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

Hiroaki Imataka, Henrik S.Olsen¹ and Nahum Sonenberg²

Department of Biochemistry and McGill Cancer Centre, McGill University, 3655 Drummond Street, Montreal, Quebec, Canada H3G 1Y6 and ¹Human Genome Sciences Inc., 9620 Medical Center, Rockville, MD, USA

²Corresponding author

Translation initiation in eukaryotes is facilitated by the cap structure, m⁷GpppN (where N is any nucleotide). Eukaryotic translation initiation factor 4F (eIF4F) is a cap binding protein complex that consists of three subunits: eIF4A, eIF4E and eIF4G. eIF4G interacts directly with eIF4E and eIF4A. The binding site of eIF4E resides in the N-terminal third of 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

Introduction

All cellular (except organellar) eukaryotic mRNAs possess a cap structure, m⁷GpppN (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 initiation factor 4G (eIF4G) serves as a scaffold for eIF4E and eIF4A to form a protein complex, eIF4F. 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 eIF4A 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.*, 1982; 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 the C-terminal two-thirds contains the binding sites for eIF4A and eIF3 (Lamphear *et al.*, 1995; Mader *et al.*, 1995). Thus, these viruses inhibit cap-dependent translation by separating eIF4E from eIF4A and eIF3. Translation of viral RNA is not affected, since the C-terminal fragment of eIF4G can support cap-independent translation (Ohlmann *et al.*, 1995, 1996). Another member of the picornaviruses, encephalomyocarditis virus, inhibits cellular translation, at least in part by inhibition of phosphorylation of 4E-BPs (Gingras *et al.*, 1996).

Here, we describe a new translational regulator with homology to the C-terminal fragment of the picornavirus-cleaved eIF4G.

