Mapping of Functional Domains in Eukaryotic Protein Synthesis Initiation Factor 4G (eIF4G) with Picornaviral Proteases

IMPLICATIONS FOR CAP-DEPENDENT AND CAP-INDEPENDENT TRANSLATIONAL INITIATION*

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Cap-dependent binding of mRNA to the 40 S ribosomal subunit during translational initiation requires the association of eukaryotic initiation factor 4G (eIF4G; formerly elf-4γ and p220) with other initiation factors, notably elf4E, elf4A, and elf3. Infection of cells by picornaviruses results in proteolytic cleavage of elf4G and generation of a cap-independent translational state. Rhinovirus 2A protease and foot-and-mouth-disease virus L protease were used to analyze the association of elf4G with elf4A, elf4E, and elf3. Both proteases bisection elf4G into N- and C-terminal fragments termed cpN and cpC. cpN was shown to contain the elf4E-binding site, as judged by retention on m7GTP-Sepharose, whereas cpC was bound to elf3 and elf4A, based on ultracentrifugal co-sedimentation. Further proteolysis of cpN by L protease produced an 18-kDa polypeptide termed cpN2, which retained elf4E binding activity and corresponded to amino acid residues 319–479 of rabbit elf4G. Further proteolysis of cpC yielded several smaller fragments. cpC2 (~887–1402) contained the elf4A binding site, whereas cpC3 (~480–886) contained the elf3 binding site. These results suggest that cleavage by picornaviral proteases at residues 479–486 separates elf4G into two domains, one required for recruiting capped mRNAs and one for attaching mRNA to the ribosome and directing helicase activity. Only the latter would appear to be necessary for internal initiation of picornaviral RNAs.

Translation of eukaryotic cellular mRNA into protein is a complex process involving nearly 200 RNA and protein components interacting in a regulated fashion to ensure timely expression of genetic information (1). Viruses often alter the host cell translational machinery to allow more efficient expression of virally encoded proteins. One of the most dramatic examples of this occurs upon picornaviral infection. The picornaviridae are small,icosahedral, positive-stranded RNA viruses of considerable clinical and veterinary importance, containing such members as poliovirus, rhinovirus, coxsackievirus, encephalomyocarditis virus, and foot-and-mouth-disease virus (FMDV)1.

* This work was supported by Research Grant GM20818 from the National Institute of General Medical Sciences, National Institutes of Health (to R. E. R.) and a grant from the Austrian Science Foundation (to T. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: FMDV, foot-and-mouth disease virus; elf, eukaryotic initiation factor; HPLC, high pressure liquid chromatography; HRV2, human rhinovirus serotype 2; MOPS, 3-N-morpholino-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

‡ The names of initiation, elongation, and termination factors were revised on April 8, 1995 by an expert panel, Dr. Marianne Grünberg-Manago, Convener, appointed by the IUBMB Nomenclature Committee. The new names are used in the present article. elf4G was formerly referred to as either p220, elf-4γ, or elf-4Gγ.
recovered from infected cells is distinctly different from that of uninfected cells, suggesting that infection disrupts macromolecular complexes important for cap-dependent initiation (28, 29). Since no alteration in other eIF3 or eIF4 polypeptides is apparent as a result of infection (6, 30, 31), cleavage of eIF4G is thought to be responsible for the observed changes. This is supported by the fact that protein complexes containing intact eIF4G restore cap-dependent translation in extracts of infected cells (23, 32, 33). Also, extracts of uninfected cells, when treated with purified viral proteases, lose their ability to translate capped mRNA, but addition of eIF4G-containing complexes restores this activity (8, 16).

Not only does cleavage of eIF4G result in inactivation of some functions important for cap recognition, but there is also evidence that eIF4G may be important for cap-independent translation of viral RNAs. Addition of the eIF4F complex stimulates in vitro translation from picornaviral internal ribosome entry site sequences (34–36). In contrast to cap-dependent initiation, cleavage of eIF4G by picornaviral proteases does not abrogate this stimulation but rather enhances it (116, 37, 38). This suggests that eIF4G cleavage products play a direct role in cap-independent translation of viral RNAs (16, 37).

Picornaviral proteases which cleave eIF4G fall into two separate classes. The 2A proteases of rhino- and enteroviruses are small thiol proteases with structural similarities to chymotrypsin and thermolysin (39–41). The primary cleavage site in rat eIF4G is Arg446, Gly487 (15), although a secondary site of unknown location has been suggested for the 2A protease of HRV2 (12). The L protease of FMDV more closely resembles papain in structure (42). It initially cleaves eIF4G at Gly479, Arg580 and subsequently at multiple sites as yet unidentified (12).

To understand better the role of eIF4G in cap-dependent and -independent initiation, we have examined the effect of proteolysis on the association of eIF4G with other initiation factors using recombinant viral proteases. We provide evidence that proteolysis of eIF4G at the primary site separates functional domains. We also report the localization of a second L protease cleavage site in eIF4G which further defines the binding region for eIF4E. These results suggest a model for the role of eIF4G cleavage products in translation of picornaviral RNA.

