A new translational regulator with homology to eukaryotic translation initiation factor 4G

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Introduction

All cellular (except organellar) eukaryotic mRNAs possess a cap structure, m^7GpppN (where N is any nucleotide) at the 5’ terminus. Multiprotein complexes are formed at or near the 5’ end of the mRNA during translation initiation. Eukaryotic translation initiation factor 4G (eIF4G) serves as a scaffold for eIF4E and eIF4A to form a protein complex, eIF4G. eIF4E binds to the cap structure to position the complex near the 5’ terminus 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 (5’-UTR) of the mRNA to facilitate ribosome binding (for reviews, see Merrick and Hershey, 1996; Pain, 1996; Sonenberg, 1996). eIF4F is a more efficient RNA helicase than free eIF4A (Rozen et al., 1990), consistent with the idea that eIF4F recycles through the eIF4F protein complex to function in unwinding (Pause et al., 1994b). eIF3 is a large protein complex consisting of at least eight polypeptides (Merrick and Hershey, 1996). The interaction between eIF3 and eIF4G juxtaposes the 40S ribosome next to the 5’ end of the mRNA (Pain, 1996).

Proteins that specifically inhibit cap-dependent translation have been described (Lin et al., 1994; Pause et al., 1994a): 4E binding proteins 1 and 2 (4E-BP1 and 4E-BP2) bind to eIF4E and prevent their association with eIF4G because 4E-BPs and eIF4G share a common site for eIF4E binding (Haghighat et al., 1995; Mader et al., 1995). Upon treatment of cells with insulin and growth factors, 4E-BPs become phosphorylated. This leads to the dissociation of 4E-BPs from eIF4E and subsequent formation of the eIF4F complex, which results in stimulation of translation (Lin et al., 1994; Pause et al., 1994a; Beretta et al., 1996).

Several members of the picornavirus family including poliovirus, whose translation is cap independent, prevent cellular mRNA translation by cleaving eIF4G into N- and C-terminal fragments (Etchison et al., 1992; Liebig et al., 1993; for review, see Belsham and Sonenberg, 1996). The binding site of eIF4E is present in the N-terminal third of the cleaved eIF4G, while eIF4A and eIF3 binding sites are present in the C-terminal two-thirds. Here, we describe a new eukaryotic translational regulator (hereafter called p97) which exhibits 28% identity to the C-terminal two-thirds of eIF4G. p97 mRNA has no initiator AUG and translation starts exclusively at a GUG codon. The GUG-initiated open reading frame (907 amino acids) has no canonical eIF4E binding site. p97 binds to eIF4A and eIF3, but not to eIF4E. Transient transfection experiments show that p97 suppresses both cap-dependent and independent translation, while eIF4G supports both translation pathways. Furthermore, inducible expression of p97 reduces overall protein synthesis. These results suggest that p97 functions as a general repressor of translation by forming translationally inactive complexes that include eIF4A and eIF3, but exclude eIF4E.

Keywords: eIF4G/initiation factor/translation/translation initiation/translational regulation

Results

The GUG-initiated open reading frame encodes a variant of eIF4G

A human cDNA clone #20881 (hereafter called clone A, nucleotide positions 473–3820 in Figure 1) from Human Genome Science Inc. was found to possess an open reading frame (ORF) encoding a protein (850 amino acids) similar to eIF4G. RNA synthesized from clone A produced no protein in a reticulocyte lysate translation system (data not shown). The ORF of clone A had no translation initiator ATG; the first in-frame ATG (nucleotide position 925) was unlikely to be the initiation codon, since a sequence upstream of this ATG (nucleotides 556–576, Figure 1) encodes the amino acid stretch ILNKLTP, which is perfectly conserved in eIF4G (amino acids 572–578, Figure 6). Thus, upstream sequences which provide the
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Fig. 1. Nucleotide and amino acid sequence of human p97. The first 11 nucleotides are derived from 5'-RACE. ATG and translation stop codons in the 5'-UTR and the poly(A) signal are underlined. The translation initiator GTG is boxed. The 5'-ends of cDNAs that were used for sequencing and expression are shown by arrows. Two peptide sequences used for antiserum production are underlined. The DDBJ/EMBL/GenBank accession No. is U73824.

