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Human Eukaryotic Translation Initiation Factor 4G (eIF4G) Possesses Two Separate and Independent Binding Sites for eIF4A

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Mammalian translation initiation factor 4F (eIF4F) consists of three subunits, eIF4A, eIF4E, and eIF4G. eIF4G interacts directly with both eIF4A and eIF4E. The binding site for eIF4E is contained in the amino-terminal third of eIF4G, while the binding site for eIF4A was mapped to the carboxy-terminal third of the molecule. Here we show that human eIF4G possesses two separate eIF4A binding domains in the middle third (amino acids [aa] 478 to 883) and carboxy-terminal third (aa 884 to 1404) of the molecule. The amino acid sequence of the middle portion of eIF4G is well conserved between yeasts and humans. We show that mutations of conserved amino acid stretches in the middle domain abolish or reduce eIF4A binding as well as eIF3 binding. In addition, a separate and nonoverlapping eIF4A binding domain exists in the carboxy-terminal third (aa 1045 to 1404) of eIF4G, which is not present in yeast. The C-terminal two-thirds region (aa 457 to 1404) of eIF4G, containing both eIF4A binding sites, is required for stimulating translation. Neither one of the eIF4A binding domains alone activates translation. In contrast to eIF4G, human p97, a translation inhibitor with homology to eIF4G, binds eIF4A only through the amino-terminal proximal region, which is homologous to the middle domain of eIF4G.

Eukaryotic translation initiation factor 4F (eIF4F) is a protein complex consisting of eIF4E, eIF4A, and eIF4G. eIF4E binds to the cap structure (m^7GpppN, where N is any nucleotide) of the mRNA. eIF4A, an RNA-dependent ATPase and ATP-dependent RNA helicase, is thought to unwind the secondary structure of the 5′ untranslated region of the mRNA to facilitate ribosome binding (for reviews, see references 16, 19, and 25). eIF4G serves as a scaffold for eIF4E and eIF4A to coordinate their functions. eIF4F exhibits a much higher RNA helicase activity than eIF4A alone (22), and dominant negative mutants of eIF4A abolish both cap-dependent and cap-independent translation (20). This is consistent with the idea that eIF4A is essential for translation of all mRNAs and that eIF4A recycles through the eIF4F complex to function in mRNA unwinding (20).

Several members of the picornavirus family, including poliovirus, cause the cleavage of eIF4G into an N-terminal third (amino acids [aa] 1 to 479 for poliovirus) and a C-terminal two-thirds fragment (aa 480 to 1396) (3, 7, 14). Extensive digestion of eIF4G with the foot-and-mouth-disease virus L protease or rhinovirus 2A protease in vitro yields several smaller fragments. One of the fragments (aa 319 to 479) binds to eIF4E (10). Mader et al. (15) localized the eIF4E binding site more precisely to the amino acid sequence ^{413}KKRYDRE FLLGFQFIF^{428}, which is well conserved between yeast and human eIF4Gs. The conserved eIF4E binding site is also found in eIF4E-binding proteins (4E-BPs) (15), and competition between eIF4G and 4E-BPs for eIF4E explains how 4E-BPs inhibit cap-dependent translation (6). Although the C-terminal third of human eIF4G was shown to contain the binding site for eIF4A (10), the yeast eIF4Gs possess no corresponding region (5). Furthermore, the central domain of human eIF4G in combination with eIF4A is sufficient to mediate internal entry of 40S ribosomes (21). These findings raise questions about the correct binding site of eIF4A on eIF4G.

Recently, we (9) and others (13, 23, 26) have cloned a new human translation regulator, p97/NAT-1/DAP-5, which is homologous to the C-terminal two-thirds of eIF4G. Since p97 interacts with eIF4A in a manner similar to that of eIF4G (9), it is very likely that p97 and eIF4G share conserved eIF4A binding motifs. Here, we show that both the middle- and C-terminal-third domains of human eIF4G bind eIF4A independently, while p97 binds eIF4A only through the N-terminal domain, which is homologous to the central domain of eIF4G.

MATERIALS AND METHODS

Construction of mutants. N-terminal deletions of p97 were made as follows. To construct a p97 mutant containing aa 55 to 907, a 5′ truncated form of p97 cDNA (nucleotide 473 to the 3′ terminus, 3820) (9) was inserted into a hemagglutinin (HA)-tagged protein expression vector, pcDNA3HA (9), resulting in pcDNA3HA-p97(55-907). To generate pcDNA3HA-p97(98-907) and pcDNA3HA-p97(275-907), DNA fragments generated by cleavage with the *Sac*I and *Sca*I restriction enzymes, respectively, were inserted into pcDNA3HA. C-terminal deletions of p97 were made as follows. An HA-tagged full-length p97 expression plasmid, pcDNA3HA-p97 (9), was cleaved with *Sac*I and blunted with T4 DNA polymerase as a template for synthesis of HA-p97(1-98) mRNA. PCR-amplified DNA fragments encoding aa 1 to 128 and 1 to 186 were inserted into pcDNA3HA to generate pcDNA3HA-p97(1-128) and -(1-186), respectively. pcDNA3HA-p97 was digested with *Sca*I, *Bgl*II, and *Nco*I for in vitro synthesis of HA-p97(1-275), HA-p97(1-333), and HA-p97(1-398) mRNAs, respectively. To construct pcDNA3HA-p97(333-907) and pcDNA3HA-p97(410-907), *Bgl*II and *Msc*I sites were used. A cluster of translation termination codons in all three reading frames, TGACTAGCTAA, was inserted into the 3′ terminus of the multicloning site of pcDNA3HA to generate pcDNA3HA-STOP. cDNA fragments encoding p97(1-333) and p97(1-410), generated by digestion with *Bgl*II and *Msc*I, respectively, were inserted into pcDNA3HA-STOP to construct pcDNA3HA-p97(1-333) and pcDNA3HA-p97(1-409).