**EXPERIMENTAL PROCEDURES**

*Materials*—The C4 column for reverse phase separation of eIF4G cleavage products was obtained from Vydac (Hesperia, CA). HPLC-grade acetonitrile was obtained from Fisher. mGTP-Sepharose was purchased from Pharmacia Biotech Inc. mGTP and antipain were obtained from Sigma. Peptide 9, consisting of the sequence KEFKEDKDDDVVFEC, was synthesized by Bio-Synthesis, Inc. (Dallas, TX). This sequence corresponds to amino acid residues 653–666 of human eIF4G (43) except that residue 664 was changed from Gln to Val to enhance the yield of peptide synthesis, and the C-terminal Cys was added to aid in coupling to a solid support. HPLC-grade trifluoroacetic acid, formic acid, N,N-dimethylformamide, and bovine serum albumin, and Freund’s complete and incomplete adjuvant were obtained from Pierce. An Affi-Gel HZ immunoaffinity kit was purchased from Bio-Rad. Elastatinal was obtained from Calbiochem. Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Vector Laboratories (Burlingame, CA). All other chemicals were of reagent grade.

*Antisera*—The production of rabbit anti-eIF4G1200–1248 (raised against a synthetic peptide corresponding to amino acid residues 1230–1248 of human eIF4G), anti-eIF4G77–81, and anti-eIF4G500–516 antisera was described elsewhere (43). A mouse monoclonal antibody against eIF4A was a generous gift from Dr. Hans Trachsel, Bern, Switzerland. A goat anti-eIF3 antibody was kindly donated by Dr. William Merrick, Case Western Reserve University. Peptide 9 was coupled to maleimide-activated keyhole limpet hemocyanin using the protocol supplied by the manufacturer and used to raise anti-eIF4G653–666 antibodies in rabbits (500 µg immunization; injections bimonthly for 3 months).

**Purification of Proteins**—Rabbit reticulocyte lysate was prepared as described (44). Preparation of ribosomal salt wash and high salt postribosomal supernatant from rabbit reticulocytes, and purification of eIF4F and eIF2eIF4F complexes from ribosomal salt wash, were as described previously (25, 45). Recombinant HRV2 2A and FMDV L proteases were expressed in bacteria and purified as described previously (12, 16).

**Reverse Phase Column Fractionation of eIF4G Cleavage Products**—For the generation of cp100 for N-terminal sequence analysis, high salt postribosomal supernatant (70 ml) from rabbit reticulocytes, dialyzed against buffer A100 (20 mM MOPS, pH 7.6, 10% glycerol, 0.1 mM EDTA, 0.25 mM dithiothreitol, 100 mM KCl) was incubated with L protease (0.5 µg/ml) for 15 min at 30 °C and then applied to a 2-ml mGTP-Sepharose column equilibrated in buffer A100 at 4 °C. The column was washed with 20 ml of buffer A100 and 10 ml of buffer A100 containing 100 µM GTP. Bound material was eluted in buffer A100 containing 70 µm mGTP. The eluate (3 ml) was further incubated with L protease (3 µg/ml) at 30 °C for 20 min to complete the conversion of cp100 to cp90 and cp72. The reaction mixture was cooled on ice, brought to 0.1% in trisfluoroacetic acid, and fractionated by HPLC using a C4 column (0.45 × 15 cm) equilibrated in buffer B (0.1% trisfluoroacetic acid). The column was developed at 1 ml/min with 5 µl of buffer B followed by a 1-ml step to 35% buffer C (0.1% trisfluoroacetic acid in 95% acetonitrile) and a linear 39-ml gradient to 60% buffer C. Fractions of 1 ml were collected throughout and those shown by SDS-PAGE and immunoblotting to contain cp90 and cp72 were pooled and reapplied to the C4 column. The column was developed with 5 µl of buffer B followed by a 1-ml step to 30% buffer C and a linear 40-ml gradient to 45% buffer C. Fractions of 0.5 ml were collected and those containing intact cp100 were removed at various times and subjected to SDS-PAGE and immunoblotting.

**Generation of eIF4G Cleavage Products and mGTP-Sepharose Column Fractionation**—For analysis of the time course of digestion, eIF4F (30 µg/ml), either in the presence or absence of eIF3 (70 µg/ml), was incubated at 30 °C with L protease (12 µg/ml) in buffer A100 (20 mM MOPS, pH 7.6, 10% glycerol, 0.1 mM EDTA, 0.25 mM dithiothreitol, 100 mM KCl) at a concentration of 140 mM. Aliquots (30 µl) were removed at various times and subjected to SDS-PAGE and immunoblotting for analysis of the eIF4G-eIF4E interaction by mGTP-Sepharose chromatography. eIF4F (27 µg/ml) was incubated in buffer A100 in the presence of either 2A (8 µg/ml) or L protease (12 µg/ml) at 30 °C for 60 min. The reactions were stopped with elastatinal (500 µM) or antipain (1 µg/ml), respectively, and placed on ice. Samples were then loaded onto a 100-µl mGTP-Sepharose column equilibrated in buffer A100, the column washed with 300 µl of buffer A100 and the bound material eluted with buffer A100 containing 70 µm mGTP.

