A Single Amino Acid Change in Protein Synthesis Initiation Factor 4G Renders Cap-Dependent Translation Resistant to Picornaviral 2A Proteases†

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ABSTRACT: Infection of cells with picornaviruses of the rhino-, aphtho-, and enterovirus groups causes a shut-off in cap-dependent translation of cellular mRNAs but permits cap-independent viral RNA translation to proceed. This shut-off is thought to be mediated in part by the proteolytic cleavage of eukaryotic initiation factor 4G (eIF4G), although there is evidence to the contrary. Cleavage of eIF4G results in the separation of the eIF4E-binding domain from the ribosome- and helicase-binding domains of the factor, thereby limiting the ability of eIF4G to function in cap-dependent recruitment of mRNAs. Previously we determined the cleavage site within eIF4G targeted by the 2A proteases from human coxsackievirus serotype B4 and human rhinovirus serotype 2 using highly purified eIF4F and recombinant proteases. To examine further the role proteolysis of eIF4G plays in shut-off of translation, we altered the 2A cleavage site in human eIF4G by site-directed mutagenesis. Strikingly, the replacement of one amino acid at the 2A cleavage site resulted in a protein that is approximately 100-fold resistant to cleavage by coxsackievirus 2A protease and 10−50-fold for rhinovirus 2A. Alteration of the cleavage site had no effect on factor activity since the variant was just as active as wild-type eIF4G in restoring cap-dependent translation to an *in vitro* translation system depleted of endogenous eIF4G. Furthermore, the presence of the variant form of eIF4G rendered *in vitro* translation reactions resistant to the 2A protease-mediated inhibition of cap-dependent translation initiation. These results support the model that 2A proteases inhibit cap-dependent translation through direct proteolysis of eIF4G.

There are two fundamentally different modes by which initiation of eukaryotic translation takes place: cap-dependent and cap-independent. In the first, the 43S initiation complex, which contains eIF3,[1] eIF2, met-tRNA$_i$, and GTP, binds at or near the 5′-terminus of the mRNA [reviewed in Merrick and Hershey (1996)]. This complex then scans until the first AUG codon in good context is encountered, after which GTP is hydrolyzed, the 60S subunit joins to make the 80S complex, and the first peptide bond is formed. Polypeptides of the eIF4 class catalyze productive binding of mRNA to the 43S initiation complex and include (i) eIF4A, an ATP-dependent RNA helicase, (ii) eIF4B, a factor which stimulates the activity of eIF4A and which also has RNA annealing activity, (iii) eIF4E, the cap-binding protein, and (iv) eIF4G (formerly eIF-4γ, eIF-4Fγ, or p220), which acts as a linker in this process, specifically complexing with eIF4E, eIF4A, and eIF3 to bring together the mRNA 5′-end, the RNA helicase activity, and the 40S ribosomal subunit (Lamphear et al., 1995). The isolated complex of eIF4E, eIF4A, and eIF4G from mammalian cells is termed eIF4F.

The best understood example of cap-independent translation occurs after infection of mammalian cells by certain picornaviruses [reviewed in Ehrenfeld (1996)]. The picornavirus family includes several pathogens of both humans (*e.g.*, poliovirus, coxsackievirus, and hepatitis A viruses) and animals (*e.g.*, foot-and-mouth disease virus and encephalomyocarditis virus). The genomes of these viruses consist of single-stranded, plus-sense RNAs which are uncapped and hence not favored by the cap-dependent pathway. More importantly, these RNAs have very long 5′-untranslated regions which contain a high degree of secondary structure and many noninitiator AUG codons. When placed between two cistrons of a bicistronic mRNA, these 5′-untranslated regions direct internal binding of ribosomes (Jang et al., 1988; Pelletier & Sonenberg, 1988). Deletion analysis indicates that a ∼450 nt region, termed an IRES, is sufficient for ribosome entry. Following infection with members of the entero-, rhino-, and aphthovirus groups, there is a dramatic switch in translational specificity. The cap-dependent pathway is inhibited, a process known as the host cell shut-off, and IRES-driven, viral translation becomes predominant. The fact that single base changes in the poliovirus IRES are responsible for the attenuated phenotype of the three Sabin strains attests to the importance of the IRES for translational efficiency and neurovirulence (La-Monica & Racaniello, 1989).

The switch from cap-dependent to IRES-driven, cap-independent translation is thought to involve changes in the intracellular quantities or the activity of eIF4G. Extracts from poliovirus-infected cells are unable to carry out cap-dependent translation, but this ability can be restored by

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[1] Abbreviations: cp$_C$, carboxyl-terminal fragment of eIF4G; cp$_N$, amino-terminal fragment of eIF4G; CVB4, coxsackievirus, serotype B4; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; eIF, eukaryotic initiation factor; FMDV, foot-and-mouth disease virus; HRV2, human rhinovirus, serotype 2; IRES, internal ribosome entry segment; MDL, messenger RNA-dependent reticulocyte lysate; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; RRL, rabbit reticulocyte lysate; SDS, sodium dodecyl sulfate.

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initiation factors from uninfected cells (Rose et al., 1978; Helentjaris et al., 1979), particularly those containing eIF4 factors (Trachsel et al., 1980; Tahara et al., 1981). eIF4G was discovered as a result of its proteolysis coincident with the loss of cap-dependent initiation during poliovirus infection (Etchison et al., 1982), and the purified eIF4F complex was shown to restore cap-dependent translation to extracts of poliovirus-infected cells (Grifo et al., 1983; Edery et al., 1983; Lee et al., 1985). eIF4F from uninfected HeLa cells differentially stimulates cap-dependent but not cap-independent translation in the RRL system, whereas the opposite is true for eIF4F from poliovirus-infected cells (Buckley & Ehrenfeld, 1987; Morley et al., 1991).