Mutation of eIF4G was performed as follows. The cDNA for human eIF4G (26) was a kind gift from R. E. Rhoads. During the course of our studies, we found several discrepancies between the published eIF4G nucleotide sequence (11, 26) and our sequence. Thus, the sequence of the entire cDNA of human eIF4G was determined by reading both strands without a mismatch (Core Fa-

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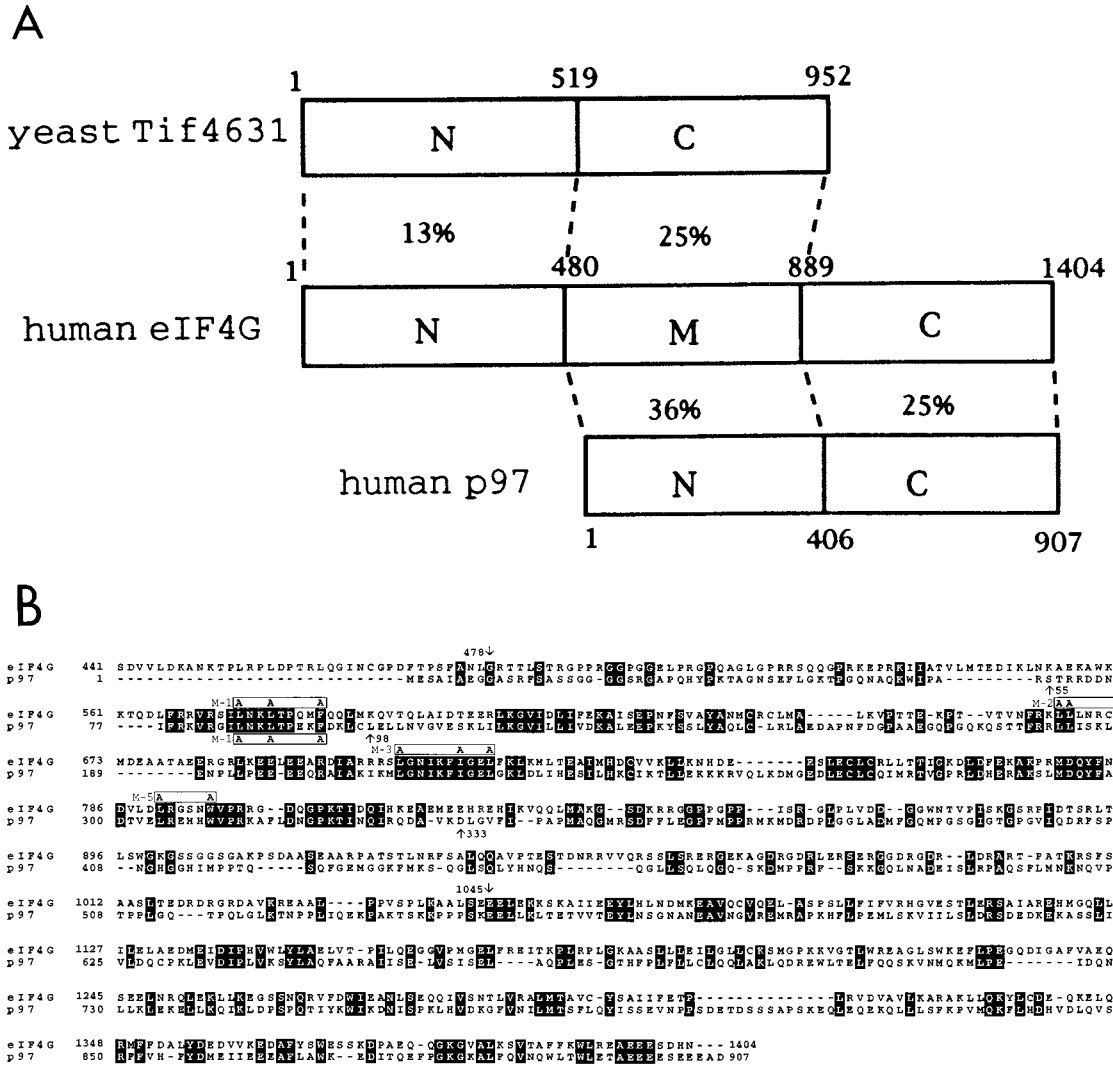

FIG. 1. Amino acid sequence of human eIF4G and p97. (A) Homology alignment of human eIF4G, p97, and yeast Tif4631(p150). eIF4G is divided into N-terminal (N), middle (M), and C-terminal (C) regions based on the results of digestion by picornaviral proteases (10). The boundaries between the N terminus and C terminus of Tif4631 and p97 are based on the alignment with the eIF4G sequence. The identity (percent) between corresponding domains is shown. (B) Alignment of the corrected human eIF4G sequence and the p97 sequence (9) by the pattern-induced multisequence alignment program (24). Conserved amino acids between eIF4G and p97 are boxed. Point mutations (M-1 to M-5) are indicated.

cility for Protein/DNA Chemistry, Queen's University, Kingston, Ontario, Canada). The deduced amino acid sequence is shown in Fig. 1. Some of the corrected amino acids of human eIF4G become identical to those of the rabbit eIF4G (GenBank accession no. P41110). An *Eco*RI-*Bam*HI fragment of the eIF4G cDNA encoding the N-terminal third of eIF4G (aa 1 to 457) (Fig. 1) was inserted into pcDNA3HA-STOP to generate pcDNA3HA-eIF4G(1-457). A *Cla*I-*Eco*RI fragment encoding the C-terminal third of eIF4G (aa 884 to 1404) was inserted into pcDNA3HA to construct pcDNA3HA-eIF4G(884-1404). To construct pcDNA3HA-eIF4G(478-883), -(884-1214), or -(1045-1404), corresponding fragments amplified by *Pfu* DNA polymerase-dependent PCR were inserted into pcDNA3HA-STOP. Point mutations were introduced by PCR with *Pfu* DNA polymerase.

In vitro translation. Capped RNAs were synthesized with T7 RNA polymerase in the presence of the cap analog, m^7GpppG. For translation, rabbit reticulocyte lysate (25 μl, final volume) (Promega) was programmed with RNA (200 ng) in the presence of [^{35}S]methionine (10 μCi) according to the manufacturer's instructions. Following translation, an aliquot of the lysate was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the amount of product was quantified with a PhosphorImager BAS2000 (Fuji). For translation in bacterial extracts, DNA fragments were cloned into pET-15b (Novagen). The resulting plasmids (4 μg) were incubated in an *Escherichia coli* extract (Promega) (50 μl, final volume) in the presence of [^{35}S]methionine (15 μCi) according to the manufacturer's instructions.