**Ultracentrifugal Sedimentation**—Reactions (30 µl) containing the eIF3-eIF4F complex (560 µg/ml) were incubated at 30 °C in buffer A100 in the presence or absence of either 2A protease (8.3 µg/ml) or L protease (12 µg/ml) at 30 °C for 30 min. The bound material was eluted with either buffer A100 or buffer A100 containing 100 µM GTP (either 50 or 5 min, respectively). Reactions were stopped with protease inhibitors as described above, placed on ice, layered onto 4.4-ml 15–30% sucrose gradients prepared in buffer A100 and centrifuged in an SW60.1 rotor at 45,000 rpm for 16 h.

**Immunologic Procedures**—For immunoblotting, unlabeled proteins were transferred to polyvinylidene difluoride membranes in a BioRad Mini Trans-Blot cell using the manufacturer’s recommendations. Detection of immunoreactive species was performed as described previously (43) except incubations were performed at 22 °C. Anti-eIF4G500–516 antisera was used at a 1:1000 dilution. Anti-eIF4G1200–1248 resin was prepared using the Affi-Gel HZ immunoaffinity kit. The antibody was purified on protein A-Sepharose and eluted with a 1–ml step to 0.1 M Tris (pH 8.0). The resin was washed with 1 ml of buffer A100 and 0.5 ml of buffer A100 containing 0.1% acetonitrile and 0.1 M Tris (pH 8.0). The resin was then treated with purified viral proteases, losing their ability to translate capped mRNA, but addition of eIF4G-containing complexes restores this activity (8, 16).
Fig. 1. Time course of cleavage of eIF4G by L protease of FMDV. eIF4F was digested with recombinant L protease in the absence (lanes 1–7) or presence (lanes 8–14) of eIF3 as described under “Experimental Procedures.” Aliquots were removed at the indicated times and subjected to SDS-PAGE on 10% gels. A, silver-stained gel. B, immunoblot probed with anti-eIF4G antibodies. C, immunoblot probed with anti-eIF4G653–666 antibodies. The positions of standard proteins of the designated molecular masses (×10⁻³) are indicated on the left. The positions of uncleaved eIF4G, the various cleavage products, eIF4A, and eIF4E are indicated on the right.

RESULTS

Cleavage of eIF4G by L Protease—A previous study indicated that, in addition to the primary cleavage of rabbit eIF4G by L protease at Gly⁴⁷⁰–Arg⁴⁸⁰, there were cleavages at several other sites (12). To characterize these secondary cleavages further, we combined purified recombinant L protease with rabbit reticulocyte eIF4F and performed a time course of digestion (Fig. 1). Proteolytic fragments were visualized by staining with silver (A) or immunoblotting with antibodies against different regions of eIF4G (B and C). Antibodies against peptides corresponding to amino acid residues 327–342, 403–416, and 1230–1248 were developed in an earlier study (43). However, the central portion of the eIF4G sequence was not represented by these antibody reagents. We therefore developed an additional antibody against residues 653–666. As observed previously (12), L protease initially makes a single cleavage, separating the N-terminal one-third of eIF4G (here designated cpN) from the C-terminal two-thirds (designated cpC; see Fig. 2). eIF4G migrates on SDS-PAGE as a series of heterogeneous bands with aberrantly slow mobility (Fig. 1, lane 1); the slowest species migrates at 220 kDa (6, 25, 48), in contrast to the 154 kDa calculated from the amino acid sequence (43). The cause of the heterogeneity and aberrant mobility is not known, but the region of eIF4G responsible for this behavior appears to be located in cpC (12, 15). Like intact eIF4G, cpN migrated abnormally slowly as a heterogeneous cluster of bands, with an apparent molecular mass of 100–130 kDa, despite its true size of 51 kDa (A and B, lanes 2–4). Further digestion with L protease caused the cpN fragments to disappear and a heterogeneous cluster of fragments, designated cpN₁, to appear (A, lanes 4–7). All members of the cpN₁ cluster arose simultaneously and therefore are likely to have resulted from cleavage of cpN at a single site. The heterogeneity of cpN₁ is similar to that of cpN and eIF4G itself, suggesting that the source of heterogeneity in eIF4G is localized to the cpN₁ region. Immunoblotting with an anti-eIF4G327–342 antibody detected a band with estimated molecular mass of 28 kDa, designated cpN₂, which appeared simultaneously with the degradation of cpN₁ (B, lanes 2–4). With further digestion, some of cpN₂ was converted to smaller species termed cpN₃ and cpN₄, which contained amino acid residues 327–416, whereas cpN₁ did not. This permitted the orientation of cpN₁, cpN₂, and cpN₃ as shown in Fig. 2.

