primary cleavage site between Arg$^{682}$ and Gly$^{683}$ (10) (Table I). N-terminal sequencing of cp$_C$ from S-eIF4G-1(590–1601)$^{SM}$ demonstrated that cleavage of S-eIF4G-1(590–1601)$^{SM}$ occurred at a site 8-aa residues upstream of the original site, between Leu$^{674}$ and Gly$^{675}$ (Table I). Cleavage at this alternate site was not because of the fact that the substrate was made in bacteria, because S-eIF4G-1(590–1601)$^{WT}$ was cleaved at the site determined previously (10) in natural rabbit eIF4G-1. These results indicate that alteration of the primary 2A$^{Pro}$ cleavage site in eIF4G-1 results in cleavage at a secondary site (labeled *2°* in Fig. 1).

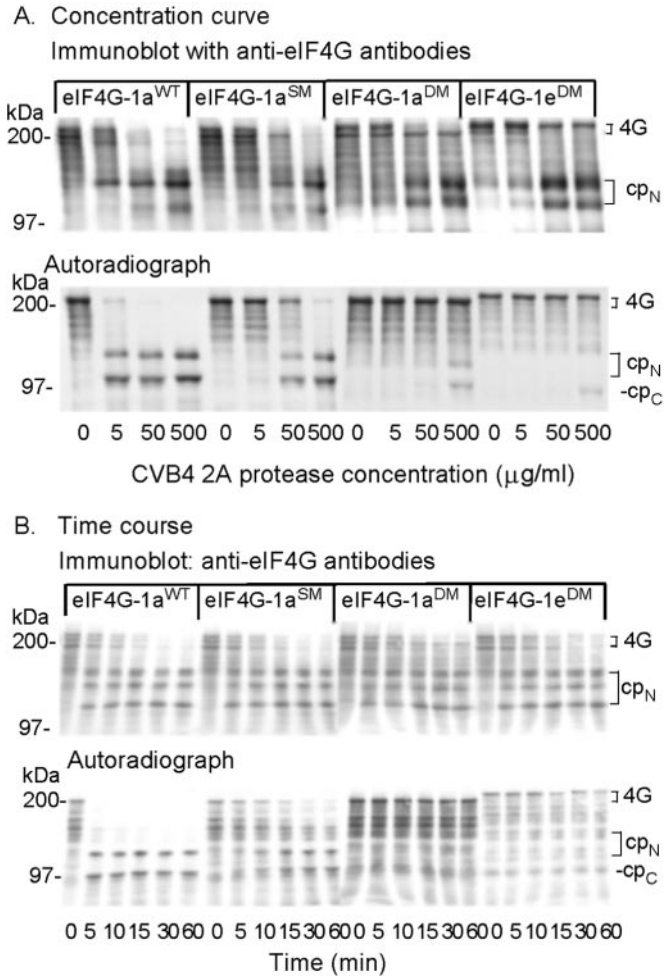


FIG. 3. **Resistance of eIF4G-1$^{SM}$ and eIF4G-1$^{DM}$ to cleavage by CVB4 2A$^{Pro}$ *in vitro*.** *A*, mRNAs encoding eIF4G-1a$^{WT}$, eIF4G-1a$^{SM}$, eIF4G-1a$^{DM}$, and eIF4G-1e$^{DM}$ were transcribed from pCITE4G$^{WT}$ (38), pCITE4G$^{G486E}$ (38), pCITE4G-1a$^{DM}$, and pCITE4G-1e$^{DM}$, respectively, and translated in an MDL system. Reactions were then incubated with the indicated concentrations of CVB4 2A$^{Pro}$ at 30 °C for 20 min, and 2-μl aliquots were subjected to SDS-PAGE on 6% gels. Proteins were transferred to polyvinylidene difluoride membranes and immunoblotted with anti-eIF4G-1 antibodies (*upper panel*). The blots were then exposed to x-ray film (*lower panel*). The *upper panel* shows both endogenous rabbit eIF4G-1 and newly synthesized human eIF4G-1, whereas the *lower panel* shows only the latter. *B*, translation reactions programmed with the same mRNAs were incubated with CVB4 2A$^{Pro}$ at 30 °C at a final concentration of 100 μg/ml. Aliquots (10 μl) were taken at the indicated times and analyzed as in *A*.