The viral component thought to be responsible for host cell shut-off is protease 2A in entero- and rhinoviruses and the L protease in aphthoviruses. Mutations affecting the 2A region of the poliovirus polyprotein causes a small-plaque phenotype and prevent the selective shut-off of host translation (Bernstein et al., 1985; O'Neill & Racaniello, 1989). Expression of protease 2A alone in HeLa cells causes shut-off of cap-dependent translation from a reporter gene (Sun & Baltimore, 1989). There is also considerable evidence that protease 2A is involved in eIF4G cleavage. *In vivo* evidence includes the fact that (i) infection with poliovirus mutated in the protease 2A-encoding region fails to cleave eIF4G (Bernstein et al., 1985; O'Neill & Racaniello, 1989); (ii) expression of protease 2A alone causes eIF4G cleavage (Sun & Baltimore, 1989); and (iii) the presence of an 18 amino acid sequence in the entero- and rhinoviral polyproteins, which is homologous to the active site of cysteine proteases, correlates with the cleavage of eIF4G during infection but the absence of cleavage during cardiovirus infection (Lloyd et al., 1988). *In vitro* evidence includes the fact that eIF4G cleavage is observed after (i) the synthesis of protease 2A in a RRL system (Kräusslich et al., 1987; Lloyd et al., 1988); (ii) the addition of bacterial extracts containing poliovirus protease 2A to HeLa extracts (Alvey et al., 1991); and the addition of purified, recombinant protease 2A from HRV2 or CVB4 to HeLa extracts or rabbit reticulocyte eIF4F (Liebig et al., 1993; Lamphear et al., 1993).

There are conflicting data, however, on the question of whether protease 2A cleaves eIF4G indirectly, through activation of a cellular protease, or directly. Evidence supporting indirect cleavage includes the fact that (i) poliovirus protease 2A does not copurify with cleavage activity through subcellular fractionation, (ii) poliovirus 2A protease is not detected by immunoblot analysis in column fractions containing cleavage activity, and (iii) antibodies against poliovirus 2A protease do not inhibit eIF4G cleavage [reviewed in Wyckoff (1993)]. On the other hand, purified HRV2 and CVB4 proteases 2A cleave purified eIF4F into characteristic proteolytic fragments (Liebig et al., 1993; Lamphear et al., 1993), as does the recombinant Lb protease of FMDV (Kirchweger et al., 1994). Similarly, the HRV2 and CVB4 proteases cleave synthetic peptides containing the substrate-recognition sequence of intact eIF4G (Sommergruber et al., 1994). Regardless of the mechanism, treatment of cell-free translation systems with 2A or L proteases mimics the *in vivo* situation of mammalian cells infected with entero-, rhino-, and aphthoviruses: eIF4G is cleaved, cap-dependent translation is inhibited, and cap-independent translation, whether initiated from IRESes in bicistronic mRNAs or from the 5′-end of uncapped monocistronic mRNAs, is stimulated [see Borman et al. (1995) and references cited therein].

Despite these correlations, there is a significant body of evidence stating that eIF4G cleavage is not responsible, or is only partially responsible, for the switch from cap-dependent to cap-independent translation. When cells are infected with poliovirus in the presence of inhibitors of viral replication, eIF4G is completely degraded, but the host shut-off is only partial (Bonneau & Sonenberg, 1987; Perez & Carrasco, 1992). Similarly, ionophores block the host shut-off but not eIF4G cleavage (Irurzun et al., 1995). Expression of poliovirus 2A protease in COS-1 cells had a much greater inhibitory effect on transcription by RNA polymerase II than on translation and led to the conclusion that eIF4G cleavage was not sufficient for complete inhibition of host translation (Davies et al., 1991). Other studies involving *in vivo* expression of protease 2A or mutations in the 2A gene that compensate for mutations in the IRES have led to the conclusion that protease 2A activates viral mRNA translation independently of its role in the host shut-off (Hambidge & Sarnow, 1992; Macadam et al., 1994). Thus, it is possible that events which accompany picornavirus infection other than eIF4G cleavage, *e.g.*, changes in the intracellular ionic environment (Irurzun et al., 1995), the phosphorylation of eIF-2α (O'Neill & Racaniello, 1989), the proteolysis of additional proteins (Ziegler et al., 1995), or the inhibition of RNA polymerase II transcription (Davies et al., 1991), are responsible, wholly or in part, for the host cell shut-off.

In the previous studies on the relationship of the L or 2A proteases to the host cell shut-off, it has been possible to show a correlation between eIF4G cleavage and the shut-off of cap-dependent translation but not a cause-and-effect relationship. Recently we determined the sites of cleavage within eIF4G for HRV2 protease 2A, CVB4 protease 2A, and FMDV protease Lb (Lamphear et al., 1993; Kirchweger et al., 1994). Previous studies with model peptides defined important elements in the substrate recognition site of the HRV2 protease 2A (Sommergruber et al., 1992, 1994). We have utilized this information to introduce a single amino acid substitution in eIF4G which sharply reduces the rate of cleavage by these proteases. This has enabled us to test directly the effect of this cleavage on cap-dependent translation.

MATERIALS AND METHODS

Materials. m^7GpppG and plasmid Bluescript pSK II(−) were obtained from Stratagene (La Jolla, CA). Plasmids pET21b and pCITE 2A were obtained from Novagen (Madison, WI). RRL and MDL were prepared from New Zealand white rabbits (Clemens, 1987) or obtained from Gibco (Gaithersburg, MD) or Promega (Madison, WI). T7 RNA polymerase, DNase I, and m^7GpppG were also obtained from Promega. [^{35}S]Met (1000 Ci/mmol) was obtained from ICN Biochemicals (Irvine, CA). Oligonucleotides used for site-directed mutagenesis [Mala (5′-CCTTGGGGGCG-CACGGGTGC-3′), Mval (5′-CCTTGGGGGAACACGGGT-GCT-3′), Marg (5′-CTTGGGGGCTCACGGGTGC-3′), Mtrp (5′-CTTGGGGGCCAACGGGTGC-3′), and Mglu (5′-CT-TGGGGGCCTACGGGTGC-3′)] were purchased from Oligos, Etc. (Wilsonville, OR). Oligonucleotides used in sequencing [plus strand primers +4G1666 (5′-AGCCA-GAGGGATTGCCACATA-3′), +4G1907 (5′-CCGAAAA-GAACCACGCAAGAT-3′), and +4G2334 (5′-GAGAAA-

GACAAAGATGATGAT-3′), and antisense primers −4G2567 (5′-CAAAGGCACTCAAGGGACTCT-3′) and −4G2325 (5′-CTCATCATCATCTTTGTCTTT-3′)] were synthesized at the University of Kentucky core facility. m^7GTP was obtained from Sigma (St. Louis, MO). Elastatinal was obtained from Calbiochem (La Jolla, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Vector Laboratories (Burlingame, CA). Immobilon P was purchased from Millipore (Bedford, MA). The production of rabbit anti-eIF4G peptide 7 antiserum (amino acids 327−342) was described elsewhere (Yan et al., 1992).