Further cleavage of the 103-kDa cpC yielded a number of smaller fragments (cpC₁—cpC₅). The relative abundance and kinetics of appearance of the fragments together with immunoblotting with anti-eIF4G653–666 (Fig. 1C) and anti-eIF4G1230–1248 (12) antibodies is consistent with the following order of events. Initial cleavage of cpC by L protease can occur at one of two nearby sites (see Fig. 2). This yields two overlapping polypeptides, cpC₁ (59 kDa) and cpC₂ (55 kDa), both of which can be seen in A, lanes 4–7, and both of which react with anti-eIF4G1230–1248 antibodies (12). Secondary cleavage of cpC₁ with other proteases at Gly⁴⁷⁰–Arg⁴⁸⁰ results in the generation of several additional smaller species (cpC₁₋₅). These species are subject to fragmentation and are detectable by immunoblotting with anti-eIF4G antibodies. The eIF4F was digested with recombinant L protease in the absence (lanes 1–7) or presence (lanes 8–14) of eIF3 as described under “Experimental Procedures.” Aliquots were removed at the indicated times and subjected to SDS-PAGE on 10% gels. A, silver-stained gel. B, immunoblot probed with anti-eIF4G antibodies. C, immunoblot probed with anti-eIF4G653–666 antibodies. The positions of standard proteins of the designated molecular masses (×10⁻³) are indicated on the left. The positions of uncleaved eIF4G, the various cleavage products, eIF4A, and eIF4E are indicated on the right.

Fig. 2. Summary of viral protease cleavage of eIF4G. Long rectangles indicate the 1402-amino acid residue rabbit eIF4G molecule or various cleavage products (proportional in length to the molecular masses of the fragments). Precise cleavage sites for 2A and L proteases in rabbit eIF4G are indicated by vertical lines through amino acid sequences. Approximate cleavage sites are indicated by lines through the rectangles. The locations in eIF4G of synthetic peptides used to generate antibodies are shown at the top with inclusive amino acid residue numbers (referring to the human sequence).
generates cp\textsubscript{C3} (note gradual loss of cp\textsubscript{C1} and appearance of cp\textsubscript{C2} in Fig. 1A). When cp\textsubscript{C} is initially cleaved to cp\textsubscript{C1}, the N-terminal half is represented by cp\textsubscript{C4} (40 kDa), which reacts with anti-eIF4G\textsubscript{653-666} antibodies (C, lanes 3–7, and Fig. 2). When cp\textsubscript{C} is initially cleaved to cp\textsubscript{C2}, the N-terminal half is represented by cp\textsubscript{C3} (45 kDa), which also reacts with anti-eIF4G\textsubscript{653-666} antibodies (C, lanes 3–7). With longer times of incubation, cp\textsubscript{C3} and cp\textsubscript{C4} disappear simultaneously with the appearance of cp\textsubscript{C5} (35 kDa; C, lanes 4–7). Neither cp\textsubscript{C6}, cp\textsubscript{C7}, nor cp\textsubscript{C8} were recognized by anti-eIF4G\textsubscript{1230-1248} (data not shown). These results indicate that cp\textsubscript{C3} and cp\textsubscript{C2} are derived from the C-terminal portion of cp\textsubscript{C} and that cp\textsubscript{C3}, cp\textsubscript{C4}, and cp\textsubscript{C5} are derived from the N terminus (Fig. 2). Based on the SDS-PAGE-derived molecular masses, cp\textsubscript{C3} and cp\textsubscript{C2} approximately correspond to amino acid residues 480–886 and 887–1402 of rabbit eIF4G, respectively.

eIF3 is a multisubunit initiation factor that associates with eIF4F (29). Previously we showed that eIF3 does not affect cleavage of eIF4F by 2A proteases from HRV2 and coxsackievirus serotype B4 (15). However, Wyckoff et al. (49, 50) have presented evidence that eIF3 is required for cleavage of eIF4G by poliovirus 2A protease. To determine if eIF3 affected either the rate or sites of cleavage of eIF4G by FMDV L protease, we repeated the time course in the presence of this factor at a concentration equivalent to that of eIF4F (Fig. 1, lanes 8–14). No cleavage of eIF4E or the various subunits of eIF3 was observed. The rate of appearance of the eIF4G cleavage products was unchanged except for a slight heterogeneity of the cp\textsubscript{C4} band; eIF3 caused a reduction in cp\textsubscript{C4} and the appearance of a new band ~1 kDa smaller in size (cf. lanes 4–7 with lanes 11–14 in C). This suggests that eIF3 may block the accessibility of a protease site or expose a new one in this region of eIF4G.

Fig. 3. m\textsuperscript{7}GTP-Sepharose column fractionation of eIF4F treated with HRV2 protease 2A. eIF4F was incubated with 2A protease and fractionated on m\textsuperscript{7}GTP-Sepharose as described under "Experimental Procedures." Aliquots of fractions were subjected to SDS-PAGE on an 8.5% gel. A, Coomassie Blue-stained gel. B, immunoblot probed with anti-eIF4G\textsubscript{327-342} antibodies. C, immunoblot probed with anti-eIF4A. Lanes U and S represent eIF4F samples either untreated or treated with protease 2A, respectively, but not subjected to m\textsuperscript{7}GTP-Sepharose chromatography. FT, flow-through (unbound) fractions. m\textsuperscript{7}GTP, fractions eluted with m\textsuperscript{7}GTP.

Fig. 4. m\textsuperscript{7}GTP-Sepharose column fractionation of eIF4F treated with FMDV protease L. eIF4F was incubated with protease and fractionated on m\textsuperscript{7}GTP-Sepharose as in Fig. 3 except that L protease was used instead of 2A protease (see "Experimental Procedures"). A, silver-stained gel. B, immunoblot of the same gel probed with anti-eIF4G\textsubscript{1230-1248} antibodies. C, immunoblot of the same gel probed with anti-eIF4G\textsubscript{327-342} antibodies.