translation initiator are lacking from clone A. The 3'-terminus is complete because of the presence of the poly(A) signal and a poly(A) tail (Figure 1). To obtain a full-length cDNA, we screened a human placenta cDNA library with a 5'-sequence of clone A. λ4-1, the longest polypeptide shorter than p97-HA (Figure 3, compare lanes 2 and 4). The apparent Mr of this shorter protein is 95 kDa, clone obtained, starts at nucleotide position 12 in Figure 1, and the sequence was extended by 11 nucleotides which is close to the size predicted from the sequence of clone A (94 kDa, ORF of clone A plus HA). These results indicate that the full-length cDNA encodes a protein which is larger than that encoded by the ORF starting from ATG-terminus is complete because of the presence of the poly(A) signal and a poly(A) tail (Figure 1). To obtain a full-length cDNA, we screened a human placenta cDNA library with a 5'-sequence of clone A. Although there is one ATG triplet in the 5'-upstream region (nucleotide position 21 in Figure 1) is the translation initiator, since it could potentially start an ORF that encodes a polypeptide of ~100 kDa, and the nucleotide sequence flanking this triplet (gccgccaaaGUGgag) is similar to the consensus sequence, To determine the capacity of the full-length cDNA to encode a protein, a modified cDNA, 4-1-A(HA), in which the hemagglutinin (HA) epitope was fused to the C-terminus of the ORF, was transfected into COS-1 and HeLa cells. Western blotting and immunoprecipitation with anti-HA antibody demonstrated that a 97 kDa protein (called, p97-HA) was synthesized (Figure 3, compare lanes 1 and 2). Transfection of a truncated cDNA, ATG-A (HA), in which an artificial ATG was inserted in-frame at position 473 to initiate the ORF of clone A, yielded a polypeptide shorter than p97-HA (Figure 3, compare lanes 2 and 4). The apparent M, of this shorter protein is 95 kDa, which is close to the size predicted from the sequence of clone A (94 kDa, ORF of clone A plus HA). These results indicate that the full-length cDNA encodes a protein which is larger than that encoded by the ORF starting from position 473. One explanation for this is that translation initiator GTG is boxed. The 5'-ends of cDNAs that were used for sequencing and expression are shown by arrows. Two peptide sequences used for antiserum production are underlined. The DDBJ/EMBL/GenBank accession No. is U73824.
p97-HA protein than the wild-type from similar amounts of RNA (Figure 4A, compare lanes 2 and 3), while the GGG mutant failed to produce any protein (lane 4). In vitro translation experiments confirmed the in vivo results (Figure 4B). When the 4-1-A(HA) RNA was translated in a rabbit reticulocyte lysate, p97-HA was synthesized as a single product (Figure 4B, lane 2). A point mutation (GUG → GGG) abolished translation (lane 3), while a mutation to AUG increased translation of p97-HA by 2-fold (lane 4). From these data, we conclude that translation of p97 mRNA derived from the clone 4-1-A cDNA starts exclusively at the GUG codon (position 307) to encode a polypeptide of 907 amino acids (Figure 1). This mode of translation apparently is not specific to the human p97 mRNA, since the cDNA sequence of the mouse p97 homolog also lacks an initiator ATG, and the GTG codon is conserved (J. Shaughnessy, accession No. U63323).

To verify the presence of p97 protein in cells, we used two different antisera raised against p97 peptide sequences (Figure 1, see underlined peptide sequences) in Western blotting. Experiments were performed with extracts from a mouse cell line, Neuro2A, since anti-GST–C-terminus pre-absorbed with GST–C-terminus peptide (INT) (lane 3) and anti-peptide (INT) after incubation with the peptide (lane 4). Extract (60 μg protein) from Neuro2A (lane 1) or extract (60 μg protein) from HeLa cells infected with vTF7-3 and transfected with pcDNA3 (lane 2) or a p97 expression vector, pcDNA3-Bam-ATG-p97 (lane 3) was resolved by SDS–PAGE. Western blotting was done with anti-p97 (INT) antiserum.