*Cleavage of eIF4G-1$^{SM}$ and Resistance to Cleavage of eIF4G-1$^{DM}$ by 2A$^{Pro}$ in Vitro*—This finding suggested that alteration of both primary and secondary cleavage sites might confer even greater resistance to CVB4 2A$^{Pro}$. We therefore performed site-

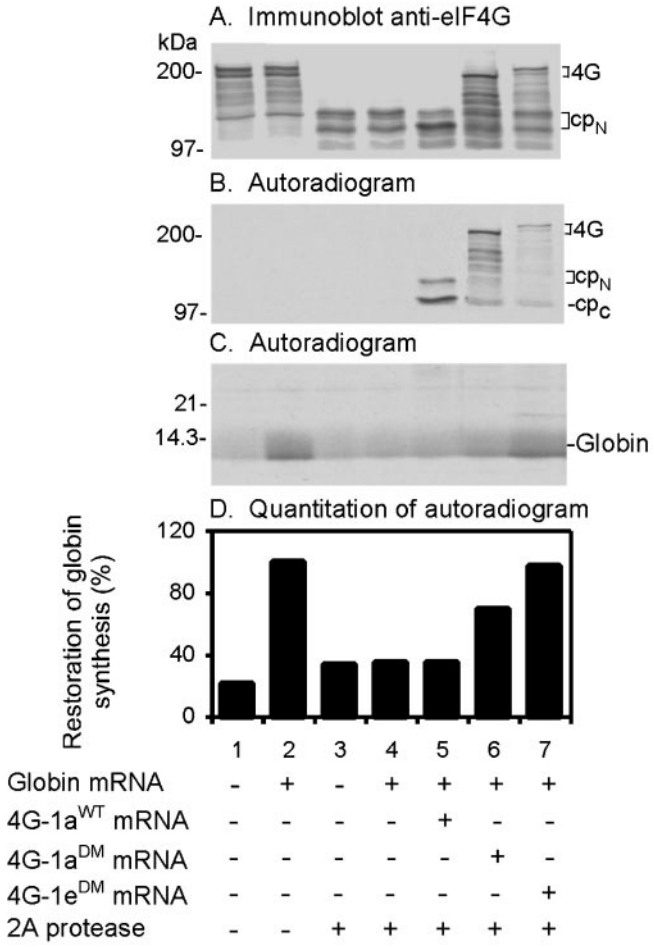


FIG. 4. **Restoration of cap-dependent translation to CVB4 2A$^{Pro}$-pretreated MDL by eIF4G-1$^{DM}$.** Two-phase *in vitro* translation reactions were performed as described under "Experimental Procedures." During phase I, either eIF4G-1a$^{WT}$, eIF4G-1a$^{DM}$, eIF4G-1e$^{DM}$, or no eIF4G-1 was 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 [$^{35}$S]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- (cp$_N$) and C-terminal (cp$_C$) 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. 3*A*, *lower panel*, lanes with no CVB4 2A$^{Pro}$), but there were also bands of faster mobility, as observed previously (43). At least some of these are due to alternative initiation at downstream AUG codons (44). The reaction mixtures were then incubated with increasing concentrations of CVB4 2A$^{Pro}$. Immunoblotting with anti-eIF4G-1 antibodies detected both endogenous rabbit and exogenous (*in vitro* synthesized) human eIF4G-1 (Fig. 3*A*, *upper panel*), whereas autoradiography detected only the exogenous eIF4G-1 (Fig. 3*A*, *lower panel*). Newly synthesized eIF4G-1$^{WT}$ was completely cleaved at 5 $\mu$g/ml CVB4 2A$^{Pro}$, whereas endogenous eIF4G-1$^{WT}$ was cleaved at 50 $\mu$g/ml. Most of the eIF4G-1$^{SM}$ was cleaved at 500 $\mu$g/ml, as observed previously (38). However, newly synthesized eIF4G-1a$^{DM}$ and eIF4G-1e$^{DM}$ were only slightly cleaved at 500 $\mu$g/ml. The degree of cleavage of eIF4G-1$^{DM}$ isoforms at 500 $\mu$g/ml was intermediate between that of eIF4G-1a$^{SM}$ at 5 and 50 $\mu$g/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$^{SM}$, eIF4G-1a$^{DM}$, and eIF4G-1e$^{DM}$ to 100 $\mu$g/ml CVB4 2A$^{Pro}$ cleavage as a function of time (Fig. 3*B*). 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$^{SM}$ was digested by 60 min (Fig. 3*B, 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$^{DM}$ isoforms were synthesized in 2A$^{Pro}$-pretreated MDL by IRES-driven translation. In phase II, the newly synthesized eIF4G-1$^{DM}$ was tested for its ability to restore translation of globin mRNA. Endogenous eIF4G-1 was cleaved in the 2A$^{Pro}$-pretreated MDL (Fig. 4*A*, *lanes 3–7*) but not in mock-treated MDL (Fig. 4*A*, *lanes 1* and *2*). eIF4G-1a$^{DM}$ (*lane 6*) and eIF4G-1e$^{DM}$ (*lane 7*) were shown by immunoblotting (Fig. 4*A*) and autoradiography (Fig. 4*B*) 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 (*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 more 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. 5*A*). 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 $\alpha$- and $\beta$-globin mRNA (Fig. 5*B*). These findings indicate that the poly(A) tract of globin mRNA was removed from the 3′-end.