Recombinant Plasmids. pSK(−)4GT7EV and pCITE4G^{wt} were constructed by ligating the *Eco*RV/*Eco*RI fragment of pSKHFC1 (nt 351−5014, which contains the entire coding region of human eIF4G; Yan et al., 1992) into the multiple cloning sites of Bluescript pSK II(−) and pCITE 2A, respectively. Site-directed mutagenesis of the coding region of eIF4G at the 2A protease-cleavage site was performed by using plasmid pSK(−)4GT7EV as template, the mutagenesis oligonucleotides described above, and the Mutagene Phagemid *in vitro* mutagenesis kit (Biorad, Hercules, CA) as described by the manufacturer. The *Apa*I restriction site at nt 1826 in the eIF4G coding region is not present in any of the mutator oligonucleotides. Potentially mutated DNAs were initially screened by restriction digestion with *Apa*I; those lacking the central *Apa*I site were sequenced to confirm the presence of the mutation. The *Bam*HI/*Hpa*I fragments of the mutated eIF4G plasmids (nt 1734−2510 of the eIF4G coding sequence) were used to replace their respective fragments from pCITE4G^{wt} to create pCITE4G^{G486E} and other mutated plasmids. Constructs were sequenced using the Sequenase T7 DNA polymerase dITP kit (U.S. Biochemical Corp.). During sequencing of the cDNA for eIF4G^{wt}, nine discrepancies with the published 5098 bp sequence (Yan et al., 1992) were observed: ^{1854}CTCGCCCGTGGCGCGCAG^{1871} should be ^{1854}CTGCCCCGTGGGCCGCAG^{1871}; ^{2003}GACAGCG^{2009} should be ^{2003}GACGGCG^{2009}; ^{2410}GAGAACG^{2416} should be ^{2410}GAGGACG^{2416}; ^{2423}GGAGGAG^{2429} should be ^{2423}GGAAGAG^{2429}; ^{2456}GCGCTGCT^{2463} should be ^{2456}GCGGCGCT^{2463}; ^{2774}TCCAAG^{2779} should be ^{2774}TCCCAAG^{2780}; ^{2812}GGAAGA^{2817} should be ^{2813}GGATAGA^{2819}; ^{2848}ATGCAAG^{2854} should be ^{2850}ATGGCCAAG^{2858}; and ^{2929}CTGACAC^{2935} should be ^{2933}CTGGAACAC^{2941}. After site-directed mutation of the cDNA for eIF4G^{wt} to eIF4G^{G486E}, no changes other than the engineered mutations were observed.

pET4G was created by inserting the *Hin*dIII/*Eco*RI fragment from pSK(−)4GT7EV, containing the entire eIF4G coding sequence, into the *Hin*dIII site of pET21b with incorporation of the *Eco*RI/*Hin*dIII polylinker sequence of pSK(−) at the 3′-end of the eIF4G cDNA. pAD4G^{wt} was created by inserting the *Xba*I/*Xho*I fragment from pET4G into the multiple cloning site of pADCMV-2. This results in a vector possessing both CMV and T7 polymerase promoters upstream of the coding region of eIF4G^{wt} cDNA, in frame with an amino-terminal T7 epitope tag for immunological detection of the product. The G486E mutation was cloned into pAD4G^{wt} by replacing the *Apa*I/*Apa*I fragment of pAD4G^{wt} (nt 381−4742 of human eIF4G; Yan et al., 1992) with the corresponding fragment from pCITE4G^{G486E} to create pAD4G^{G486E}.

Purification of Proteins. eIF4F was purified from rabbit reticulocyte lysate (Lamphear & Panniers, 1990). Recombinant HRV2 and CVB4 proteases 2A were expressed in *E. coli* and purified as described elsewhere (Liebig et al., 1993). Recombinant eIF4E was expressed in *E. coli* (Stern *et al*., 1993). Briefly, 2 L of BL21(DE3)pLysS bearing plasmid pET4E was grown in LB media containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol to an OD_{600nm} of 0.3, and expression was induced with 0.4 mM IPTG for 4 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 buffer A (50 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 50 mM KCl, and 5% glycerol), and lysis was completed by sonication (3−6 bursts of 10 s each). Insoluble cell debris was removed by centrifugation at 28000*g* for 30 min. The supernatant was made 10% in streptomycin sulfate, and precipitated nucleic acids were removed by centrifugation at 15000*g* for 20 min. The supernatant was applied directly to a 10 mL m^7GTP−Sepharose column equilibrated in buffer A and washed and eluted as described (Lamphear & Panniers, 1990).

Treatment of RRL with 2A Proteases. For preparation of 2A- and mock-treated MDL, RRL was incubated in the presence or absence of HRV2 protease 2A (160 μg/mL) at 20 °C for 10 min. Reactions were then supplemented with 1 mM $CaCl_2$, micrococcal nuclease (100 units/mL) was added, and incubation was continued for another 17 min at 20 °C. Reactions were stopped by the addition of EGTA (2 mM) and elastatinal (500 μM). Treatment of RRL with 2A protease was as described above except that CVB4 protease 2A (100 μg/mL) was used, incubation was for 20 min, and reactions were stopped with 1.5 mM elastatinal. Lysates were either used immediately or stored at −80 °C. Cleavage of eIF4G was monitored by SDS−PAGE followed by immunoblotting with anti-eIF4G$^{327-342}$ antiserum. Globin synthesis was measured by incorporation of [^{35}S]Met into protein.

Cell-Free Protein Synthesis. mRNA transcription and cell-free translation were performed essentially as described previously (Joshi et al., 1994) except 2 mM cAMP was also present. For synthesis of ^{35}S-labeled eIF4G, reactions were programmed either with uncapped mRNA transcribed from *Xho*I-linearized pCITE4G^{wt} or pCITE4G^{G486E} or with capped mRNA transcribed from *Xho*I-linearized pAD4G^{wt} or pAD4G^{G486E}. Reactions contained 50% MDL (Pelham & Jackson, 1976) and 60 mM KCl and were incubated for 90 min at 30 °C. For measurement of cap-dependent synthesis of globin in the HRV2 protease 2A-treated MDL, reactions contained 80 mM KCl.