Fig. 2. Expression of the human p97 mRNA. Cell line (A) and human tissue (B) poly(A)+ RNA blots (CLONTECH) were probed with clone A (nucleotides 473–3822, Figure 1) (upper panel) and with β-actin (lower panel).

Fig. 3. Expression of proteins from cDNAs. Vector alone (lane 1), HA-tagged full-length cDNA, 4-1-A(HA) (lane 2), or its truncated versions; 6-4-A(HA) (lane 3) and ATG-A(HA) (lane 4; an artificial ATG in this construct was inserted at position 473 in Figure 1) were transfected into COS-1 or HeLa cells which previously had been infected with vTF7-3 as described in Materials and methods. Cells were processed for Western blotting (W.B.) with anti-HA antibodies or labeled with [35S]methionine for immunoprecipitation (I.P.) using anti-HA antibodies. cDNAs are schematically shown above.

Fig. 4. Mutational analysis of the GUG translation initiation codon. GTG (nucleotide position 307, Figure 1) of 4-1-A(HA) (Figure 3) was mutated into GGG or ATG. (A) HeLa cells were infected with vTF7-3 and transfected with pcDNA3 (lane 1), pcDNA3-4-1-A(HA) (lane 2), pcDNA3-4-1-A(HA)-ATG (lane 3) or pcDNA3-4-1-A(HA)-GGG (lane 4). Cells were processed for Western blotting with anti-HA antibodies (upper panel) and for RNase protection assay with an antisense sequence to the HA sequence (lower panel). (B) An equal amount of in vitro transcribed RNA from the indicated plasmids was translated in a reticulocyte lysate in the presence of [35S]methionine, and radioactive products were analyzed by SDS–PAGE. Lane 1, no RNA.

Fig. 5. Identification of p97 protein in cells. (A) Extract (60 μg protein) from Neuro2A cells was resolved by SDS–PAGE. Western blotting was done with the following antisera: anti-GST–C-terminus (lane 1), anti-GST–C-terminus pre-absorbed with GST (lane 2), anti-peptide (INT) (lane 3) and anti-peptide (INT) after incubation with the peptide (lane 4). (B) Extract (60 μg protein) from Neuro2A (lane 1) or extract (60 μg protein) from HeLa cells infected with vTF7-3 and transfected with pcDNA3 (lane 2) or a p97 expression vector, pcDNA3-Bam-ATG-p97 (lane 3) was resolved by SDS–PAGE. Western blotting was done with anti-peptide (INT) antiserum.
The 65 kDa polypeptide is likely to be a cross-reacting material, since another serum, anti-peptide (INT) serum, did not detect this band (Figure 5A, lane 3). In contrast, the 97 kDa band was also detected by the latter serum (lane 3), and disappeared by treatment of the serum with the peptide (INT) (lane 4). Thus, the 97 kDa polypeptide is the only common polypeptide that is recognized specifically by the two different antisera. To substantiate further the authenticity of the 97 kDa polypeptide, we expressed non-tagged p97 from a CDNA (Figure 5B). For this experiment, HeLa cells were employed because of their superior transfection efficiency. The amount of p97 was increased by 4-fold following transfection with a non-tagged p97 expression plasmid, pcDNA3-Bam-ATGp97 (Figure 5B, compare lanes 2 and 3). Therefore, clearly, p97 is translated from the endogenous mRNA.