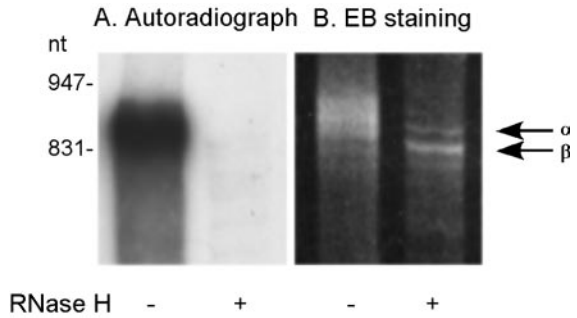


FIG. 5. **Deadenylation of globin mRNA.** Globin mRNA labeled at the 3′-end with [5′-$^{32}$P]pCp was mixed with unlabeled globin mRNA, hybridized to oligo(dT), and treated with RNase H. The RNA was separated on a 6% denaturing polyacrylamide gel and analyzed by autoradiography (*A*) and ethidium bromide staining (*B*). α and β refer to the positions of α-globin and β-globin mRNAs.

The untreated and deadenylated globin mRNAs were then used to test the activity of eIF4G-1$^{DM}$ isoforms. Either eIF4G-1a$^{DM}$ or eIF4G-1e$^{DM}$ was synthesized in phase I (Fig. 6*A*). In phase II, the newly synthesized eIF4G-1a$^{DM}$ and eIF4G-1e$^{DM}$ were used to translate untreated or deadenylated globin mRNA (Fig. 6*B*). Deadenylated globin mRNA was translated at the same efficiency in the presence of either eIF4G-1a$^{DM}$ or eIF4G-1e$^{DM}$ (*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-1e$^{DM}$ compared with eIF4G-1a$^{DM}$ (*lane 8 versus 7*, $p < 0.008$, $n = 3$). Furthermore, polyadenylated and deadenylated globin mRNAs were translated with the same efficiency with eIF4G-1a$^{DM}$ (*lane 7 versus 9*), presumably due to the absence of a PABP-binding site in eIF4G-1a$^{DM}$.