Protein interaction assay. Following translation, reticulocyte lysate or bacterial lysate (5 to 12 μl, depending on samples) containing an equal amount of radioactivity was adjusted to 12 μl with unprogrammed reticulocyte lysate or bacterial lysate and mixed with FLAG (Kodak)-eIF4A bound to anti-FLAG resin (20 μl) (Kodak) (9). Following incubation for 30 min on ice, the mixture was washed with buffer A, consisting of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.15 M NaCl, and 0.1% Nonidet P-40 (1 ml, three times). Bound proteins were eluted with 30 μl of buffer A containing 100 μg of FLAG peptide (Kodak) per ml and resolved by SDS–12.5% PAGE. The gel was exposed to an X-ray film overnight. Radioactivity of each polypeptide was quantified with a PhosphorImager (BAS2000; Fuji).

Immunoprecipitation. HeLa cells (6-cm dish) were infected with vaccinia virus vTF7-3 (4) and then transfected with the plasmids indicated in the figure legends by using Lipofectin (Gibco BRL). Twenty hours later, cells were lysed in 1 ml of buffer B (100 mM KCl, 0.5 mM EDTA, 20 mM HEPES-KOH [pH 7.6], 0.4% Nonidet P-40, 20% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg of pepstatin per ml, 5 μg of leupeptin per ml). After centrifugation, the supernatant was mixed with anti-HA antibody (2 μg) (12CA5; a kind gift from M. Tremblay) for 4 h in the cold room. Protein G-Sepharose (30 μl of a 50% slurry) was added, and the mixture was incubated for 2 h. After washing with buffer A (0.5 ml, three times), immunoprecipitates were collected by centrifugation and proteins were dissolved in Laemmli buffer. Half of the sample was boiled, and proteins were resolved by SDS–10% PAGE and transferred to an

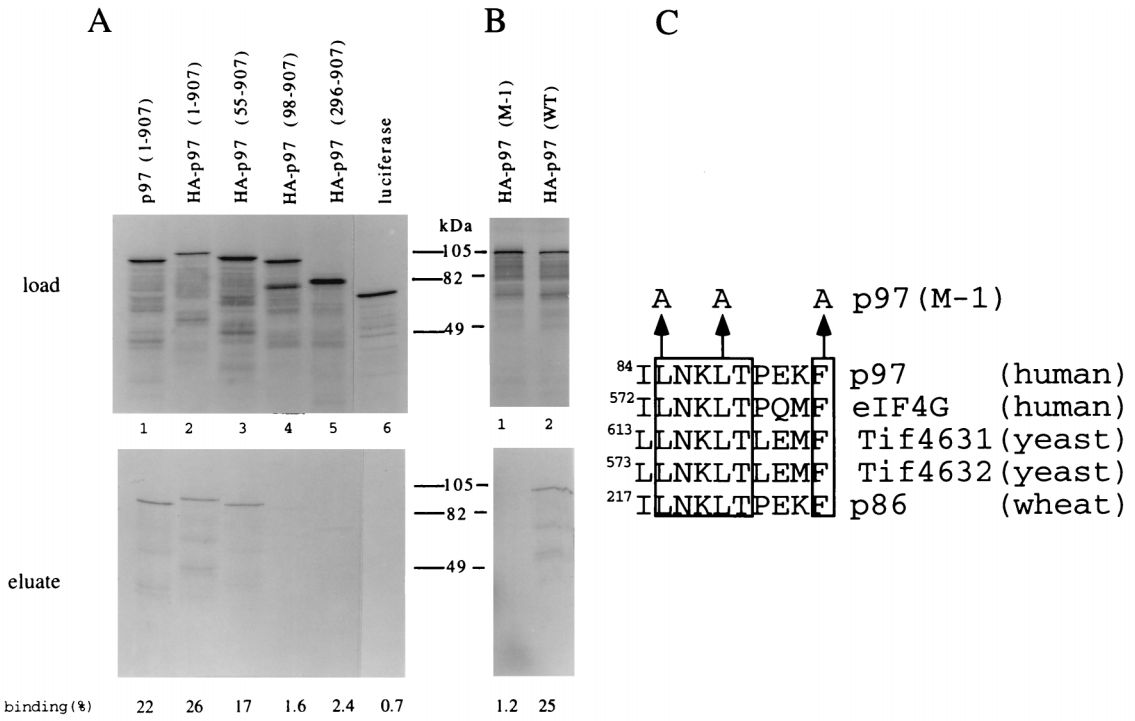

FIG. 2. Binding of N-terminal deletion fragments of p97 to eIF4A. (A) p97 (lane 1), HA-p97 (lane 2), N-terminal deletion fragments of p97 (lanes 3, 4, and 5), and luciferase (lane 6) were synthesized in the presence of [^{35}S]methionine in a reticulocyte lysate and mixed with FLAG-eIF4A resin as described in Materials and Methods. Proteins bound to the resin were eluted with FLAG peptide and resolved by SDS–10% PAGE. The percentage of eluted protein out of the load is indicated at the bottom. (B) Binding of HA-p97 (M-1) (lane 1) and HA-p97 (wild type [WT]) (lane 2) to eIF4A was determined as for panel A. (C) Amino acids mutated in p97 (M-1) and the homologous regions of human eIF4G (Fig. 1), yeast eIF4Gs p130 (Tif4632) and p150 (Tif4631) (5), and the large subunit of wheat eIF(iso)4F, p86 (1). Conserved amino acids are boxed.

Immobilon polyvinylidene difluoride membrane (Millipore) for Western blotting with anti-eIF4A (a gift from H. Trachsel), anti-eIF3 (a gift from J. Hershey), and anti-eIF4E (12). Protein bands were visualized on an X-ray film by an enhanced chemiluminescence detection system.