The N-terminal Region of eIF4G Contains the eIF4E-binding Site—As noted above, the eIF4F complex consists of eIF4A, eIF4E, and eIF4G. To determine which portions of eIF4F bind to eIF4E, we digested purified rabbit eIF4G with HRV2 protease 2A and subjected the reaction products to chromatography on m\textsuperscript{7}GTP-Sepharose (Fig. 3). Analysis of column fractions by SDS-PAGE followed by either Coomassie Blue staining (A) or immunoblotting with an anti-eIF4G\textsubscript{327-342} antibody (B) or a monoclonal anti-eIF4A antibody (C) indicated that neither cp\textsubscript{C} nor eIF4A were retained on the column, but rather were found in the flow-through (FT) fractions (A and C, lanes 1–3). However, the cp\textsubscript{C} bands were retained on the column and co-eluted with eIF4E (A and B, lanes 6 and 7). This suggests that the cp\textsubscript{N} region of the protein contains the eIF4E-binding site.

A similar analysis of eIF4F treated with L protease was performed (Fig. 4). In this case, proteins in column fractions were resolved by SDS-PAGE and visualized by either silver staining (A) or immunoblotting with an anti-eIF4G\textsubscript{1230-1248} (B) or anti-eIF4G\textsubscript{327-342} antibodies (C). (The fact that cp\textsubscript{N} stains much more strongly than cp\textsubscript{C} with silver has been documented previously (15), but the reason is not known; it may be due to groups of charged amino acid residues observed in the cp\textsubscript{N} portion of eIF4G (43) or posttranslational modifications which introduce hydrophylic or charged groups.) cp\textsubscript{C}, fragments of cp\textsubscript{C}, and eIF4A were not retained on the column but were found in the flow-through fractions (A and B, lanes 1–3). cp\textsubscript{N} was also detected in the flow-through (A, lanes 1–3). Most of the cp\textsubscript{N}1, however, was retained on the column and co-eluted with eIF4E (A and C, lanes 6–8), indicating that the eIF4E-binding site is contained within this region of eIF4G. The fact that some of the cp\textsubscript{B} and cp\textsubscript{N2} are found in the flow-through as well (lanes 1–3) may indicate dissociation from eIF4E during chromatography.

In order to define more precisely the region of eIF4G that binds to eIF4E, we determined the L protease cleavage site at the cp\textsubscript{N1}/cp\textsubscript{N2} junction. Fragments of eIF4G produced by a brief...
digestion with L protease were subjected to reverse phase HPLC on a C4 column (Fig. 5). The peaks in fractions 40–50 contained cpN2 and cpN3 and were well resolved from the peak in fractions 51–56, which contained cpN1. Although cpN2 and cpN3 eluted in the same general region, it was possible to resolve the majority of the cpN2 (fractions 44–47) from the cpN3 (fractions 40–43). cpN2 was subjected to N-terminal sequence analysis by automated Edman degradation (Table I). Alignment of the resultant sequence with the rabbit polypeptide sequence determined by cDNA cloning indicated that L protease cleaves at Lys319, which corresponds to Lys318.

C-terminal Region of eIF4G Contains the eIF4A- and eIF3-binding Sites—In intact eIF4F, the eIF4A subunit is retained on m7GTP-Sepharose even though only eIF4E has affinity for m7GTP (23–25, 48). This has been interpreted to mean that both eIF4E and eIF4A have affinity for eIF4G, although another study has indicated that eIF4E and eIF4A can bind to each other directly (51). The results in Figs. 3 and 4 demonstrate that eIF4A is no longer retained on m7GTP-Sepharose after treatment with 2A or L protease. This could mean either that eIF4A has been released as a free protein by proteolytic digestion of eIF4G or that it is bound to a portion of eIF4G which is not retained on m7GTP-Sepharose. To distinguish between these possibilities, we took advantage of the fact that eIF3, an eight-subunit initiation factor with molecular mass of over 500 kDa (20), can be isolated in a complex with eIF4F and purified by m7GTP-Sepharose chromatography (45). Proteolytic fragments of eIF4G that remain associated with eIF3 should co-sediment with this factor in sucrose gradients, whereas nonassociated components should remain near the top of the gradient.

The eIF3-eIF4F complex was isolated, digested with either 2A or L protease, and the products subjected to sedimentation analysis (Fig. 6). The sedimentation of eIF3 was indicated by immunoreactivity of the 110-kDa eIF3γ subunit. In the absence of protease, eIF4G and eIF3η sediments at approximately 18 S with the eIF3-eIF4F complex (A, lanes 6–9). The majority of the eIF4A (70%) co-sedimented with this complex as well, although some apparently dissociated during sedimentation. Cleavage of eIF4G disrupted the eIF3-eIF4F complex. When eIF4G was cleaved by 2A protease, the two fragments of eIF4G did not co-sediment. Rather, all of the cpN3 sedimented near the top of the gradient (B, lanes 1–2), whereas all of the cpC and most (75%) of the eIF4A co-sedimented with eIF3 (lanes 6–9). This indicates that cpC and cpD remain associated with eIF3 after 2A cleavage of eIF4G, but cpN3 does not.