It was possible that the deadenylated mRNA was less stable in the translation system, accounting for its lower activity with eIF4G-1e$^{DM}$. 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-1$^{DM}$ Restores Cap-dependent Translation in Vivo*—In order to test the translational activity of eIF4G-1$^{DM}$ variants *in vivo*, we established HeLa cell lines stably transfected with plasmids expressing either eIF4G-1a$^{DM}$ or eIF4G-1e$^{DM}$ under control of a tetracycline-regulated promoter. To distinguish endogenous eIF4G-1 from eIF4G-1$^{DM}$, we treated cell extracts with CVB4 2A$^{Pro}$ before immunoblotting with anti-eIF4G-1 antibodies. The level of exogenous eIF4G-1a$^{DM}$ (Fig. 7*A*, + *lanes*) and eIF4G-1e$^{DM}$ (Fig. 7*B*, + *lanes*) increased with increasing doxycycline concentration. Unfortunately, the expression level of exogenous eIF4G-1$^{DM}$ 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-1a$^{DM}$ and eIF4G-1e$^{DM}$ by co-transfecting the stable cell lines with vectors expressing GFP and CVB3 2A$^{Pro}$. 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 2A$^{Pro}$ also contained an EMCV IRES, so the protease was produced regardless of the cleavage status of eIF4G. eIF4G-1$^{DM}$ isoforms were induced with doxycycline for 24 h. Cells were then transiently co-transfected with the GFP- and 2A$^{Pro}$-expressing vectors.

It was necessary to control for differences in GFP mRNA

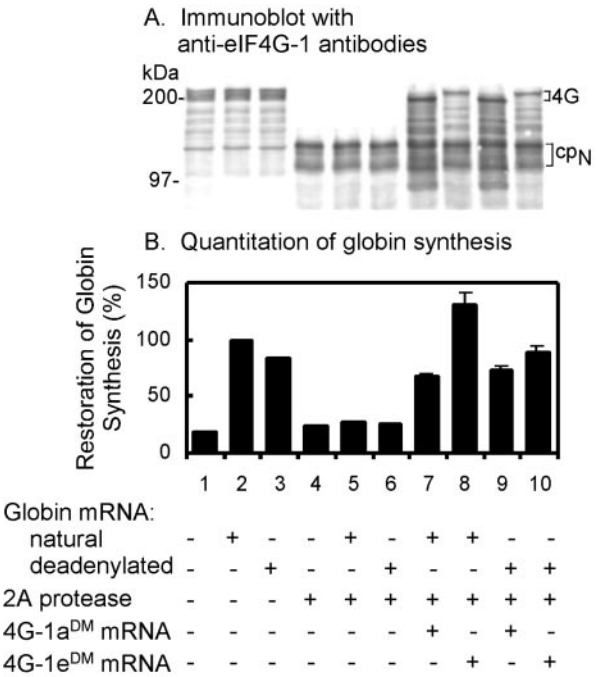


FIG. 6. **Translational activity of eIF4G-1a$^{DM}$ and eIF4G-1e$^{DM}$ using polyadenylated and deadenylated mRNA.** The two-phase *in vitro* protein synthesis system described in Fig. 4 was utilized except that either polyadenylated or deadenylated rabbit globin mRNA was translated. Aliquots were removed after 30 min of the phase II reaction and subjected to SDS-PAGE on 6 and 15% gradient gels. *A*, the 6% gel was immunoblotted with anti-eIF4G-1 antibodies. *B*, globin synthesis was quantitated from the autoradiograph of the 15% gel.

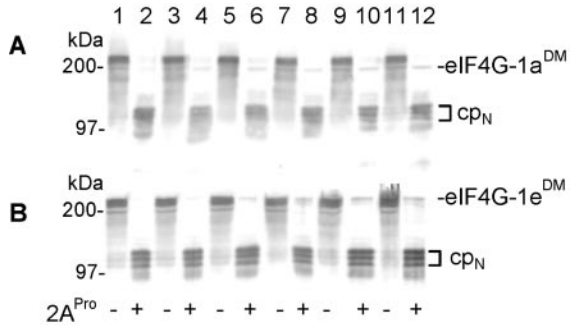


FIG. 7. **Synthesis of eIF4G-1a$^{DM}$ and eIF4G-1e$^{DM}$ in stably transfected HeLa cells as a function of increasing doxycycline.** Cells expressing either eIF4G-1a$^{DM}$ (*A*) or eIF4G-1e$^{DM}$ (*B*) under the control of a tetracycline-regulated promoter were cultured for 24 h with no doxycycline (*lanes 1* and *2*) or doxycycline at 100 (*lanes 3* and *4*), 200 (*lanes 5* and *6*), 300 (*lanes 7* and *8*), 500 (*lanes 9* and *10*), or 1000 (*lanes 11* and *12*) ng/ml. Cell lysates were either treated (+) or mock-treated (−) with CVB4 2A$^{Pro}$ on ice for 2 h. Samples were subjected to SDS-PAGE on 6% gels, transferred to a polyvinylidene difluoride membrane, and immunoblotted with anti-eIF4G-1 antibodies.