**p97 binds to eIF4A and eIF3, but not to eIF4E.**

Alignment of human p97 and eIF4G amino acid sequences reveals that p97 exhibits overall 28% identity and 36% similarity to the C-terminal two-thirds of eIF4G (Figure 6). The N-terminal third of eIF4G, to which eIF4E binds (Lampheear et al., 1995; Mader et al., 1995), bears no similarity to p97. Therefore, no canonical eIF4E binding site (Mader et al., 1995) is found in p97. Lampheear et al. (1995) showed that the C-terminal two-thirds of the poliovirus protease-cleaved eIF4G contains the binding sites for eIF4A and eIF3. Thus, it is predicted that p97 would bind to eIF4A and eIF3, but not to eIF4E. To examine this, HA-tagged p97 and eIF4G were expressed in HeLa cells, and cell extracts were immunoprecipitated with anti-HA antibody. The immunoprecipitates were assayed by Western blotting for eIF4A (Figure 7A, upper panel), eIF3 (middle panel) and HA-tagged protein expression (lower panel). Both eIF4A and eIF3 were co-precipitated with p97 and eIF4G (Figure 7A, lane 3 and 4; the p115 subunit of eIF3 is shown, because it exhibits the strongest reactivity towards anti-eIF3 antibody among all subunits), while an unrelated RNA binding protein, La autoantigen (Chambers et al. 1988) failed to precipitate either factor (lane 5).

The light chain of the HA-tagged antibody co-migrates with eIF4E on SDS–PAGE, rendering detection of immunoprecipitated eIF4E difficult. To circumvent this problem, we co-expressed FLAG-tagged eIF4E, which migrates more slowly than non-tagged eIF4E (Figure 7B, upper panel), with HA-tagged p97, eIF4G or La. Cell extracts were immunoprecipitated with anti-HA, and the immunoprecipitates were examined by Western blotting for eIF4E (middle panel) and HA-tagged proteins (lower panel). No detectable FLAG-eIF4E was co-precipitated with p97 (Figure 7B, lane 3) or with La (lane 5), while eIF4G was able to precipitate FLAG-eIF4E (lane 4). The amount of FLAG-eIF4E (Figure 7B, upper panel, lane 3) is 2.5-fold less than that in lane 4, since p97 suppresses translation in general, as shown in Figure 9. However, no FLAG-eIF4E was detectable in the immunoprecipitates of p97, even upon a longer exposure of the X-ray film (data not shown).

To substantiate these results further, p97 or eIF4G was synthesized in vitro and mixed with bacterially expressed FLAG-eIF4E or FLAG-eIF4A bound to the anti-FLAG resin. Proteins bound to the anti-FLAG resin were eluted with the FLAG peptide, p97 and eIF4G specifically bound to eIF4A (Figure 8, upper panel, lanes 1 and 2, respectively). In contrast, binding of p97 to eIF4E was not detectable (lower panel, lane 1), while an interaction between eIF4G and eIF4E was evident (lower panel, lane 2). La failed to bind to either resin (lane 3). We were not able to perform similar experiments for eIF3, since it consists of multi-subunits, and it is not known which subunit(s) interact(s) with eIF4G or p97. Thus, we conclude that p97 forms a protein complex which includes eIF4A and eIF3, but excludes eIF4E.

**p97 suppresses cap-dependent and cap-independent translation**

Ohlmann et al. (1996) showed that the C-terminal two-thirds fragment of eIF4G supports cap-independent translation. Based on its homology to eIF4G, p97 might also promote cap-independent translation. To explore this possibility, we expressed p97 and eIF4G in HeLa cells together with a reporter CAT (chloramphenicol acetyltransferase) mRNA, whose ORF is preceded by the encephalomyocarditis virus internal ribosome entry site (EMCV-IRES). Translation of EMCV-IRES-CAT mRNA was repressed 2-fold by expression of p97 (Figure 9A, lane 2). In contrast, eIF4G stimulated cap-independent translation by 2-fold (lane 4; these experiments were repeated four times with <10% variation between the results). Moreover, the p97-induced repression of translation was not apparent in cells containing an increased amount of eIF4G (compare lanes 2 and 3), consistent with the idea that p97 inhibits cap-independent translation by competing with eIF4G. To study the effect of p97 on cap-dependent translation, CAT mRNA was used as the reporter. Similarly to its effect on cap-independent translation, p97 inhibited cap-dependent translation by 2-fold (Figure 9B, lane 2) and expression of eIF4G stimulated translation (lane 4).