Functional analysis of eIF4G and p97. HeLa cells (6-cm dish) were infected with vaccinia virus vTF7-3 and then cotransfected with an HA-tagged protein expression plasmid (4.8 μg) and pGEM-EMCV-IRES-CAT (0.2 μg). Twenty hours later, two-thirds of the cells recovered from the dish were processed for RNA extraction followed by an RNase protection assay to quantify chloramphenicol acetyltransferase (CAT) RNA (9). The remaining cells from the same dish were suspended in a buffer (100 mM KCl, 0.5 mM EDTA, 20 mM HEPES-KOH [pH 7.6], 20% glycerol, 1 mM phenylmethylsulfonyl fluoride) and disrupted by freezing and thawing (three times). After centrifugation, the supernatant was used for CAT assays (9) and Western blotting with anti-HA antibody. Translation activity was calculated by dividing CAT activity by the amount of CAT mRNA.

Nucleotide sequence accession number. The accession number of the sequence shown in Fig. 1 is AF012088.

RESULTS

eIF4A binding domain on p97. Figure 1 shows the alignments of yeast and human eIF4G with p97. Also shown is the amino acid sequence alignment between human eIF4G and p97. To localize the binding site for eIF4A on p97, the full-length protein and N-terminal deletion fragments were synthesized in the presence of [^{35}S]methionine in a reticulocyte lysate and mixed with FLAG-eIF4A bound to anti-FLAG resin (9). Following washing, proteins bound to the resin were eluted with FLAG peptide. Seventy percent of the p97 loaded was recovered in the unbound fraction (data not shown). About 90% of the bound material (22% of the loaded p97) was eluted from the eIF4A resin with FLAG peptide (Fig. 2, lane 1), while only a small percentage (7% of the loaded material) remained on the resin (a similar ratio of bound versus unbound material was observed for eIF4G and its fragments [see below]). Less than 0.2% of loaded p97 was eluted with the FLAG peptide from the anti-FLAG resin alone (data not shown), and only 0.7% of luciferase protein was bound and eluted from the eIF4A resin (lane 6). When a similar experiment was performed with eIF4E resin, 0.5% of p97 was eluted with FLAG peptide (9). These results confirm earlier results for specific binding of p97 to eIF4A (9). Most of the mutants used in this study contain an HA epitope at the N terminus, which did not interfere with the interaction between p97 and eIF4A (compare lanes 1 and 2). While deletion of 54 aa from the N terminus of p97 did not significantly affect eIF4A binding, a further deletion of 43 aa considerably decreased binding (about 90%; compare lanes 3 and 4). The sequence between aa 55 and 98 contains an amino acid stretch which is well conserved not only between human p97 and eIF4G (Fig. 1B) but also among eIF4G proteins of yeasts, plants, and humans (Fig. 2C). A combination of three point mutations in this amino acid stretch, ILNKLTPEKF into IANKATPEKA (M-1), abolished eIF4A binding (Fig. 2B; compare lanes 1 and 2). These results suggest that this conserved sequence is important for eIF4A binding, although the possibility of the distortion of p97 structure by the mutation cannot be excluded.

The sequence containing aa 1 to 98 of p97 is not sufficient, however, for eIF4A binding. The N-terminal fragment, aa 1 to 98 or even aa 1 to 275, exhibited no binding to eIF4A (Fig. 3, lanes 2 to 5), while the fragment extending to aa 333 bound to eIF4A as well as wild type (compare lanes 1 and 6). The region between aa 275 and 333 also contains several amino acid stretches conserved between p97 and eIF4G (Fig. 1). Thus, the region encompassing aa 55 to 333 of p97 is the minimal

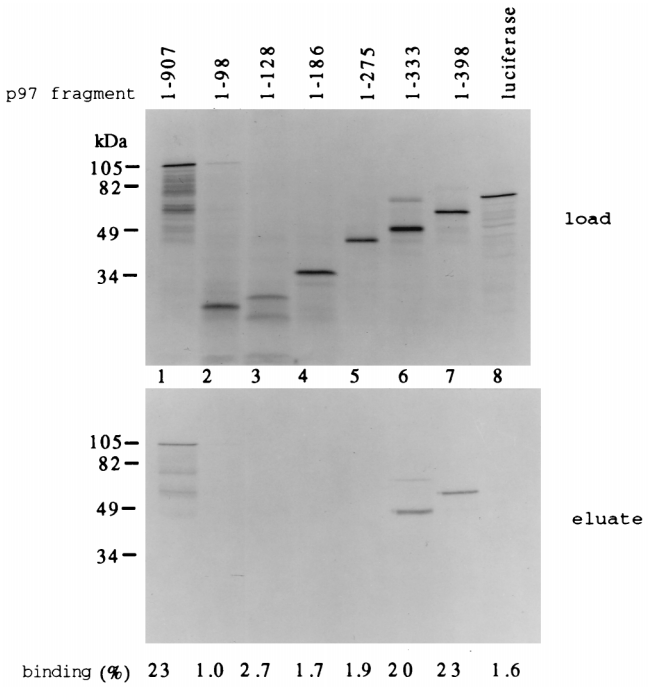

FIG. 3. Binding of C-terminal deletion fragments of p97 to eIF4A. p97 (lane 1), C-terminal deletion fragments (lanes 2 to 7), and luciferase (lane 8) were synthesized in the presence of [^{35}S]methionine in a reticulocyte lysate and mixed with FLAG-eIF4A resin as described in Materials and Methods. Proteins bound to the resin were eluted with FLAG peptide and resolved by SDS–10% PAGE. The percentage of eluted protein out of the load is indicated at the bottom. All products except luciferase were HA-tagged proteins.

sequence that is required and sufficient for binding to eIF4A.

Two eIF4A binding domains on eIF4G. The eIF4A binding domain of p97 is highly conserved through evolution among eIF4G proteins (13) and corresponds to the middle region (region M, aa 483 to 818) of eIF4G (Fig. 1). However, Lamphear et al. (10) showed that the C-terminal-third fragment (aa 890 to 1404) of eIF4G was the only fragment that bound to eIF4A. To address this discrepancy, we dissected the human eIF4G into three domains, eIF4G (aa 1 to 457), eIF4G (aa 478 to 883), and eIF4G (aa 884 to 1404), and determined their capacity to bind to eIF4A (Fig. 4). Surprisingly, both the middle region (aa 478 to 883) (lane 3) and the C-terminal region (aa 884 to 1404) (lane 4) as well as the full-length eIF4G (aa 1 to 1404) (lane 1) bound to eIF4A to a significant extent, while the N-terminal region (aa 1 to 457) or luciferase did not (lanes 2 and 5).