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 ($C_T$) for all GAPDH cDNA samples fell between 18 and 20 (Fig. 8*A*), whereas those for GFP cDNA fell between 15 and 18 (Fig. 8*B*). 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

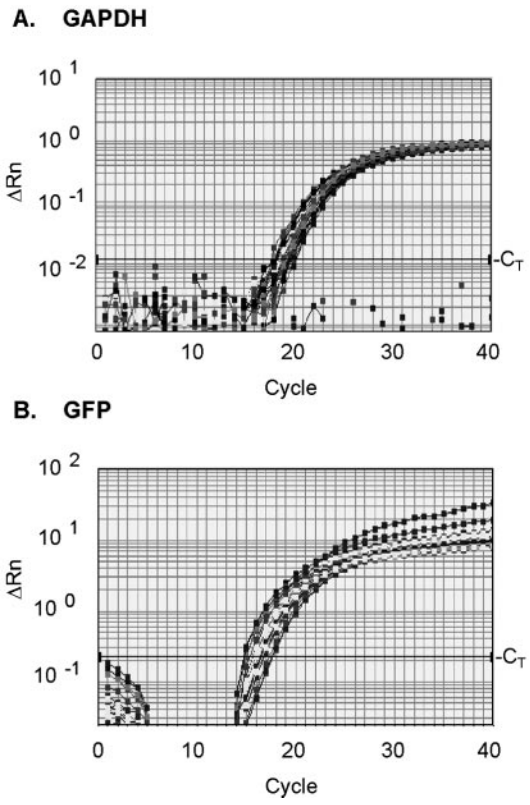


FIG. 8. **Measurement of GFP mRNA levels by real time PCR.** RNA was isolated from cells transiently transfected with plasmids expressing GFP or co-transfected with plasmids expressing GFP and $2A^{Pro}$ and subjected to reverse transcription using oligo$(dT)_{15}$ as primer. Real time PCR was used to amplify the endogenous control GAPDH cDNA (*A*) and GFP cDNA (*B*) as described under "Experiment Procedures." $\Delta Rn$ and $C_T$ are defined in the ABI PRISM® 7700 users manual and correspond to the difference of fluorescence intensity between reactions containing or not containing template, and the cycle in which there occurs a significant increase of fluorescence intensity, respectively.

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-$1a^{DM}$ cells not expressing $2A^{Pro}$ (Fig. 9*A*, *GFP*, *open versus filled bars*; $0.5 < p < 0.8$; eight degrees of freedom). This was also true for eIF4G-$1e^{DM}$ cells (Fig. 9*B*, GFP; $0.5 < p < 0.8$). Expression of $2A^{Pro}$ dramatically decreased GFP synthesis in eIF4G-$1a^{DM}$ cells uninduced with doxycycline (Fig. 9*A*, *open bars*, GFP + 2A *versus* GFP; $p < 0.05$). This was also true for eIF4G-$1e^{DM}$ cells (Fig. 9*B*, *open bars*, GFP + 2A *versus* GFP; $p < 0.02$). However, induction of eIF4G-$1a^{DM}$ significantly increased GFP expression (Fig. 9*A*, *GFP + 2A*, *open versus filled bars*, $p < 0.01$). Similarly, induction of eIF4G-$1e^{DM}$ significantly increased GFP expression (Fig. 9*B*, *GFP + 2A*, *open versus filled bars*; $p < 0.001$).