Limited digestion of eIF4G by L protease disrupted its interaction with both eIF4A and eIF3 (C). Whereas all remaining intact cpN, and some cpN4A still co-sedimented with eIF3 (lanes 5–8), most of the eIF4A (80%), as well as all of the cpC1 and cpN3, shifted to the top of the gradient (lanes 1–4). The proportion of eIF4A at the top of the gradient was approximately equal to the proportion of cpC cleaved. Longer digestion with L protease resulted in complete cleavage of cpN and all of the eIF4A shifting to the top of the gradient (data not shown). The cpC3 fragment co-sedimented with eIF3 (lanes 6 and 7), whereas cpC4 and cpC5 were released and sedimented near the top of the gradient (data not shown). These results suggest that the eIF3-binding site is located within cpC3.

Since eIF4A co-sedimented with cpC, the eIF4A binding site is likely to be contained within this region. The fact that eIF4A shifts to the top of the gradient when cpN is further digested with L protease (C) could mean that either eIF4A is free or is bound to a fragment of eIF4G which has shifted to the top of the gradient, e.g., cpC1 or cpC2. To determine whether eIF4A has affinity for cpC1 or cpC2, we covalently attached purified anti-eIF4G antibodies to agarose beads and used this immunoaffinity resin to test for physical association between eIF4A and fragments of eIF4G. L protease was incubated with either eIF4A alone (Fig. 7, lane 1) or eIF4A mixed with eIF4G (lanes 2 and 3), the reactions subjected to immunoadsorption, and the bound fractions analyzed (lanes 4–6). (eIF4A was added to the eIF4F preparation because eIF4F prepared by the method of Lampheear and Panniers (25) is substoichiometric for eIF4A.) The immobilized antibody bound cpC1, cpC3, and cpC5 as expected (A, cf. lanes 2 and 3 with lanes 5 and 6); the strong band
Domain Structure of eIF4G

### Table I

| Source of sequence | Sequence<sup>a</sup> |
|--------------------|----------------------|
| Edman degradation of rabbit eIF4G cp<sub>P<sub>2</sub></sub> | XRKKIKELNKEAVGD |
| Rabbit eIF4G cDNA<sup>b</sup> | 311-QVAVSVPK |
| Human eIF4G cDNA<sup>c</sup> | 310-QVAVSVPK |
| CP<sub>C3</sub> | RRRKKIKELNKEAVGD-334 |
| CP<sub>C4</sub> | RRRKKIKELNKEAVGD-333 |

<sup>a</sup> X indicates that an unambiguous assignment could not be made.

<sup>b</sup> R. Yan, W. He, and R. E. Rhoads, manuscript in preparation. GenBank™ accession number L22090.

<sup>c</sup> Ref. 43.

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**Fig. 6. Ultracentrifugal fractionation of the protease-treated eIF3-eIF4F complex.** The eIF3-eIF4F complex was incubated in the absence (A) or presence of the 2A (B) or L (C) proteases, layered onto 15–30% sucrose gradients, and centrifuged as described under "Experimental Procedures." Gradients were fractionated and aliquots subjected to SDS-PAGE on 8.5% gels. 3<sub>θ</sub>, immunoblot probed with anti-eIF3 antibodies; 4A, immunoblot probed with anti-eIF4A antibodies; 4G, cp<sub>C3</sub>, cp<sub>C4</sub>, and cp<sub>C5</sub>, immunoblot probed with anti-eIF4G<sub>1230–1248</sub> antibodies; cp<sub>C3</sub>, immunoblot probed with anti-eIF4G<sub>327–342</sub> antibodies; cp<sub>C5</sub>, immunoblot probed with anti-eIF4G<sub>653–666</sub> antibodies. Lane 6 represents the starting material which was layered onto each gradient. Fraction 1 corresponds to the top of the gradient.

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below cp<sub>C3</sub> migrating at 50 kDa is the highly immunoreactive IgG heavy chain, which probably results from insufficient removal of the immunoaffinity resin from eluted samples). eIF4A alone was not bound to the immunoaffinity resin (B, lane 4) unless eIF4G fragments were present (B, lanes 5 and 6). In a similar experiment in which the digestion with L protease was continued until all of the cp<sub>C1</sub> was converted to cp<sub>C2</sub>, eIF4A was also retained on the immunoaffinity resin (data not shown), indicating that the ~4 kDa which is removed from cp<sub>C1</sub> by the secondary L protease cleavage does not contain the eIF4A binding site.