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

```

1  cag cag tga gtc gga gct cta tagg agg tgg cag cgg gta cgg agt ggc ggc tgc agc agc 60
gac tcc tca tagg cta agt tga agt ccc tcc ccc tcc tcc tcc ccc tcc tcc tcc tcc tcc 120
ttt ttt ttt ttt ccc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc 180
gga ggc agc tgc ggt ggc agc tgc tga att ctc tcc tcc tcc tcc tcc tcc tcc tcc tcc 240
cct ccc ccc ccc ccc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc tcc 300
ccc aaa ggg gag agt agt agt gca gaa ggg ggt ggt ggt ggt ggt ggt ggt ggt ggt ggt 360
      V E S A I A E G G A S R F S A S S G 18

gga gga gga agt agt ggt gca cct cag cat ccc aag act ggc aac agc gag ttc 420
G G G S R G G A P Q C H Y P K T A G N S E F 38

ctg ggg aaa acc cca ggg caa aac gct cag aaa tgg att cct gca cga agc act aga cga 480
L G K T P G Q N A Q K W I P A R S T R R 58
gat gac aac tcc gca gca aac aac tcc gca aac gaa aaa cga cat gat gca tcc ttc 540
D D N S A A N N S A N E K E R H D A I F 78
agg aaa gta aga gga ata aat aag ctt act cct gaa aag ttt gac agc cta tgc ctt 600
R K V R I L N K L P E K F D K L C L 98
gag ctc ctc aat grg ggt gta gat ctt aaa cct ctt aaa ggg gtc ata ctg ctg att 660
E L L N V G V E S K L I L K G V I L L L I 118
gtg gca aaa gcc cta gaa gag cca aag yat agc tca ctg tat gct cag cta tgc ctg cga 720
V D K A L E E P K Y S S L Y A Q L C L R 138
LT gca gaa gat gca cca aac ttt gat ggc cca gca gaa gag ggt caa cca gga cag aag 780
L G A E D A P N F D G P A A E G Q P G Q K 158
caa agc acc cca ttc aga cgc ctc ctt att tcc aaa tta caa gat gaa ttt gaa aac cga 840
Q S T T F R R L L I S K L Q D E F E N R 178
act aga aat gtt gat gtc tat gat aag cgt gaa aat ccc ctc ctc ccc gag gag gag gaa 900
T R N V D Y D K R E N P L L P E E S E 198
cag aga gcc att gct agc atc agt tgg gga aac atc aaa ttc att gga gag ctt ggc 960
Q R A I A K I K M L G N I K P I G E L G 218
aag ctt gat ctt att cag gaa tct atc ctt cag agt tgc atc aaa cca ctt ttg gaa aag 1020
K L D L I H E S I L H K C I K T L L E K 238
aag aag aga gtc caa ctc aaa gat atg gga gag gat ttg gag tgc ctc tgc ata atg 1080
K K R V Q L K D M G E D L E C L C Q I M 258
agg aca gtg gga cct aga tta gac cat gaa cga gcc aag tcc tta atg gat cag tat ttt 1140
R T V G P R L D H E R A K S L M D Q Y F 278
ggc cga att tgc tcc ttg att tta agt aag gaa tgc cca gca agt att cgt ttc ctg ctg 1200
A R M C S L M L S K E L P A R I R F L L 298
cag gat acc gta gag ttg gca gaa acc cat tgg gtt cct cgc aag gct ttt ttg gac aat 1260
Q D T V E L R E H H V P R K A F L D N 318
gga cca aag agc atc aat caa att cgt cea gat gta aaa gat cta ggg ggt ttt att 1320
G P K T I N Q I R Q D A V K D L G V F I 338
cct gct cct atg gct caa ggg att aga agt gac ttc ttg gga cga cgc ttc atg cca 1380
P A P M A G G M R S D P F L E G P P M P 358
ccc agt atg aaa atg gat agg gac cca ctt gga gga ctt gct gat atg ttt gga caa atg 1440
P R M K M D R D P L G G L A D M F G Q M 378
caa ggt agc gga att ggt act ggt cca gga gtt atc cag gat aga ttt tca ccc acc att 1500
P G S G I G T G P G V I Q D R F S P T M 398
gga cgt cat cgt tca aat cca ctc ttc aat ggc atg ggg gga cac atc act ccc aca 1560
G R H R S N Q L H H G H Q G H I M P T 418
caa cgt cag ttt gga gag att gga ggc aag ttt atg aaa agc cag ggc cta agc cag ctc 1620
S Q F G E M G C K P M K S Q G L S Q L 438
tac cat aac cag agt cag gga gtc tta ccc cag ctg caa gga cag tgc aag gat atg cca 1680
Y H N Q Q S Q G L L S Q Q L Q G Q S K D M P 458
cct cgt ttt tct aag aaa gga cag ctt aat gca gat gat agt agc ctg agc cct cag 1740
R P F S K K G Q L N A D E I S L R P A Q 478
tcg ttc cta atg aat aaa aat caa gtc cca aag ctt cag ccc cag ata act agt att cct 1800
S F L M N K N K Q V P K L Q P Q I T M I P 498
cct agt gca caa cca cca cgc act cca cca cct ctg gga cag aca cct cag ctt ggt 1860
P S A Q Q P P R T Q T P L Q Q T P L Q 518
ctc aaa act aat cca ctt atc cag gaa agc cct gcc aag acc agc aaa gga cca cca 1920
L K T N P P L I Q E K P A K T S K K P P 538
ctc tca aag gaa gaa ctc ctt aaa cta act gaa act gtt ggt act gaa tat cta aat agt 1980
P S K E E L L K L T E T V V T E Y L N S 558