To study how p97 expression affects overall protein synthesis in cells, we established a cell line that expresses p97 under a tetracycline-regulatable promoter (Beauparlant et al., 1996). Withdrawal of tetracycline from the medium increased the amount of p97 by ~4-fold without a noticeable change in the amounts of eIF4G, eIF4E or eIF4A (Figure 10A, compare lanes 1 and 2). Overexpression of p97 decreased the rate of protein synthesis by 20–25% as determined by incorporation of [3H]leucine (Figure 10B). We performed similar labeling experiments with [35S]methionine and obtained essentially similar results (data not shown). These functional assays, combined with the binding results (Figures 7 and 8), suggest that p97 is a general suppressor of translation by forming a translationally inactive protein complex that includes eIF4A and eIF3, but excludes eIF4E.

**Discussion**

We have cloned a new translational regulator, p97, which is homologous to the C-terminal two-thirds of eIF4G. This region of eIF4G contains binding sites for eIF4A and eIF3, while the binding site for eIF4E is present in the N-terminal third of the protein (Lampheear et al., 1995; Mader et al., 1995). As expected from sequence homology, p97 binds to eIF4A and eIF3, but not to eIF4E (Figures 7 and 8). While the C-terminal two-thirds fragment of
C-terminal variant of eIF4G

Fig. 6. Alignment of human p97 and eIF4G amino acid sequences. The pattern-induced multi-sequence alignment program (Smith and Smith, 1992) was used to align p97 and eIF4G (accession No. D12686) amino acid sequences. Conserved amino acids are boxed. Arrows indicate the cleavage sites by the 2A proteases of Coxsackie- and rhinoviruses (Lamphear et al., 1993) and by the L protease of foot-and-mouth disease virus (Kirchweger et al., 1994). The eIF4E binding site in eIF4G (Mader et al., 1995) is underlined.

eIF4G is able to support translation initiation from the IRES of hepatitis C virus and Thiel’s murine encephalomyelitis virus (Ohlmann et al., 1996), p97 inhibits EMCV-IRES-dependent translation. It is unlikely that the opposite effects on translation are due to the different IRES elements used in the two studies, since poliovirus IRES-mediated translation was promoted by the C-terminus of eIF4G, required for cap-dependent and cap-independent translation (Pause et al., 1994b) and eIF3 is essential for while transient expression of p97 repressed translation of poliovirus IRES-CAT mRNA (H. Imataka, unpublished observations). Thus, it is probable that p97 generally inhibits IRES-dependent translation, while the C-terminal two-thirds of eIF4G generally supports IRES-dependent translation.

What is the mechanism of translational inhibition by p97? Sequestering eIF4A and eIF3 away from eIF4G is one of the plausible explanations. eIF4A is absolutely required for cap-dependent and cap-independent translation (Pause et al., 1994b) and eIF3 is essential for recruitment of ribosomes to mRNA (Pain, 1996). However, eIF4A binding to eIF4G was not decreased after inducible
Fig. 7. Immunoprecipitation of p97 and eIF4G with translation factors. (A) HA-tagged protein expression plasmids, pcDNA3-HA (lane 2), pcDNA3-HA-p97 (lane 3), pcDNA3-HA-eIF4G (lane 4) and pcDNA3-HA-La (lane 5) were transfected into HeLa cells after infection with vTF7-3. Extracts were immunoprecipitated with anti-HA antibodies and immunoprecipitates were resolved by SDS–PAGE. Western blotting performed with anti-eIF4A (upper panel), anti-eIF3 (middle panel) or anti-HA (lower panel). Mock-infected cell extract (60 μg protein) (lane 1). (B) FLAG-eIF4E expression plasmid, pAR(DR1)[59/60]-eIF4E was co-transfected with pcDNA3-HA (lane 2), pcDNA3-HA-p97 (lane 3), pcDNA3-HA-eIF4G (lane 4) or pcDNA3-HA-La (lane 5). An aliquot of the extract was removed for Western blotting with anti-eIF4E (upper panel). The remaining portion was used for immunoprecipitation with anti-HA antibodies. Western blotting of immunoprecipitates was performed with anti-eIF4E (middle panel) or anti-HA (lower panel). Mock-infected cell extract (60 μg protein) (lane 1).