As a control, the anti-eIF4G<sub>653–666</sub> antibody was used to test whether cp<sub>C3</sub>, cp<sub>C4</sub>, or cp<sub>C5</sub> were bound to the immunoaffinity resin. If this were the case, e.g. that cp<sub>C3</sub> remained bound to cp<sub>C2</sub> after proteolytic cleavage of cp<sub>C1</sub>, it would not be possible to distinguish whether eIF4A were bound to cp<sub>C2</sub> or cp<sub>C3</sub>. The experiment indicated that cp<sub>C3</sub>, cp<sub>C4</sub>, or cp<sub>C5</sub>, though detectable in the starting material (Fig. 7C, lanes 2 and 3), was not retained by the resin (lane 5). (Contamination by the IgG heavy chain did not permit analysis of cp<sub>C3</sub> in lane 6, but cp<sub>C4</sub> or cp<sub>C5</sub> were clearly absent.) cp<sub>C2</sub>, on the other hand, which contains the epitope for both anti-eIF4G<sub>1230–1248</sub> and anti-eIF4G<sub>653–666</sub> antibodies, was detected in the bound fractions (Fig. 7C, lanes 5 and 6). Thus, the immobilized anti-eIF4G<sub>1230–1248</sub> antibody selectively adsorbs cp<sub>C3</sub> and cp<sub>C2</sub>, but not cp<sub>C3</sub>, cp<sub>C4</sub>, or cp<sub>C5</sub>. In a similar experiment using anti-eIF4G<sub>27–342</sub> antibodies, it was determined that cp<sub>C2</sub> did not bind to the immunoaffinity resin (data not shown). In summary, these results indicate that there is a binding site for eIF4A in the cp<sub>C2</sub> fragment of eIF4G.

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**DISCUSSION**

Previous studies have established that eIF4G can form complexes with initiation factor polypeptides whose activities are associated with each of the events necessary for recruitment of host cell mRNA for translation. These events are cap recognition, a property of eIF4E (reviewed in Ref. 52); ATP-dependent unwinding of mRNA secondary structure, which is a property of eIF4A alone but one which is greatly enhanced by the pres-
ence of eIF4B and eIF4E (53, 54); and ribosome binding, which requires eIF3 (55–57). Purification of a high salt ribosomal wash by gel filtration (23) or m7GTP-Sepharose chromatography (25) leads to co-elution of eIF4A, eIF4B, eIF4E, eIF4G, and eIF3 in a large macromolecular complex. The present study indicates that treatment with proteases which have high specificity for eIF4G separates these major functional activities into two complexes, one containing the cap recognition function and one containing the helicase and ribosome binding functions (Figs. 3, 4, 6, and 7). In vivo, the 2A and L proteases appear to carry out only the cleavage event which generates cpC and cpN (12). Therefore, the activities of these fragments are most relevant to viral replication. However, further proteolysis by L protease in vitro, again specific for eIF4G, results in the separation of the ribosome binding function (eIF3) and the helicase function (eIF4A; Fig. 6). This suggests that these three functional activities of initiation factors involved in mRNA recruitment (cap binding, RNA helicase, and ribosome binding) are brought together principally through an interaction with eIF4G rather than with each other.

cpN is the region of eIF4G responsible for binding eIF4E, representing amino acid residues 1–486 of rabbit eIF4G in the case of 2A protease cleavage (1–479 in the case of L protease). Finer mapping with L protease narrows this region to amino acids 319–479. The criterion in either case is retention on m7GTP-Sepharose. Previously it was reported that poliovirus-induced cleavage products of eIF4G, as detected by a monoclonal antibody, were retained on cap-animals columns, but the location of the epitope was unknown (27–29). With the availability of antibodies to defined regions of eIF4G, it is now possible to conclude that the monoclonal antibody used earlier recognizes the same cleavage products as antipeptide antibodies against amino acid residues 327–416 (43). The validity of m7GTP-Sepharose retention as a basis for assigning the eIF4E-binding region to cpN is reinforced by the observation that cpN in the absence of eIF4E is not retained on m7GTP-Sepharose (28).

cpC, representing amino acids 480–1402, is the region of eIF4G which binds to eIF4A and eIF3, as demonstrated by co-sedimentation (Fig. 6) and immunoadsorption (Fig. 7). cpC3 contains the eIF3-binding site, based primarily on co-sedimentation, but also supported by the observation that eIF3 alters cleavage within cpC3 (Fig. 1C). cpC2 contains the eIF4A binding site, since both cp4A and cpC2 are released from eIF3 by L protease and co-sediment (Fig. 6C). Also, eIF4A specifically binds to the anti-eIF4G1230–1248 affinity resin, but only in the presence of cpC2 or larger fragments containing cpC2 (Fig. 7B).

Putting these two sets of data together suggests a domain model for eIF4G wherein the cap recognition function is in the N-terminal one-third of the molecule and the unwinding and ribosome binding functions are in the C-terminal two-thirds (Fig. 8). The region separating the N- and C-terminal domains may be a flexible hinge or loop which is more exposed to proteases. In support of this prediction, analysis of secondary structure motifs suggests that this region has a high probability of β-turns (58). Furthermore, a synthetic peptide based on a different portion of the eIF4G sequence is cleaved by HRV2 protease 2A (17), but this site is not cleaved in intact eIF4G (15), suggesting that it is in the region of the molecule which is not as accessible as the putative hinge region. Finally, fragments of eIF4G similar in size to cpN are observed upon raising Ca2+ levels in extracts of uninfected cells, suggesting that the junction between cpN and cpC is more susceptible to normal intracellular proteases (59).