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

## DISCUSSION

Comparison of the cleavage sites for $2A^{Pro}$ in the viral polyproteins of 22 entero- and rhinoviruses yields the consensus sequence Leu-Thr-Thr-*X*↓Gly-Pro . . . (P4-P3-P2-P1↓P1′-P2′ . . .; 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

TABLE II
*Cap-dependent and -independent synthesis of GFP in cells transfected with vectors producing CVB4 2A as a function of $eIF4G^{DM}$ induction*

| GFP synthesis[a] | Cells | Doxycycline[b] | CVB4 2A[c] | GFP fluorescence[d] | GFP mRNA[e] | GFP synthesis[f] |
|---|---|---|---|---|---|---|
| Cap-dependent | eIF4G-$1a^{DM}$ | − | − | 907 ± 51 | 10.7 ± 2.0 | 85 ± 21 |
| | | + | − | 881 ± 37 | 8.9 ± 0.8 | 100 ± 13 |
| | | − | + | 162 ± 12 | 5.3 ± 0.2 | 30 ± 4 |
| | | + | + | 533 ± 42 | 7.3 ± 1.5 | 73 ± 21 |
| | eIF4G-$1e^{DM}$ | − | − | 718 ± 29 | 14.3 ± 3.0 | 50 ± 13 |
| | | + | − | 745 ± 19 | 17.3 ± 2.3 | 43 ± 7 |
| | | − | + | 62 ± 6.8 | 7.1 ± 0.5 | 8.8 ± 1.9 |
| | | + | + | 228 ± 7 | 7.4 ± 0.7 | 31 ± 3.7 |
| IRES-dependent | eIF4G-$1a^{DM}$ | − | − | 226 ± 44 | 9.8 ± 1.5 | 23 ± 9.5 |
| | | + | − | 281 ± 57 | 7.9 ± 0.4 | 36 ± 14 |
| | | − | + | 360 ± 32 | 3.7 ± 0.01 | 98 ± 17 |
| | | + | + | 317 ± 51 | 3.7 ± 0.5 | 86 ± 30 |
| | eIF4G-$1e^{DM}$ | − | − | 349 ± 44 | 7.5 ± 0.4 | 47 ± 11 |
| | | + | − | 153 ± 33 | 6.4 ± 0.8 | 24 ± 11 |
| | | − | + | 392 ± 27 | 3.2 ± 0.1 | 122 ± 16 |
| | | + | + | 336 ± 41 | 9.1 ± 0.3 | 37 ± 8.6 |

[a] Cells were transfected with either pEGFP-C1, for which GFP synthesis is cap-dependent, or pIRES2-EGFP, for which GFP synthesis is cap-independent due to the presence of an EMCV IRES in the mRNA, 24 h before fluorescence measurements.
[b] Doxycycline was added to the indicated cells 24 h before transfection.
[c] The indicated cells were transfected with the pVP12A vector expressing CVB4 $2A^{Pro}$ 24 h before fluorescence measurements.
[d] The number of fluorescent cells was measured at five random sites in the cell culture well using 16 adjacent microscopic fields for each site, using a minimum threshold of 1000. The averages ± S.E. are given.
[e] GFP mRNA levels were measured by real time PCR and normalized to endogenous GAPDH mRNA levels.
[f] GFP synthesis rate over 24 h was determined by dividing the number of fluorescent cells by the GFP mRNA level. The S.E. for the quotient was calculated from the formula

$$\text{S.E.}_{\frac{F}{M}} = \frac{F}{M}\sqrt{\left(\frac{\text{S.D.}_F^2}{F^2} + \frac{\text{S.D.}_M^2}{M^2}\right)\left(\frac{1}{n_F} + \frac{1}{n_M}\right)}$$

where $F$ and $M$ refer to number of fluorescent cells and GFP mRNA level, respectively.