Two-phase translation reactions were performed as follows: For testing restoration of globin synthesis, MDL reactions (10 μL) containing either no mRNA or mRNA transcribed from pAD4G^{wt} or pAD4G^{G486E} were initially incubated in the absence of [^{35}S]Met, and contained 70 mM KCl. After 5 min, either buffer or CVB4 protease 2A (1.0 μg) was added to the indicated reactions to cleave endogenous eIF4G. At 13 min, elastatinal (500 μM) was added to inhibit 2A protease activity. After 50 min, the globin synthesis phase was initiated by addition of a second translation reaction (5 μL) containing 50% CVB4 protease 2A-treated RRL, 200 μM of each amino each except Met, 12.5 μM hemin, 0.1 mg/mL creatine phosphokinase, 7 mM creatine phosphate, 2 mM cAMP, 1 mCi/mL [^{35}S]Met, and 100 mM KCl to bring the final concentration of KCl in the second-phase reaction to 80 mM. Aliquots were analyzed by SDS−PAGE followed by immunoblotting and autoradiography. Immunoblots of eIF4G from aliquots taken at the

initiation of second-phase synthesis looked essentially the same as those taken after the globin synthesis phase, indicating that eIF4G cleavage was unchanged during the period of globin synthesis. It was found that the presence of exogenous eIF4E slightly stimulated globin synthesis in both 2A-treated and untreated two-phase reactions; eIF4E was therefore added to all reactions at a final concentration of 1 μg/mL.

For testing protection of globin synthesis, two-phase reactions were performed as described above for the restoration of globin synthesis except CVB4 protease 2A was added 35 min after initiation of first-phase synthesis, and elastatinal was not added.

Electrophoresis. SDS−PAGE was performed for 90 min at 100 V using a Hoefer minigel electrophoresis system (Laemmli, 1970). Proteins were visualized by autoradiography or Western blotting (Lamphear et al., 1995). For Western blotting analysis, unstained proteins were transferred to Immobilon P membranes using a Bio-Rad Mini Trans-Blot cell. Image analysis was by PhosphorImager detection with quantitation using ImageQuant (Molecular Dynamics) or by scanning on a Hewlett Packard ScanJet with quantitation by NIH Image v1.5 software.

RESULTS

In order to determine the role of eIF4G cleavage by picornaviral 2A proteases in impairment of cap-dependent translation, we altered the 2A protease cleavage site in human eIF4G by site-directed mutagenesis. Both HRV2 and CVB4 proteases cleavage the Arg^{486}−Gly^{487} bond in rabbit eIF4G which corresponds to the Arg^{485}−Gly^{486} bond in human eIF4G (Lamphear et al., 1993; Yan et al., 1992). Since the amino acid sequence of human eIF4G at this site is very similar to the rabbit sequence, and since peptides corresponding to the human sequence of eIF4G can be cleaved *in vitro* (Sommergruber et al., 1994), it is likely that the same site is recognized in the human protein as well.

The P1′ Gly (first position on the carboxyl-terminal side of the scissile bond) was chosen as the site most likely to influence protease cleavage based upon two lines of evidence. First, the most common element in the 2A substrate recognition sequence in all entero- and rhinovirus polyproteins is a Gly at the P1′ position. Second, the P1′ Gly is essential for recognition of model peptides by 2A proteases *in vitro* (Sommergruber et al., 1992, 1994). Therefore, we altered the corresponding site in human eIF4G from Gly^{486} to one example of each amino acid group: Ala, in the small side chain group; Val, in the short chain aliphatic group; Trp, in the aromatic group; Arg, in the cationic group; and Glu, in the anionic group. Alteration of the P1′ Gly reduced the rate of cleavage by HRV2 protease 2A with an order of effectiveness of E > R > W > V > A > G (data not shown). We therefore chose to characterize further the most promising variant, $eIF4G^{G486E}$.

In vitro translation reactions were programmed with mRNA encoding either the wild-type form of eIF4G ($eIF4G^{wt}$) or the $eIF4G^{G486E}$ variant. After synthesis of eIF4G was complete, the reactions were incubated with CVB4 protease 2A (Figure 1). Both endogenous and exogenous (*in vitro*-translated) eIF4G can be seen on the immunoblot (panel A), while in the autoradiogram (panel B), only the *in vitro*-synthesized products are seen. The primary translation product migrates at ∼205 kDa, although some incomplete

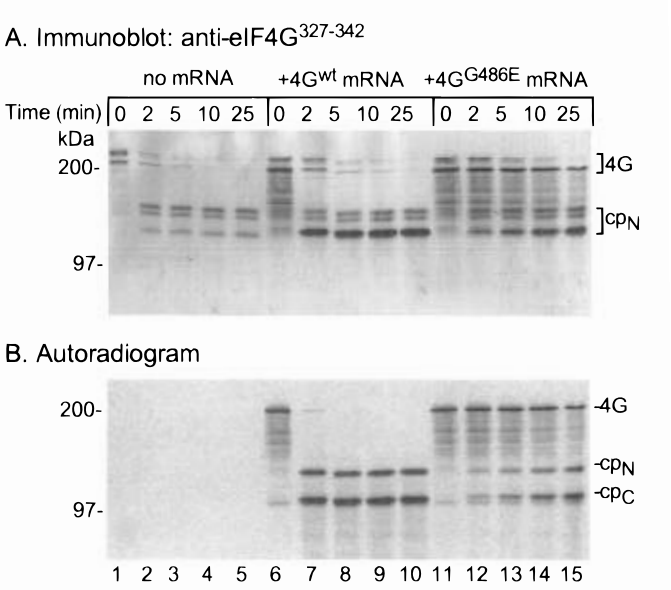

FIGURE 1: Time course of digestion of *in vitro*-synthesized $eIF4G^{wt}$ and $eIF4G^{G468E}$ by CVB4 protease 2A. mRNAs encoding $eIF4G^{wt}$ and $eIF4G^{G486E}$ were transcribed from $pCITE4G^{wt}$ and $pCITE4G^{G486E}$, respectively, and translated *in vitro* as described under Materials and Methods. Translation reactions (10 μL) which had been programmed with either no mRNA, $eIF4G^{wt}$ mRNA, or $eIF4G^{G486E}$ mRNA were incubated with CVB4 protease 2A (225 μg/mL). Aliquots were taken at the indicated times and subjected to SDS−PAGE on a 6% gel. (A) Immunoblot probed with anti-$eIF4G^{327-342}$ antibodies. (B) Autoradiogram of the same blot. The positions of protein standards are indicated on the left. The positions of the intact endogenous (panel A) and *in vitro*-synthesized (panel B) eIF4G are indicated by "4G", and the corresponding cleavage products, by "cp_N" and "cp_C".