Fig. 8. p97 binds to eIF4A, but not to eIF4E. p97 (lane 1), eIF4G (lane 2) and La (lane 3) proteins were synthesized in vitro in the presence of [35S]methionine and mixed with FLAG-eIF4A resin (upper panel) or with FLAG-eIF4E resin (lower panel). After washing, the bound proteins were eluted with the FLAG peptide and resolved by SDS–10% PAGE.

overexpression of p97 (H.Imataka, unpublished observation). This result is not surprising, as eIF4A is the most abundant initiation factor (Duncan et al., 1987). It is thus more likely that sequestration of eIF3 might explain the translational inhibition by p97. This model of translational inhibition is reminiscent of the mechanism by which 4E-BPs inhibit translation. 4E-BP-1 competes with eIF4G for binding to eIF4E, and thereby inhibits formation of the whole eIF4F complex (Haghighat et al., 1995). While 4E-BP-1 and eIF4E were reported to exist in reticulocyte lysate at an ~1:1 molar ratio (Rau et al., 1996), we could not determine the molar ratio of p97 to other translation factors, because of the difficulty in obtaining pure recombinant protein. The relative ratios of eIF4A, eIF4G (Duncan et al., 1987) and eIF3 (Meyer et al., 1982; Mengod and Trachsel, 1985) to ribosomes in HeLa cells have been reported to be 3, 0.2 and 0.5, respectively. Another mechanism by which p97 inhibits translation is based on recent results by Tarun and Sachs (1996), who showed that in yeast the poly(A) binding protein enhances translation by interacting with eIF4G. If this mechanism operates also in the mammalian system, p97 could potentially compete with eIF4G for interaction with the poly(A) binding protein.
Plants have two different eLF4F complexes. One is a complex of two polypeptides, p220 and p26, which are homologs of mammalian eLF4G and eLF4E. The other complex, called eLF(iso)4F, consists of p28, another homolog of mammalian eIF4G and eIF4E. The binding site for eLF4E is conserved in the N-terminus of p82 (Mader et al., 1995). eLF(iso)4F, like eLF4F, stimulates translation in vitro (Abramson et al., 1988). Yeast also has two genes encoding eLF4G homologs, TIF4631 and TIF4632 (Goyer et al., 1993). Although both contain an eLF4E binding site (Mader et al., 1995), there seems to be a functional difference between two proteins, since TIF4631-disrupted strains exhibited a slow growth phenotype, while disruption of TIF4632 failed to show any phenotype. Double gene disruption engendered lethality (Goyer et al., 1993). It is possible that p97 has evolved from eLF(iso)4F to become a repressor by losing the binding site for eLF4E.

p97 mRNA has no initiator AUG and translation starts exclusively at a GUG codon. The nucleotide sequence surrounding the initiator GUG is GCCAAAGUGGGAG, which matches the consensus rule that purines are favorable at positions –3 and +4 (the first nucleotide of the initiation codon is defined as +1) (Kozak, 1989). More importantly, p97 mRNA has adenine at the +5 position. Translation starting at a non-AUG is efficient when the second codon is GAA, where G at +4 and A at +5 are more important than U at +4 (Boeck and Kolakowski, 1994; Grunter and Jackson, 1994).

Why is GUG employed instead of AUG? Several important regulatory genes including c-myc (Hann et al., 1988), int-2 (Acland et al., 1990), pim-1 (Saris et al., 1991), FGF-2 (Florkiewicz and Sommer, 1989) and WT-1 (Bruno and Pelletier, 1996) have non-AUG initiators in addition to a downstream and in-frame AUG initiation codon, so that non-AUG-initiated translation generates amino-terminally extended proteins. Some of the extended proteins show intracellular localization different from their shorter counterparts (Acland et al., 1990; Bugler et al., 1991). In contrast, there are no multiple products from p97 mRNA, since the GUG is the only initiator. Translation initiation at CUG of c-myc mRNA was enhanced when culture medium was deprived of methionine (Hann et al., 1992). For FGF-2, eLF4F seems to activate utilization of CUG more than that of AUG (Kevil et al., 1996). The expression of p97 might also be translationally controlled.