Is such a domain model compatible with current ideas about cap-dependent initiation? Two models for the mRNA-binding step of initiation have been proposed, here referred to as the stepwise assembly model and the preformed complex model. Recruitment of capped mRNAs requires the coupling of cap recognition, unwinding, and scanning. The models differ primarily in which of these functions occur on the ribosome. The division of eIF4G into functional domains, however, is compatible with either model. The stepwise assembly model states that eIF4E first recognizes the cap as a free polypeptide, i.e. not in the eIF4F complex, whereas eIF4G is present in the 43 S initiation complex prior to mRNA recruitment, bound to eIF3 (Ref. 60; see Fig. 8). eIF4A, although largely found in the 43 S initiation complex and thus also be present in the 43 S initiation complex. The joining of eIF4E to eIF4G brings mRNA to the 40 S ribosomal subunit and to the unwinding machinery. In this model, the cpC region would serve to anchor eIF4G to the ribosome via eIF3 and provide a binding site for eIF4A (and possibly eIF4B as well), whereas the cpN domain would provide a flexible arm to receive the incoming eIF4E:mRNA complex (Fig. 8A). Cleavage of eIF4G by viral proteases would permit eIF4E to bind the cap

![Diagram](image.png)
and the eIF4E-mRNA complex to bind cpC (Fig. 8B, upper portion), but these events would occur apart from the unwinding machinery and the 40 S ribosomal subunit. Hence, no recruitment of capped mRNA to the ribosome would result. The preformed complex model states that eIF4F exists prior to formation of any complexes between mRNA, initiation factors, or ribosomes (3, 21, 22, 61). This eIF4F complex recognizes the cap of free mRNA and unwinds its secondary structure. When a sufficiently long stretch of secondary structure is unwound, the 40 S ribosomal subunit binds and begins scanning. A domain structure for eIF4G as proposed here would suggest that eIF4E, bound to the cpCg region of eIF4G, first recognizes the cap and directs the mRNA to the unwinding machinery which is bound to the cpC region. Cleavage of eIF4G by viral proteases would yield separate complexes capable of cap recognition and unwinding, but since unwinding would not be directed to the 5'-end of the mRNA, ribosome binding to single-stranded RNA would be frequently unproductive.

Is such a domain model for eIF4G compatible with current ideas about cap-independent initiation? Translation of picornaviral RNA requires eIF4A, and inactive variants of eIF4A have a dominant negative effect on translation of both capped and picornaviral mRNAs (36). This inhibition can be overcome by the addition of normal eIF4A, but the addition of eIF4F is six times more effective, underscoring the importance of eIF4G for unwinding. Binding of picornaviral RNA to the ribosome requires an internal ribosome entry site sequence (reviewed in Ref. 62). As eIF3 is involved in mRNA binding to ribosomes (see above), it must be presumed that translation of picornaviral RNA requires eIF3. In summary, translation of picornaviral RNA requires factors involved in unwinding (eIF4A, eIF4B) and ribosome binding (eIF4E) but not cap recognition (eIF4F). Cleavage of eIF4G by viral proteases separates the functions required for cap-independent translation (those attached to cpCg) from those which are not (those attached to cpC; see Fig. 8B). It is interesting in this light that the primary cleavage sites in eIF4G for 2A protease (15) and L protease (12) are only seven amino acid residues apart, despite the fact that the proteases are structurally distinct and that the amino acid sequences surrounding the cleavage sites bear little similarity. Thus, there has been evolutionary conservation among the enterovirus, rhino-, and aphthovirus functions (separation of cpCg from cpC) rather than recognition of a specific amino acid sequence.

cpCg is thus likely to contain the functions required for cap-independent initiation. A binding site for eIF3 in cpCg permits attachment to the ribosome. Consistent with this, we have observed that cpC sediments with ribosomes in a micrococcal nuclease-treated rabbit reticulocyte lysate (data not shown). A binding site for eIF4A in cpCg suggests that unwinding functions can take place in the absence of cpCg. The unwinding activity eIF4A is greatly enhanced when it is present in the eIF4F complex (53, 54); based on the location of the eIF4A binding site, it is likely that this stimulatory activity of eIF4F residues in cpCg. Also, there is a proposed RNA-binding region in eIF4G which is within cpC (63), and this region may be important for recruitment of mRNAs to the ribosome or stabilization of mRNA-ribosome interactions during translation of cellular and viral mRNAs. The proposed requirement of cpCg for internal initiation may explain a paradoxical observation: picornavirion infection “inactivates” eIF4F (6, 64), yet eIF4F has been shown to stimulate internal cap-independent initiation from viral sequences (34, 35, 65). The proposed domain model would state that the added eIF4F is supplying functions contained in the cpCg region which are needed for cap-independent translation; eIF4F is “inactivated” by 2A or L protease only with respect to cap-dependent translation. Based on this consideration, one would predict that cpC alone would stimulate cap-independent initiation through its eIF3, eIF4A, and possibly RNA binding properties.

Acknowledgments — We thank Ai-Li Cai for excellent technical assistance during HPLC purity tests of eIF4F cleavage products. Dragana Jugovic for purification of FMDV L protease, Dr. Hans Trachsel for mouse anti-eIF4A monoclonal antibody, and Dr. William Merrick for goat anti-eIF3 antibodies.

Note Added in Proof—Mader et al. (Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995) Mol. Cell. Biol., in press) have recently shown in agreement with our results that eIF4E interacts with the N-terminal portion of eIF4G. They have mapped the eIF4E-binding region to within amino acids 408–457 and have identified a conserved motif between amino acids 413–424 that is important for the interaction.

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