To confirm that these interactions also occur in vivo, HA-tagged fragments of eIF4G or p97 were expressed in HeLa cells, and the proteins were immunoprecipitated with anti-HA antibody. Immunoprecipitates were examined by Western blotting with an antibody against eIF4A (Fig. 5). The antibody detected eIF4A in extracts from HeLa cells (lane 1), whereas an anti HA-antibody did not precipitate endogenous eIF4A (lane 2). In agreement with the results of the in vitro binding assay, the full-length eIF4G and the middle and C-terminal fragments of eIF4G bound to eIF4A (Fig. 5A, lanes 3, 5, and 6, respectively), while the N-terminal-third fragment did not (lane 4). The results obtained with p97 were different from those with eIF4G. While the N-terminal fragment (aa 1 to 333) of p97, which corresponds to the middle domain of eIF4G, bound eIF4A (Fig. 5B, lane 4), the C-terminal fragment (aa

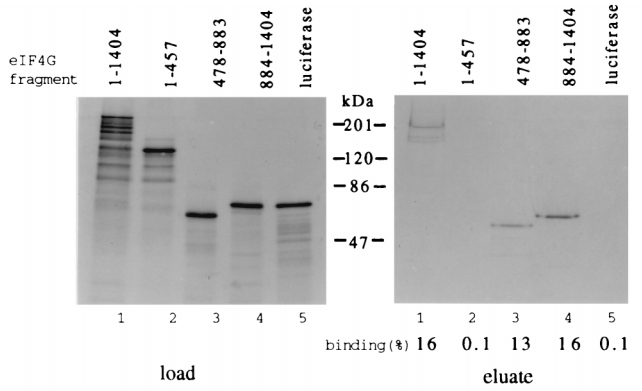

FIG. 4. The middle- and C-terminal-third fragments of eIF4G bind to eIF4A. Full-length eIF4G (aa 1 to 1404) or fragments containing the N-terminal third (aa 1 to 457), middle third (aa 478 to 883), or C-terminal third (aa 884 to 1404) of eIF4G were synthesized in the presence of [^{35}S]methionine in a reticulocyte lysate and mixed with FLAG-eIF4A resin as described in Materials and Methods. Proteins bound to the resin were eluted with FLAG peptide and resolved by SDS–10% PAGE. The percentage of eluted protein out of the load is indicated at the bottom. All products except luciferase were HA-tagged proteins.

333 to 907) failed to bind to eIF4A (lane 5). These results are in agreement with the in vitro binding data (Fig. 2 and 3).

To delimit the eIF4A binding domain in the C-terminal region of eIF4G (aa 884 to 1404), two fragments (aa 884 to 1214 and aa 1045 to 1404) were tested for eIF4A binding. The carboxy-proximal fragment containing aa 1045 to 1404 as well as the entire C-terminal region (aa 884 to 1404) bound to eIF4A, as detected by using the eIF4A resin (Fig. 6A; compare lanes 1 and 3) and also by the HA-tagged protein immunoprecipitation system (Fig. 6B, lanes 4 and 6). In contrast, the fragment with aa 884 to 1214 exhibited no binding activity (Fig. 6A, lane 2, and 6B, lane 5). These results indicate that the C-terminal eIF4A binding site does not overlap with the middle eIF4A binding domain (aa 478 to 883) sequence.

In an attempt to localize the eIF4A binding site in the middle-third region (aa 457 to 934) of eIF4G, we mutated several amino acid stretches conserved not only between

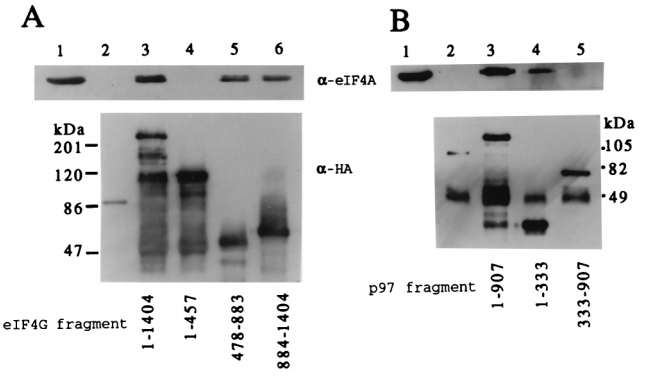

FIG. 5. Coimmunoprecipitation of eIF4G and p97 fragments with eIF4A. Plasmids were transfected into HeLa cells following infection with vTF7-3. Proteins were immunoprecipitated with anti-HA antibody, and immunoprecipitates were resolved by SDS–10% PAGE as described in Materials and Methods. Western blotting was performed with anti-eIF4A (upper panels) or anti-HA (lower panels). HeLa cell extract (60 μg of protein) was used for lanes 1. (A) pcDNA3HA (lane 2), pcDNA3HA-eIF4G (lane 3), pcDNA3HA-eIF4G(1-457) (lane 4), pcDNA3HA-eIF4G(478-883) (lane 5), and pcDNA3HA-eIF4G(884-1404) (lane 6). (B) pcDNA3HA (lane 2), pcDNA3HA-p97 (lane 3), pcDNA3HA-p97(1-333) (lane 4), and pcDNA3HA-p97(333-907) (lane 5).