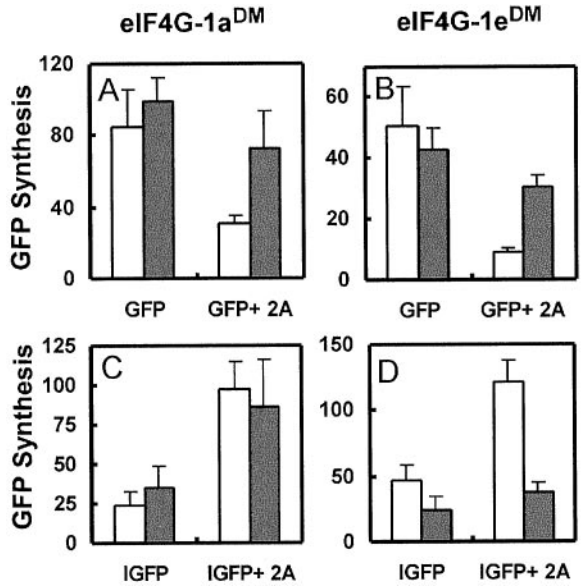


FIG. 9. **GFP synthesis in stable cell lines expressing eIF4G-1$^{DM}$ isoforms.** GFP synthesis, as calculated in Table II, was measured in cells stably expressing either eIF4G-1a$^{DM}$ (*A* and *C*) or eIF4G-1e$^{DM}$ (*B* and *D*). Expression of eIF4G-1 was either uninduced (*open bars*) or induced with doxycycline for 24 h (*filled bars*). Cells were then co-transfected with vectors expressing 2A$^{Pro}$ (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.

cases. The 2A$^{Pro}$ of HRV2 and CVB4 cleave eIF4G-1 initially at Leu$^{679}$-Ser-Thr-Arg ↓ Gly, a sequence that contains both of these consensus aa residues (10). Our previous observation that the G683E variant eIF4G-1a$^{SM}$ was still cleaved (38), albeit 100-fold more slowly than eIF4G-1a$^{WT}$, meant either that CVB4 2A$^{Pro}$ 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 Leu$^{674}$ and Gly$^{675}$, 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-1$^{WT}$, but our method of analyzing cp$_C$ 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 Gly$^{675}$ and Arg$^{676}$ (46), only 1-aa residue from the secondary cleavage site of CVB4 2A$^{Pro}$. Thus, this region of eIF4G-1 may be particularly accessible to proteases. Because of this proximity of sites, the eIF4G-1$^{DM}$ variant may have some resistance to Lb protease as well.

Careful examination of the immunoblots in Fig. 3 reveals that the newly synthesized eIF4G-1a$^{WT}$ 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 2A$^{Pro}$ than rabbit eIF4G-1, because human matches the consensus CVB4 2A$^{Pro}$-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 2A$^{Pro}$ 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-1e$^{DM}$ to restore translation to 2A$^{Pro}$-pretreated MDL was significantly higher than that of eIF4G-1a$^{DM}$ when the system contained natural globin mRNA. By contrast, the activities of eIF4G-1a$^{DM}$ and eIF4G-1e$^{DM}$ were similar with deadenylated globin mRNA. This is most 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 2A$^{Pro}$-pretreated MDL lacks both functional eIF4G-1 and PABP, because the latter is also cleaved by 2A$^{Pro}$ (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 2A$^{Pro}$ resulted in only one-third cleavage of PABP but complete cleavage of eIF4G-1 (data not shown). The fact that eIF4G-1e$^{DM}$ is able to fully restore translation of globin mRNA indicates that (i) any hypothetical changes to the translational system caused by 2A$^{Pro}$ pretreatment other than eIF4G cleavage do not affect the overall rate of translation, and (ii) the 2-aa substitution in eIF4G-1e$^{DM}$ does not affect its activity for *in vitro* translation of capped and polyadenylated mRNA.

Co-transfection of stable cell lines expressing eIF4G-1$^{DM}$ isoforms with plasmids that express GFP and 2A$^{Pro}$ indicated that eIF4G-1$^{DM}$ isoforms substantially protect cap-dependent protein synthesis from inhibition by 2A$^{Pro}$ *in vivo*. The response of GFP synthesis to 2A$^{Pro}$ was opposite depending on how translation of GFP was initiated. Cap-dependent GFP synthesis was dramatically inhibited by 2A$^{Pro}$ 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$_C$ 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 2A$^{Pro}$ makes limiting components of the translational machinery available for IRES-driven synthesis. Doxycycline-induced expression of eIF4G-1e$^{DM}$ substantially reversed the effects of 2A$^{Pro}$ in both cases; inhibition of cap-dependent translation by 2A$^{Pro}$ was relieved (Fig. 9*B*), and stimulation of IRES-dependent translation by 2A$^{Pro}$ was suppressed (Fig. 9*D*). For reasons we do not understand, eIF4G-1a$^{DM}$ was able to restore cap-dependent translation (Fig. 9*A*) but not suppress the IRES-dependent stimulation (Fig. 9*C*).

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-1$^{DM}$ 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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