translation products can also be seen at higher mobilities. [Completion of the 154-kDa eIF4G polypeptide requires approximately 60 min (Joshi et al., 1994), so the ratio of nascent to completed chains at the 90 min time point is substantial.] In addition, there is a noticeable absence of more slowly migrating forms (panel B), consistent with the earlier observation that *in vivo*-synthesized eIF4G is heterogeneous, ranging from an apparent molecular mass of 206 to 220 kDa, but *in vitro*-synthesized migrates as a single predominant band (Joshi et al., 1994) (panel A, lanes 6 and 11).

The rate of cleavage of $eIF4G^{G486E}$ was much slower than that of the wild-type form. Most of the *in vitro*-synthesized $eIF4G^{wt}$ was cleaved by 2 min (Figure 1, panel B, lane 7), but the majority of the $eIF4G^{G486E}$ variant persisted throughout the 25 min time course (panel B, lanes 11−15). This suggests that substitution of a single amino acid residue at the P1′ position inhibits the rate of cleavage of eIF4G, confirming our earlier assignment of the rabbit eIF4G cleavage site location (Lamphear et al., 1993) and also indicating that human eIF4G is cleaved at the Arg^{485}−Gly^{486} bond. However, despite the slower rate, the $eIF4G^{G486E}$ variant was still cleaved. It is unclear whether the cleavage is occurring at the same location or at a nearby secondary site. In support of the latter possibility, the cp_N and cp_C fragments from $eIF4G^{G486E}$ appear to migrate slightly differently than their wild-type counterparts.

Although less obvious than $eIF4G^{G486E}$ *vs* $eIF4G^{wt}$, the *in vitro*-synthesized $eIF4G^{wt}$ was cleaved more rapidly by CVB4 protease 2A than endogenous eIF4G (*cf.* panel A and panel B). The reason for this is unknown, but it may reflect the absence of additional associated proteins (*e.g.*, eIF4E,

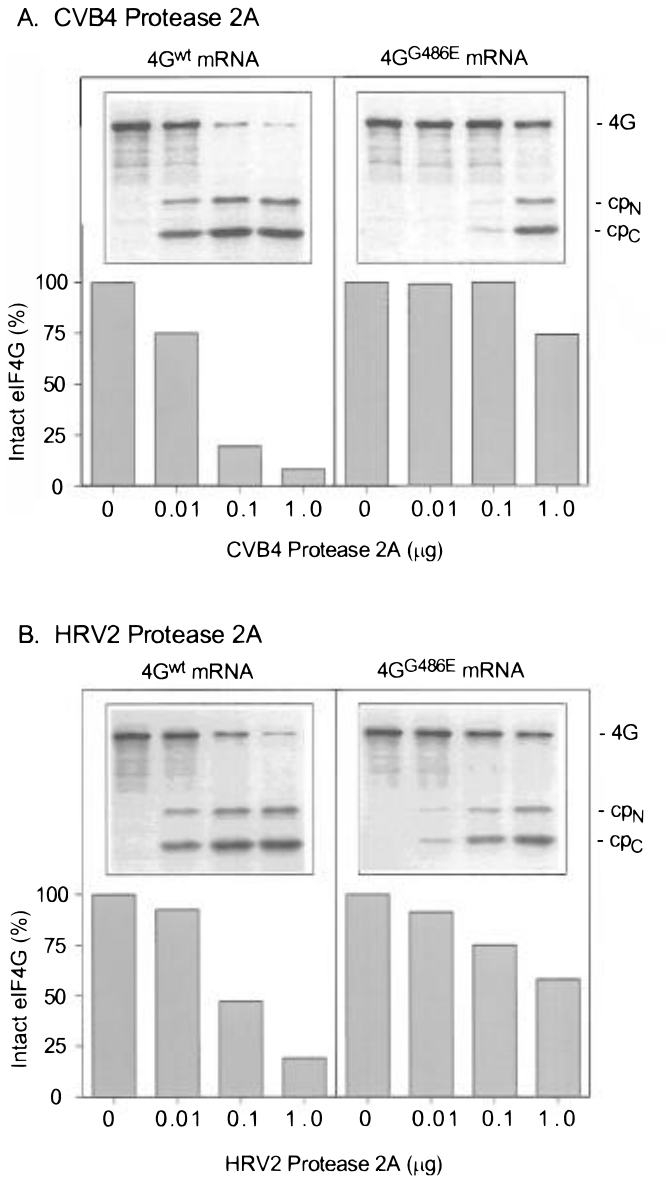

FIGURE 2: Comparison of CVB4 and HRV2 proteases 2A for cleavage of *in vitro*-synthesized eIF4G^{wt} and eIF4G^{G486E}. mRNAs encoding eIF4G^{wt} and eIF4G^{G486E} were transcribed from pAD4G^{wt} and pAD4G^{G486E}, respectively, and translated *in vitro* as described under Materials and Methods. Translation reactions (6 μL) were then incubated with the indicated amounts of either CVB4 (panel A) or HRV2 (panel B) protease 2A. Aliquots were taken after 20 min and subjected to SDS−PAGE on 7.5% gels. Autoradiograms (insets) were scanned and the results presented in the bar graphs.

eIF4A, eIF4B, eIF3) which might mask the cleavage site, conformational differences between endogenous and exogenous proteins, or posttranslational modifications present in the endogenous but not the exogenous eIF4G. Endogenous eIF4G was cleaved as effectively in the presence of eIF4G^{G486E} as eIF4G^{wt} (*cf.* the 220-kDa region of panel A, lanes 7−10 with lanes 12−15). This suggests that the presence of eIF4G^{G486E} does not sequester or inhibit the 2A protease activity.