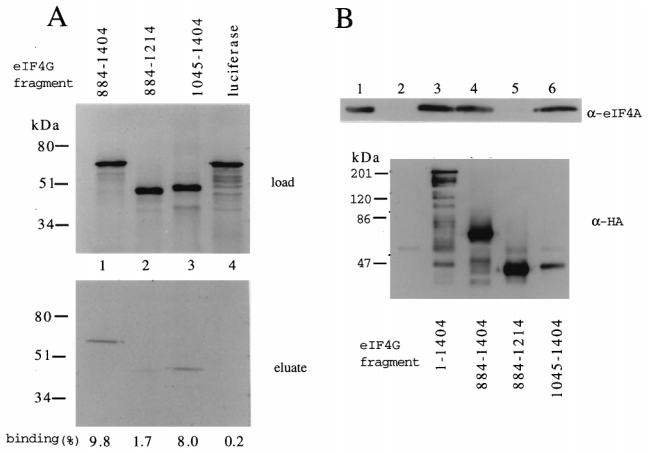

FIG. 6. Demarcation of the C-terminal eIF4A binding domain in eIF4G. (A) eIF4G(884-1404) (lane 1), eIF4G(884-1214) (lane 2), eIF4G(1045-1404) (lane 3), and luciferase (lane 4) were synthesized in the presence of [^{35}S]methionine in a reticulocyte lysate and mixed with FLAG-eIF4A resin. Proteins bound to the resin were eluted with FLAG peptide and resolved by SDS–10% PAGE. The percentage of eluted protein is indicated at the bottom. All products except luciferase were HA-tagged proteins. (B) The following plasmids were transfected into HeLa cells after infection with vTF7-3: pcDNA3HA (lane 2), pcDNA3HA-eIF4G (lane 3), pcDNA3HA-eIF4G(884-1404) (lane 4), pcDNA3HA-eIF4G (884-1214) (lane 5), and pcDNA3HA-eIF4G(1045-1404) (lane 6). Proteins were immunoprecipitated with anti-HA antibody, and immunoprecipitates were resolved by SDS–10% PAGE. Western blotting was performed with anti-eIF4A (upper panel) or anti-HA (lower panel). HeLa cell extract (60 μg of protein) was used in lane 1.

eIF4G and p97 (Fig. 1) but also between yeast and human eIF4Gs (5, 13) and examined their effects on eIF4A binding. Mutation of the conserved amino acid stretch ^{572}ILNKLT PQMF^{582} of eIF4G (^{84}ILNKLTPEKF^{93} in the case of p97) into IANKATPQMA (M-1) abolished binding to eIF4A (Fig. 7A; compare lanes 3 and 4). In addition, mutations of other conserved amino acid stretches, M-2, M-3, and M-4 (Fig. 1), abolished or dramatically decreased binding to eIF4A (Fig. 7A, lanes 5, 6, and 7, respectively). One mutation, M-5, showed a relatively small effect on eIF4A binding (2.5-fold decrease as determined by densitometry) (lane 8). Likewise, binding to eIF3, which was shown previously to be mediated by the middle domain (10), was severely decreased by mutations M-1 to M-4 (Fig. 7A, middle panel, lanes 4 to 7; p170 and p115 subunits of eIF3 are shown, since they exhibit the strongest reactivity towards the anti-eIF3 antibody; p115 is hPRT1, as the same band is detected by anti-hPRT1 [data not shown]), while the effect of M-5 was moderate (lane 8). Binding of eIF3 and eIF4A to p97 was also abolished by a mutation (M-1) (Fig. 7B; compare lanes 3 and 4). In contrast, the same mutations (M-1 to M-5) in an eIF4G fragment with the N-terminal region (aa 1 to 934) did not affect binding to eIF4E to a significant extent (Fig. 7C; the light chain of anti-HA antibody comigrates with eIF4E on SDS-PAGE; to circumvent this problem, the sample was not boiled in order to retain the disulfide bonds between the light and heavy chains of the antibody for Western blotting with anti-eIF4E), indicating that the mutations did not cause global distortion of the protein structure. These mutations exhibited the same effects on eIF4A binding as mutations in the fragment from aa 457 to 934 (compare Fig. 7A and C). Thus, the binding site of eIF4A and eIF3 in the eIF4G middle domain could not be mapped to a defined stretch of amino acids. This is in contrast to the N-terminal domain of eIF4G, where a 49-aa region is necessary and sufficient for eIF4E binding (15).

To study the effects of the mutations of eIF4G and p97 on translation, we expressed HA-tagged eIF4G and p97 mutants in HeLa cells together with a reporter CAT mRNA, whose open reading frame is preceded by the encephalomyocarditis virus internal ribosome entry site (EMCV-IRES). The HA-tagged proteins were expressed to similar levels as determined by Western blotting (Fig. 8, lower panel). The C-terminal two-thirds of eIF4G (aa 457 to 1404) activated translation by 2.2-fold (Fig. 8; compare lanes 1 and 2), as determined by CAT activity normalized to CAT mRNA amount. In contrast, the eIF4G fragment (aa 457 to 1404) harboring the M-1 mutation, which abolishes eIF4A and eIF3 binding (Fig. 7), failed to activate translation (Fig. 8, lane 3). The eIF4G fragment (aa 457 to 934), which lacks the C-terminal eIF4A binding site but retains the eIF4A binding site in the middle domain, did not activate translation either (lane 4), suggesting that the region (aa 934 to 1404) containing the C-terminal eIF4A binding site is necessary for translation. p97 decreased translation to 33% of that of the control (compare lanes 1 and 5), while p97 (M-1), which abolishes eIF4A and eIF3 binding (Fig. 7), affected translation only moderately (decrease by 15%) (lane 6), consistent with the idea that sequestering eIF4A and/or eIF3 is one of the mechanisms by which p97 suppresses translation (9).

To exclude the possibility that binding of eIF4G and p97 to eIF4A is mediated by eIF3 or other eukaryotic proteins in HeLa cells or reticulocyte lysate, fragments of eIF4G and p97 were expressed in a bacterial in vitro translation system and examined for binding to eIF4A. The middle (aa 478 to 883) and C-terminal (aa 884 to 1404) fragments of eIF4G and the N-terminal (aa 1 to 333) fragment of p97 exhibited significant binding to eIF4A (Fig. 9, lanes 1, 2, and 3), while the C-