What is the biological significance of p97? Levy-Strumpf et al. cloned p97 as a putative modulator of interferon-β-induced progranule cell death (Levy-Strumpf et al., 1997). Also, apoptosis has been shown to be affected by protein synthesis inhibitors (Martin et al., 1988; Ledda-Columbano et al., 1992; Polunovsky et al., 1994), and overexpression of eLF4F in NIH3T3 cells prevents apoptosis induced by serum depletion (Polunovsky et al., 1996). Future research on p97 should shed light on the role of translational inhibition in regulating apoptosis. We have also learned recently that p97 mRNA is heavily edited when the apolipoprotein B mRNA editing protein is overexpressed in the liver of transgenic mice (S.Yamanaka and T.Innerarity, personal communication), suggesting that the amount of p97 in the cell might be controlled by an editing mechanism.

Materials and methods

Cloning of cDNAs

The cDNA #20881 was obtained from a human embryonic DNA library by random cloning. A human placenta cDNA library in λgt11 was screened with a fragment (nucleotide positions 473–1200) of #20881. 5' RACE (rapid amplification of cDNA ends, Gibco-BRL) was performed with HeLa poly(A) + RNA and sequence-specific primers (594–614 and 643–664 in Figure 1) according to the manufacturer’s instructions.

Construction of plasmids

To generate the C-terminally HA-tagged cDNAs, an antisense primer composed of the sequences encoding the C-terminal six amino acids of p97 followed by the HA epitope peptide, YPYDVPDYAG, and nucleotides corresponding to the XhoI site was used for PCR with a primer sense (nucleotides 2527–2549 in Figure 1). pcDNA3 (Invitrogen), which contains a human cytomegalovirus (CMV) and T7 RNA polymerase promoters, was used as an expression vector for most of the experiments. pcDNA3-3-1-A(HA) and pcDNA3-3-4-A(HA) contain the corresponding p97 cDNA sequences downstream of nucleotide positions 12 and 30 in Figure 1, respectively. pcDNA3-3-ATG-A(HA) contains the sequence downstream of nucleotide 473 and a part of the sequence of transcription factor BTEB (5’ to 12, including the initiator ATG) (Imataka et al., 1992).

For an N-terminally HA-tagged construct, the initiator ATG codon and three copies of the HA sequence (Mader et al., 1995) were inserted into pcDNA3 to generate the parental vector, pcDNA3-HA. A PCR-amplified fragment from the GTG initiation codon to a SacI site (nucleotide 600) was ligated to a fragment from SacI to the 3' terminal of #20881 to construct pcDNA3-HA-p97. An EcoRI fragment of the human eLF4G cDNA (kindly provided by Dr. Rhoads; Yan et al., 1992) was used to construct pcDNA3-HA-eLF4G. HA-La (kindly provided by A.Craig, unpublished) was inserted into pcDNA3 to obtain pcDNA3-HA-La.

For expression of non-tagged p97, a fragment from BamHI (nucleotide 172) to the 3' terminal, in which the initiator GTG had been mutated into ATG, was inserted into pcDNA3 to generate pcDNA3-Bam-ATGp97. A point mutation (GTG → ATG in a cap-independent manner, the Renal ATGp97 and the EcoRI fragment of eLF4G cDNA was inserted into pcDNA3.

The poliovirus IRES was inserted into pSP72 (Promega), which contains the T7 RNA polymerase promoter, to generate pSP72IRES. For expression of p97 or eLF4G in a cap-independent manner, the Renal ATGp97 and the EcoRI fragment of eLF4G cDNA were inserted downstream of the IRES to construct pSP72IRES-p97 and pSP72IRES-eLF4G.

Transient transfection

HeLa cells were infected with recombinant vaccinia virus vTF7-3 (Fuerst et al., 1996), and then transfected with plasmids (5 μg) using Lipofectin (Gibco-BRL). For expression in COS-1 cells, plasmids (10 μg) were transfected by electroporation (Bio-Rad Gene Pulser II, 1200 V, 25 μF).