In order to compare and quantitate the cleavage of eIF4G^{wt} and eIF4G^{G486E} by two different picornaviral proteases, we incubated *in vitro*-synthesized proteins with different amounts of either HRV2 or CVB4 protease 2A (Figure 2). The eIF4G^{G486E} variant was approximately 100-fold resistant to CVB4 protease 2A, based on the observation that cleavage of the former by 1 μg of protease was comparable to cleavage

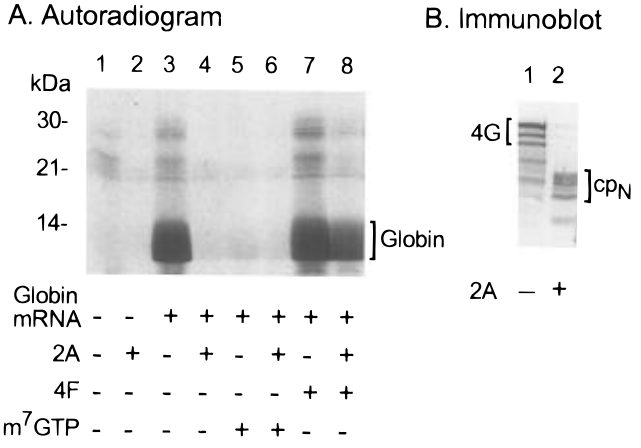

FIGURE 3: Treatment of RRL with 2A protease reduces globin mRNA translation. RRL was treated with HRV2 protease 2A and the resulting translation system programmed with globin mRNA as described under Materials and Methods. (A) Aliquots of translation reactions were subjected to SDS−PAGE on a 15% gel, and incorporation of [^{35}S]Met into globin was analyzed by autoradiography. Reactions were run with either mock (−) or 2A protease-treated (+) RRL either in the absence (−) or in the presence (+) of globin mRNA (10 μg/mL), eIF4F (3 μg/mL), or m^{7}GTP (1 mM). The positions of globin and protein standards are indicated. (B) Mock (−) and 2A protease-treated (+) RRL were subjected to SDS−PAGE on a 6% gel followed by immunoblotting with anti-eIF4G$^{327-342}$ antibodies. The positions of eIF4G and cp$_N$ are indicated.

of the latter by 0.01 μg of protease over the same time period (panel A). The resistance of the eIF4G^{G486E} variant to cleavage by HRV2 protease 2A was less dramatic (10−50-fold), since the extent of cleavage of eIF4G^{G486E} by 1.0 μg of protease was intermediate between that achieved with 0.01 and 0.1 μg with eIF4G^{wt} (panel B). This difference between the two proteases on eIF4G substrates was observed with all eIF4G variants tested and may reflect either a greater tendency for the HRV2 protease to cleave at a nearby secondary site or else its greater reliance upon additional sequence determinants (other than P1′) for substrate recognition.

In order to correlate eIF4G cleavage with its participation in protein synthesis, it was necessary to develop an assay for eIF4G activity. Addition of HRV2 protease 2A to a HeLa cell-derived *in vitro* translation system results in the cleavage of endogenous eIF4G and a corresponding inhibition of cap-dependent translation (Liebig et al., 1993). We adapted this to the more efficient RRL translation system (Figure 3). Pretreatment of RRL with HRV2 protease 2A converted the endogenous eIF4G from its characteristic heterogeneous cluster of bands which migrate between 206 and 220 kDa to a series of immunoreactive amino-terminal fragments (cp$_N$; panel B). Globin was efficiently synthesized in the mock-treated MDL (panel A, lane 3) but not in the 2A-treated lysate (lane 4). This inhibition was similar to that observed in the presence of the mRNA cap analogue m^{7}GTP (lane 5), indicating that the system is highly cap-dependent. The inhibition by 2A protease was not due to a general effect on the translational apparatus since translation from an mRNA containing an EMCV IRES was not affected (data not shown). The translation from globin mRNA was restored by the addition of eIF4F purified from RRL (lane 8 *vs* lane 4). Similar results were also observed with CVB4 protease 2A (data not shown). This *in vitro* system is therefore sensitive to the presence of intact eIF4G for cap-dependent translation.

All studies to date have utilized complexes of eIF4 factors (sometimes with additional proteins as well) to restore cap-dependent translation after picornaviral protease action. To determine if eIF4G alone, especially the *in vitro*-synthesized form of it, is capable of restoring cap-dependent translation to a 2A protease-treated MDL system, we developed a two-phase translation assay. In the first phase, translation of eIF4G mRNA was initiated, and then the 2A protease was added to deplete endogenous eIF4G. Then the protease was inhibited prior to completion of eIF4G synthesis. (This was necessary since initiation of translation from the capped eIF4G transcript requires intact eIF4G protein, but the continued presence of active protease would cleave the nascent eIF4G chains as soon as the substrate-recognition sequence was synthesized.) Under these conditions, the majority of the exogenous eIF4G was synthesized after the endogenous eIF4G was depleted. The second phase of synthesis measures the cap-dependent translation potential and was performed by supplementing the system with a 2A protease-treated RRL (which contained endogenous globin mRNA and cleaved, endogenous eIF4G) and [^{35}S]Met. The incorporation of radioactivity into globin then measures cap-dependent synthesis *after* the depletion of endogenous eIF4G and its replacement by exogenous eIF4G.

As shown in Figure 4, treatment with CVB4 protease 2A during the first phase depleted the lysate of endogenous eIF4G (panel A; *cf*. the slowest migrating eIF4G bands in lane 1 *vs* lane 2 and lane 3 *vs* lane 4) and inhibited efficient initiation of globin synthesis during the second phase in reactions containing no eIF4G^{wt} mRNA (panel C, lane 1 *vs* lane 2). *In vitro* synthesis of eIF4G^{wt} caused a slight stimulation of globin synthesis in reactions not treated with protease 2A (panels B and C, lane 3 *vs* lane 1) but a substantial increase in reactions treated with protease 2A (panels B and C, lane 4 *vs* lane 2). These results suggest that *in vitro*-synthesized eIF4G is active in supporting globin synthesis.

To determine if the eIF4G^{G486E} variant was also active in restoring translation, the two-phase assay system was programmed with mRNA encoding either eIF4G^{wt} or eIF4G^{G486E} (Figure 5A). In both cases, a strong band of intact, immunoreactive eIF4G was observed with only a minimal amount of the slower migrating forms characteristic of endogenous eIF4G (panel A, inset). The variant was indeed active, and, in fact, slightly more so than eIF4G^{wt} (lane 3 *vs* lane 2), possibly because eIF4G^{G486E} is resistant to cleavage by any CVB4 protease 2A that fails to be completely inhibited by elastatinal.