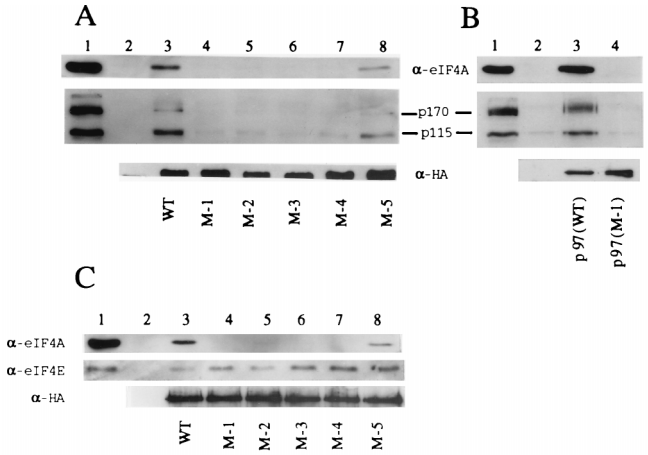

FIG. 7. Analysis of eIF4A and eIF3 binding sites in the middle domain of eIF4G. (A) The following plasmids were transfected into HeLa cells after infection with vTF7-3: pcDNA3HA (lane 2), pcDNA3HA-eIF4G(457-934) (lane 3), and pcDNA3HA-eIF4G(457-934) harboring mutations M-1 (lane 4), M-2 (lane 5), M-3 (lane 6), M-4 (lane 7), and M-5 (lane 8) (see Fig. 1 for mutated amino acids). Proteins were immunoprecipitated with anti-HA antibody, and immunoprecipitates were resolved by SDS-PAGE as described in Materials and Methods. Western blotting was performed with anti-eIF4A (upper panel), anti-eIF3 (middle panel), or anti-HA (lower panel). HeLa cell extract (60 μg of protein) was used in lane 1. (B) pcDNA3HA (lane 2), pcDNA3HA-p97 (lane 3), or pcDNA3HA-p97 (M-1) (lane 4) was transfected into HeLa cells, and extracts were processed for Western blotting as for panel A. HeLa cell extract (60 μg of protein) was used in lane 1. (C) Plasmids pcDNA3HA (lane 2), pcDNA3-eIF4G(1-934) (lane 3), and pcDNA3HA-eIF4G(1-934) harboring mutations M-1 (lane 4), M-2 (lane 5), M-3 (lane 6), M-4 (lane 7), and M-5 (lane 8) were transfected, and cell extracts were processed as for panel A. Western blotting was performed with anti-eIF4A (upper panel), anti-eIF4E (middle panel), or anti-HA (lower panel). HeLa cell extract (60 μg of protein) was used in lane 1. WT, wild type.

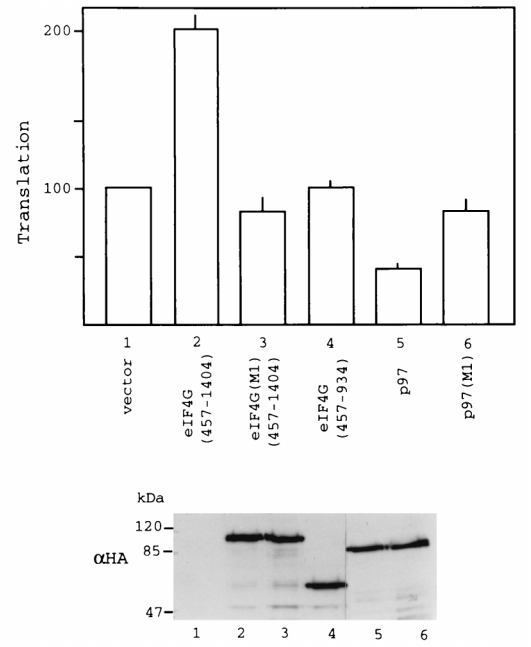

FIG. 8. Functional analysis of eIF4G and p97 mutants. HA-tagged expression vectors, pcDNA3HA (lane 1), pcDNA3HA-eIF4G(457-1404) (lane 2), pcDNA3HA-eIF4G(457-1404) (M-1) (lane 3), pcDNA3HA-eIF4G(457-934) (lane 4), pcDNA3HA-p97 (lane 5), and pcDNA3HA-p97 (M-1) (lane 6) were transfected together with pEMCV-IRES-CAT into HeLa cells following infection with vaccinia virus vTF7-3. Cells were processed for CAT assay, RNase protection assay with antisense CAT probe, and Western blotting with anti-HA antibody as described in Materials and Methods. The level of CAT protein in cells transfected with the empty vector (lane 1) was set at 100. The columns and bars represent the means and standard errors, respectively, of four independent transfections.

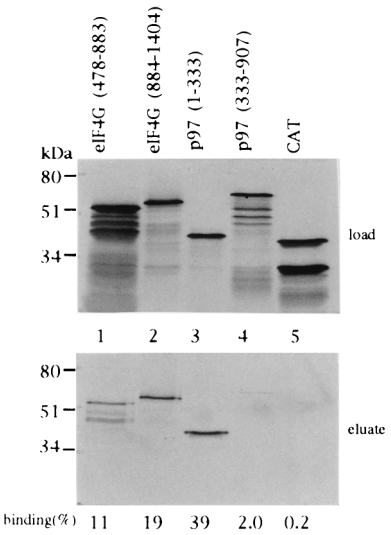

FIG. 9. Direct binding of eIF4G and p97 to eIF4A. Proteins eIF4G(478-883) (lane 1), eIF4G(884-1404) (lane 2), p97(1-333) (lane 3), and p97(333-907) and a negative control protein (Pin Point/CAT; Promega) (lane 5) were synthesized in the presence of [^{35}S]methionine in a bacterial in vitro translation system and mixed with FLAG-eIF4A resin as described in Materials and Methods. Proteins bound to the resin were eluted with FLAG peptide and resolved by SDS–10% PAGE. The percentage of eluted protein is indicated at the bottom.

terminal fragment (aa 333 to 907) of p97 bound very weakly to eIF4A (lane 4) and the CAT protein, which served as a negative control, did not bind (lane 5). These results are in agreement with those obtained in the reticulocyte lysate in vitro translation system. It is possible that the interaction of eIF4A or eIF3 with eIF4G is mediated by RNA. However, RNase treatment of the eIF4A resin or the bacterially expressed proteins did not alter the binding of any of the fragments to eIF4A (data not shown). Thus, the data suggest that both the middle and C-terminal fragments of eIF4G as well as the N-terminal fragment of p97 bind directly to eIF4A.