Immunoprecipitation

After transfection, HeLa and COS-1 cells were cultured for 16 and 48 h, respectively, and then labeled with [35S]methionine (100 μCi/ml) for 1 h in 3.5 cm dishes. Cells were lysed in 0.5 ml of buffer A [50 mM NaCl, 1% NP-40, 0.1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 50 mM Tris–HCl, pH 7.4]. After centrifugation, the supernatant was mixed with anti-HA antibody (2 μg/ml), washed with buffer B (1 ml, three times), and resolved by SDS–10% PAGE. After centrifugation, an aliquot (0.5 ml) was mixed with anti-HA antibody (2 μg/ml). Immunoprecipitates were washed with buffer B (1 ml, three times), and resolved by SDS–10% PAGE.
PAGE, except for eIF4E experiments where 12.5% polyacrylamide gels were used.

**Western blotting and antibodies**

Immunoprecipitates or cell extracts (60 μg protein) were resolved by SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membrane (Millipore). Protein bands were visualized by chemiluminescence (Amersham). Quantification was done with a laser densitometer (LKB).

The peptide SDTEDSSAPKSEQ (called INT for internal amino acids 788–802) conjugated with keyhole limpet hemocyanin was used to raise anti-peptide (INT) antibody in rabbits. For absorption experiments, serum (4 μl) was pre-incubated with the INT peptide (10 μg) on ice for 1 h, and was used for Western blotting. A fusion protein GST–C–terminus, i.e. GST linked to the peptide ETAAEEEEEED (amino acids 895–907), was produced in *Escherichia coli* for immunization in rabbits. The resulting serum was passed through a GST or GST–C–terminus column for adsorption.

Anti-eIF4E, anti-eIF3 and anti-eIF4G antibodies were kind gifts from Drs. H. Trachsel, J. Hershey and L. Carrasco, respectively. Anti-eIF4E antibody was described (Lazaras-Karatzas et al. 1992).

**CAT assay and RNase protection assay**

The CAT assay was performed as described (Gorman et al. 1990). The RNase protection assay was done as described (Imataka et al., 1994) with modifications as follows: as an internal control, in vitro synthesized unlabeled BTEB RNA and radiolabeled antisense RNA to the BTEB sequence (Imataka et al., 1992) were mixed with antisense CAT probe. The intensities of the CAT and BTEB signals were quantified by PhosphorImager BAS 2000 (Fuji). The amount of CAT mRNA was normalized to that of the internal control BTEB RNA.

**In vitro transcription and translation**

Capped RNA was synthesized by T7 RNA polymerase in the presence of the cap analog, m’GpppG. Rabbit reticulocyte lysate (25 μl, final volume) (Promega) was programed with 0.2 μg of mRNA in the presence of [35S]methionine (20 μCi) according to the manufacturer’s recommendation.

**Binding assay for in vitro translated factors**

Following translation, 5 μl of the lysate was incubated on ice for 30 min with anti-FLAG resin (Kodak) (20 μl) to which FLAG-eIF4E or FLAG-eIF4A had been bound. After washing with buffer C consisting of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.15 M NaCl and 0.1% NP-40 (1 ml, three times), bound proteins were eluted with 40 μl of buffer C containing 100 μg/ml FLAG peptide. pAR(DRI)[59/60] (Blanar and Rutter, 1992) was used to express FLAG-eIF4E (Pause et al., 1994a) and FLAG-eIF4A in *E. coli*.

**Inducible expression of p97**

p97 cDNA (nucleotides 36–3810 in Figure 1), in which the initiator GTG codon was converted to ATG, was inserted into a tetracycline-dependent expression vector, pREP9-CMVt (Beaurapart et al., 1996) to construct pREP9-CMVt-p97. An NIH3T3-derived cell line, S2-6 (Shockett et al. 1995), was transformed with pREP9-CMV (control) or with pREP9-CMVt-p97 using G418 (400 μg/ml). S2-6 and the established transformants were maintained in the presence of 1 μg tetracycline/ml. To induce p97 expression, cells were cultured in medium without tetracycline for 40 h. After induction, cells were processed for Western blotting or labeled with [2,3,5-3H]leucine (20 μCi/ml). Cells were lysed in buffer B, and extracts (20 μg protein) were applied to filter paper (Whatman). After washing with trichloroacetic acid (5%), radioactivity remaining on the paper was counted.

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**References**


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