With the knowledge that both exogenous eIF4G^{wt} and eIF4G^{G486E} were active in restoring cap-dependent initiation to 2A protease-treated MDL (Figure 4 and Figure 5, panel A), it was possible to test for protection of the translational system against protease action. The assay protocol was modified slightly by performing the first phase (eIF4G synthesis) in the absence of elastatinal. In this situation, the 2A protease was able to act on both endogenous and exogenous eIF4G. The results demonstrated that both endogenous eIF4G and *in vitro*-synthesized eIF4G^{wt} were depleted by 2A (Figure 5, panel B, inset, lanes 4 and 5) but eIF4G^{G486} was resistant to digestion (lane 6). The level of globin synthesis showed that eIF4G^{wt} mRNA afforded little protection of cap-dependent translation against the protease (panel B, bar graphs, lane 5 *vs* lane 4) whereas eIF4G^{G486E} mRNA allowed cap-dependent translation to continue (lane 6). Repeating this experiment with a higher level of CVB4 protease 2A caused further cleavage of all eIF4G forms but a clear protection of globin synthesis by eIF4G^{G486E} (panel C). This indicates that a single amino acid change in eIF4G protects cap-dependent translation against CVB4 protease 2A.

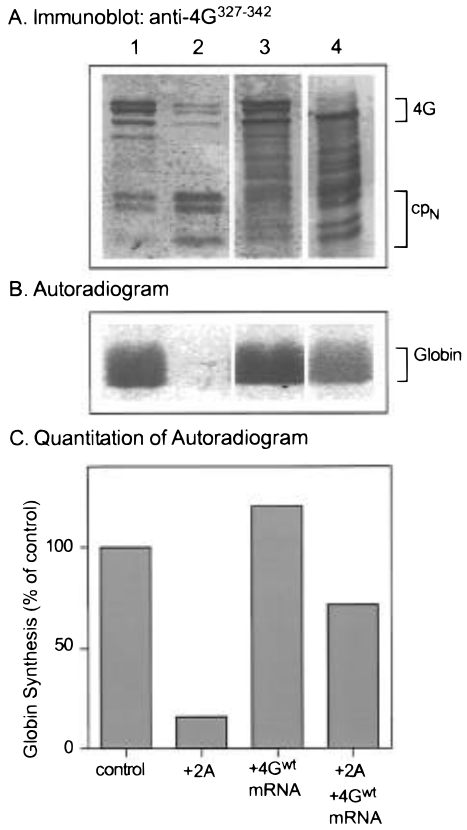

FIGURE 4: Restoration of cap-dependent translation to a 2A protease-treated rabbit reticulocyte lysate by *in vitro* synthesis of eIF4G^{wt}. Two-phase MDL reactions were performed as described under Materials and Methods. Reactions were initiated in either the absence or the presence of mRNA encoding eIF4G^{wt}. After 50 min, globin synthesis was initiated as described under Materials and Methods. Aliquots were removed after 80 min and subjected to SDS−PAGE on 7.5% (panel A) and 15% (panel B) gels. (A) Immunoblot probed with anti-eIF4G$^{327-342}$ antibodies. (B) Autoradiogram of the gel showing globin translation product. (C) Globin synthesis quantitated from autoradiogram. The positions of intact endogenous eIF4G (bracket labeled "4G") and exogenous eIF4G (fastest band within bracket) as well as cleavage products (cp_N) and globin are indicated.

DISCUSSION

Before it was possible to test for a cause-and-effect relationship between protease 2A-mediated cleavage of eIF4G and the inhibition of cap-dependent translation, it was necessary to demonstrate that *in vitro*-synthesized eIF4G was active in protein synthesis. There is a difference in mobility of *in vitro*- and *in vivo*-synthesized eIF4G, with the latter exhibiting a heterogeneous pattern and slower mobility (Joshi et al., 1994). The predicted molecular mass of eIF4G from the cDNA sequence is 154 kDa (Yan et al., 1992), but the natural protein migrates on SDS−PAGE as a cluster of four bands from 206 to 220 kDa, while the *in vitro*-synthesized protein migrates as a single band of 205 kDa (Joshi et al., 1994). The reasons for this heterogeneity and aberrant mobility are not known, but it does not appear to be due to multiple genes (Yan & Rhoads, 1995) and is more likely due to posttranslational modification. It was not known prior

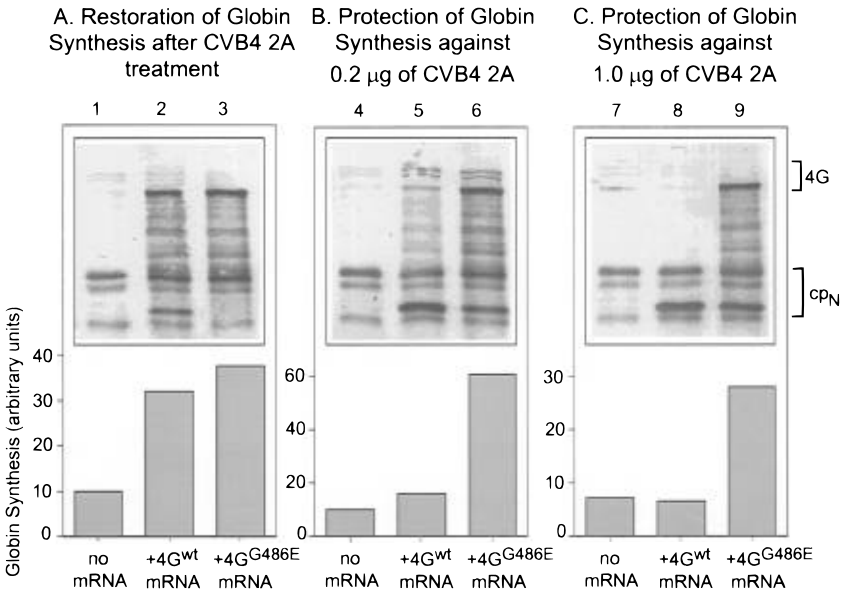

FIGURE 5: Protection of cap-dependent translation from CVB4 2A protease by synthesis of eIF4G^{G486E} *in vitro*. Two-phase MDL translation reactions were performed as described under Materials and Methods. Reactions were initiated in the presence of either no mRNA or mRNA encoding eIF4G^{wt} or eIF4G^{G486E}. For testing restoration of globin synthesis (panel A), CVB4 protease 2A was added after 5 min to cleave endogenous eIF4G, and after 13 min, elastatinal was added to stop 2A protease activity. For testing protection of globin synthesis (panels B and C), the indicated amount of CVB4 protease 2A was added after 35 min to cleave both endogenous and synthesized eIF4G. Globin synthesis was initiated after 50 min as described under Materials and Methods. After 75 min, aliquots were removed and subjected to SDS−PAGE on 7.5% gels for immunodection of eIF4G and cleavage products (insets) or on 15% gels followed by autoradiography for quantitation of globin synthesis (bar graphs).