DISCUSSION

Previously, Lamphear et al. (10) mapped the eIF4A binding site to the C-terminal-third fragment of eIF4G (aa 890 to 1404). However, yeast eIF4Gs possess no corresponding region (5), but eIF4A is required for translation in yeast (2). In addition, a combination of the middle domain (aa 457 to 935) of human eIF4G and eIF4A is sufficient to mediate binding of ribosomes to the EMCV RNA (21). These discrepancies are now explained by our demonstration that human eIF4G has two separate and nonoverlapping binding sites for eIF4A, one in the middle part (aa 478 to 883) and the other in the C-terminal region (aa 1045 to 1404).

Metz and Browning (17) showed that p86, which is the homolog of eIF4G in wheat eIF(iso)4F, lost eIF4A binding activity upon removal of 90 aa from the N terminus. The authors concluded that this sequence was at least part of the eIF4A binding site. The N-terminal 90-aa region of p86, which exhibits homology to the N-terminal third of eIF4G, contains the binding site for eIF4E but does not overlap with the middle domain of human eIF4G or the N-terminal part of p97. Thus, the results of Metz and Browning do not seem to agree with our data. Since their experiments were performed by the yeast two-hybrid system, it is not clear that the deletion affected the binding to eIF4A rather than other functions, such as protein stability or nuclear import.

The amino acid sequence of the middle region is conserved between human, yeast (Fig. 1), and plant eIF4Gs (17). Mutations of several conserved amino acids within this domain abolished binding to eIF4A and eIF3 (Fig. 7) and translational activity (Fig. 8), indicating that this domain is essential for eIF4A and eIF3 binding and therefore for translational activ-

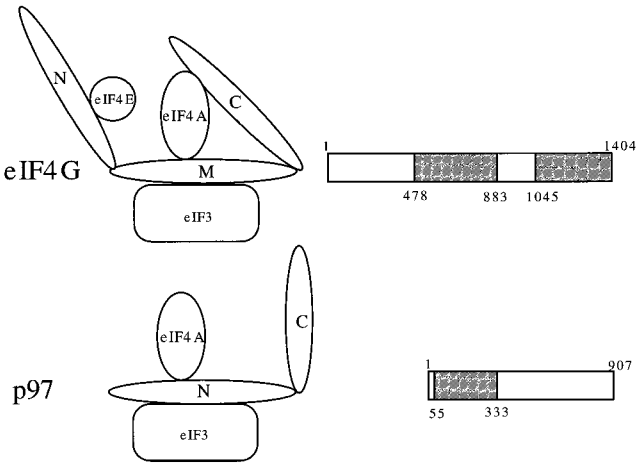

FIG. 10. Model for the interaction of eIF4G and p97 with translation initiation factors. (Left) Human eIF4G binds eIF4A, eIF4E, and eIF3, while p97 binds eIF4A and eIF3. (Right) eIF4A binding domains (shaded areas) in eIF4G and p97 are schematically indicated.

ity. Binding of eIF4A to this domain is not through eIF3, as it occurs in the absence of eIF3 in an *E. coli* extract (Fig. 9). The middle domain is likely to bind eIF4A and eIF3 independently and simultaneously, since, in an in vitro binding assay, absence of eIF4A or addition of an excess of eIF4A did not abrogate eIF3 binding to p97 (8), which binds eIF4A only through the conserved domain. It is possible, however, that mutations (M-1 to M-4) of the conserved amino acids (Fig. 7) cause drastic changes in the structure of the middle domain of eIF4G. Co-crystallization of this domain with eIF4A may be required to elucidate how eIF4A binds to the middle domain.

The existence of the C-terminal eIF4A binding domain (aa 1045 to 1404) in eIF4G is not universal. Yeast eIF4Gs possess no corresponding region (5), and wheat p86 possesses part of the domain with low homology (12%) to mammalian eIF4G (1, 13), suggesting that, in contrast to the middle domain, the C-terminal eIF4A binding domain of mammalian eIF4G functions as a modulator of translation in a manner that is not conserved through evolution. Furthermore, a C-terminal eIF4A binding activity is not exhibited by the translational inhibitor, p97 (see Fig. 10). Although the C-terminal regions of eIF4G and p97 encompassing aa 1045 to 1404 and aa 542 to 907, respectively, exhibit 28% identity, sequences in eIF4G that are not conserved in p97 must be involved in eIF4A binding. It remains to be determined whether the translational inhibition by p97 is due to the difference from eIF4G in the C-terminal region. Studies to determine the affinities between the two different eIF4A forms (eIF4AI and eIF4AII) (18) for eIF4G and p97 are also required to better understand the different biological activities of these molecules.

Our functional analysis showed that the C-terminal-third region of eIF4G (aa 934 to 1404) containing an eIF4A binding site is necessary for EMCV-IRES-dependent translation (Fig. 8). In contrast, using a reconstituted ribosome binding assay, Pestova et al. showed that a middle portion of eIF4G (aa 457 to 934) is sufficient to mediate internal entry of 43S preinitiation complexes to EMCV-IRES (21). However, it is not clear whether the complex is a translationally active precursor. They also showed that eIF4A cross-linked weakly to EMCV-IRES RNA in the presence of the middle portion of eIF4G but that cross-linking was considerably stimulated in the presence of the C-terminal two-thirds fragment (aa 457 to 1404). It is conceivable that the C-terminal fragment, by binding to eIF4A, engenders a conformational change that promotes better interaction of eIF4A with RNA. This would then lead to enhanced translation. In vitro reconstitution systems may allow translation with only the middle portion of eIF4G, because of the use of unusually high concentrations of translation factors. It remains to be determined, however, whether the necessity of the C-terminal region for translation is due solely to its eIF4A binding activity or to another unknown function(s).

Our results raise an important question. How many eIF4A molecules are bound to eIF4G? There are two models to explain our results. One model is depicted in Fig. 10: eIF4G binds one eIF4A molecule through two different binding sites on eIF4G and eIF4A. The alternative model is that two eIF4A molecules bind to one eIF4G molecule, one to the middle third and one to the carboxy-terminal third. Mapping of the eIF4G binding domain(s) on eIF4A will help to distinguish between these two possibilities.

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