to the present study whether eIF4G lacking this putative posttranslational modification was active in protein synthesis. Previous studies had established that *in vitro*-synthesized eIF4G is capable of participating in two partial reactions of protein synthesis initiation, binding to eIF4E and binding to the 43S initiation complex (Joshi et al., 1994). The results of Figures 4 and 5, however, indicate that globin synthesis, which presents the culmination of all of the individual steps of protein synthesis, is fully restored by *in vitro*-synthesized eIF4G in a translation system depleted of endogenous eIF4G. Thus, the putative posttranslational modifications are apparently not important for full eIF4G function in protein synthesis.

The finding that both globin synthesis and loss of the 205-kDa band of eIF4G are resistant to protease 2A when the eIF4G^{G486E} variant is employed (Figure 5) indicates a direct effect. Though the protease may have other targets in the whole-cell lysate, such targets would not seem to be necessary for the translational shut-off. An important consideration in making such a statement, however, is whether the protease has been inactivated, *e.g.*, by being sequestered in a complex with eIF4G^{G486E}. Proof that this has not occurred is the finding that endogenous eIF4G (indicated by the immunoreactive material migrating slower than 205 kDa) is cleaved with the same kinetics in the presence or absence of eIF4G^{G486E} (Figure 1). Thus, the enzyme is active and would be able to cleave any other hypothetical targets.

The results presented provide additional evidence for a direct *vs* indirect cleavage of eIF4G by protease 2A. There is a considerable body of evidence on both sides of this question (see the Introduction). It is important to note that the cleavage sites which were mapped for 2A and Lb proteases were for purified eIF4F, treated *in vitro* with purified, recombinant proteases (Lamphear et al., 1993; Kirchweger et al., 1994). The sites for eIF4G cleavage in a whole cell extract treated with 2A or Lb proteases have not been determined, primarily because proteolysis of eIF4G separates cp_N, which binds eIF4E and is needed for retention of eIF4F on m^7GTP−Sepharose, from cp_C, which would be needed to perform Edman degradation. Hence, cp_C has not yet been purified from whole cell extracts nor sequenced. It is possible that the viral protease activates a cellular protease which cleaves eIF4G. The results of the present study show, however, that if such a cellular protease were responsible for the loss of cap-dependent translation, it is inhibited by the G486E substitution, suggesting that it cleaves eIF4G at the identical site (Arg^{485}−Gly^{486}) as the viral 2A protease.

The finding that Glu substitution for Gly at position 486 retards protease 2A action is consistent with previous findings with model peptide substrates based on the VP1−2A junction of a variety of entero- and rhinovirus serotypes. Despite considerable variations among the genera, a Gly residue is always present at position P1′. HRV2 protease 2A cleaves 16 amino acid peptides spanning the VP1−2A junction sequence of 7 rhinovirus and poliovirus serotypes with varying efficiencies, but peptides based on serotypes which do not contain Thr at position P2 or Gly at position P1′ are not cleaved (Sommergruber et al., 1992). The G486E substitution occurs at position P1′ and hence would be predicted to prevent cleavage. In another study, 16 amino acid peptides were made with substitutions at each position of the native VP1−2A junction sequences of HRV2 and CVB4, with the result that acidic residues in the P3−P8 region reduced cleavage (Sommergruber et al., 1994). The deleterious effect of a negative charge in the general region of cleavage may explain our findings that Glu was more effective than Arg, Trp, Val, or Ala at position 486.

It is not clear, however, why eIF4G^{G486E} is cleaved at all. In the 76 synthetic peptides tested with HRV2 or CVB4 proteases 2A (Sommergruber et al., 1992, 1994), Gly at position P1′ was always required for cleavage. Yet we observed cleavage of eIF4G^{G486E} at high protease concentrations and long incubation times (Figures 1 and 2). This

suggests that cleavage may be occurring at a site other than $Arg^{485}-Gly^{486}$. Interestingly, the Lb protease of FMDV, which represents a papain-like protease as opposed to the chymotrypsin-like 2A proteases, cleaves eIF4G at a different site, seven amino acid residues closer to the N-terminus ($Gly^{479}-Arg^{480}$; Kirchweger et al., 1994). Thus, two unrelated proteases, with completely different substrate specificities, cleave eIF4G in the same region. This and other considerations have led to the hypothesis that cp_N and cp_C are separated by a flexible hinge region which may be more susceptible to protease action than other parts of this molecule (Lamphear et al., 1995). The 2A proteases may be cleaving eIF4G at another site within this putative hinge rather than at $Arg^{485}-Gly^{486}$. Thus, it may be possible to create an even more resistant eIF4G molecule by determining the site and performing additional site-directed mutagenesis.

The ability to produce a resistant form of eIF4G should prove useful in *in vivo* studies. Picornaviruses cause a variety of cellular changes in addition to cleavage of eIF4G, *e.g.*, in membrane permeability (Carrasco et al., 1989), transcription (Davies et al., 1991), and the cytoskeleton (Joachims & Etchison, 1992). Since eIF4G is presumably involved in the synthesis of all cellular proteins, any of these alterations could occur secondary to the loss of key proteins and hence be downstream of eIF4G cleavage. Alternatively, they may be independent events, caused by other actions of 2A proteases, by 3C proteases, or by other picornavirus proteins. *In vivo* studies with $eIF4G^{G486E}$ or similar molecules should allow one to distinguish between these alternatives.

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