```

```

gga aat gca aat gac gct gtc aat ggt gta aga gaa atg agg gct cct aaa cac ttt ctt 2040
G N A N E A V N G V R E M R A P K H F L 578
cct gag atg tta agc aaa gta atc atc tgc tca cta gat aga agc gat gaa gat aaa gaa 2100
P E M L S K V I I L S L D R S D E K E 598
aaa gca agt tct ttg atc agt tta ctc aaa cag gaa ggg ata gcc aca agt gac aac ttc 2160
K A S S L I S L L K Q G E I A T S D N F 618
atg cag gct ttc ctg aat gta ttg gac cag tgc ccc aaa ctg gag gtt gac atc ctt ttg 2220
M Q A F L N V L D Q C P K L E V D I P L 638
vtg aaa tcc tat tta gca cag ttt gca cgt ggc act ttc tca gat ggt agc att 2280
V K S Y L A Q F A A R A I I S E L V S I 658
tca gaa cta gct caa cca cta gaa agt ggc acc cat ttt cct ctc ttc cta ctt tgt ctt 2340
S E L A Q P L E S G T H F P L F L L C L 678
cag cag tta gct aaa tta caa gat cga gaa tgg tta aca gaa ctt ttt caa cca agc aag 2400
Q Q L A K L Q D R E W L T E L F Q Q S K 698
gtc aat atg cag aaa atg ctc cca gaa att gat cag aat aag gac cgc atg ttg gag att 2460
V N M Q K M L P E I D Q N K D R M L E I 718
ttg gaa gga aag gaa ctg agt ttc tta ttc cca ctc ctc aaa ttg gag aag gaa ctg ttc 2520
L E G K C L S F L F P L L K L E K E L 738
aag caa ata aag ttg gat cca tcc cct caa acc ata aaa tgg att aag gat aac atc 2580
K Q I K L D P S P Q T I Y K W I K D N I 758
tct ccc aaa ctt cat gta gat aaa gga ttt gtc agt cta tta atg act agt ttc tta cag 2640
S P K L H V D K G F V N I L M T S F L Q 778
tac att tct agt gaa ata ccc ccc agc gat gaa aca gat tca tcc tct gct cct ctc 2700
Y I S S E V N P P S D E T D S S A P S 798
aaa gaa cag tta gac cag gaa aaa caa cta cta tct ttc aag cca gta atg cag aaa 2760
K E O L E Q E K Q L L L S F K P V M Q K 818
ttt ctt cat gat cag gtt gat cta caa gct agt gcc ctg tat gct ctc cag gtc cag tgc 2820
F L H D H V D L Q V S A L Y A L Q V H C 838
tat aac agc aac tcc cca aaa ggc atg tta ctt cgc ttt ttt gtc cag ttc tat gat agt 2880
Y N S N F P K G M L R F V H F Y D M 858
gaa att att gaa gaa gaa gct ttc ttg gct tgg aaa gaa gat ata acc caa gag ttt cgg 2940
E I I E E A F L A W K E D I T Q E F P 878
gga aaa ggc aag cgt ttg ttc cag gtc aat cag tgg cta acc ttg gaa act gct gaa 3000
G K G K A L F Q V N Q W L T W L E T A E 898
gaa gaa gaa tca gag gaa gac gct gac taa aga acc agc cca gac ctt aaa ttg tgc aaa 3060
E E E S E E E A D *
aca tac tgt tgc tat gat gta act gca ttt ttg gac cta acc act cgc aaa att cat tcc gct 3120
gta att ttt tca caa tat tta aag cag aag cag gtc agt gat gat ttc ctt ctg cat aag 3180
gtt ttt ttg gat tgt aat gtc tta atc ata gtc tat cat caa ata ttt tag gag tat ctt 3240
taa ttt tta gat agt ata tta gca gca tgc aat aat tca atc ata agt tct caa gca gag 3300
gca gtc tat tgc aag gac ctt ctt tgc tgc cag tta cta agt gtt tta agt gat ttt 3360
act gaa tag caa cca tga ata ctg tag aaa tgc act ttg ctc agt aat cta gtt gtt 3420
gca ata ttt gat tat cca ttt ggt tgt tgc aga aaa att ctt aac tgc aat tga tgg ttt 3480
ttg cgc taa tag tat att ggc tgt att tct acc tct agt aat ggc ctt tat gtc cta gat 3540
ttt aat atc ctt gac cct ggg cca gtc cag aag tct ttt taa aag aaa cat ggt tta ctt 3600
gca caa aac tga tca gtt ttg aga gat cgt taa tgc cct tga agt ggt ttt tgt ggt tgt 3660
gaa aca aat gct gag aat ttg aat tgg tcc ctc cta tta gat tat tga aat taa gtc tat 3720
tta att tat gtc atg ttc atg ccc tga ttt tat ata ctg tta tct atc aat aaa cat 3780
tgt gat act tga aaa aaa aaa aaa aaa aaa aaa 3820

```

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. λ 4-1, the longest clone obtained, starts at nucleotide position 12 in Figure 1, and the sequence was extended by 11 nucleotides by 5' RACE (Figure 1). The longest sequence (3820 nucleotides) (Figure 1) is close to full-length, since Northern blotting showed that the mRNA is ~3.8 kb in length (Figure 2). We have performed numerous 5' RACE experiments but have not obtained any further 5' sequence. In addition, the 5' end of cDNA for the mouse p97 homolog, which recently has been deposited into the DDBJ/EMBL/GenBank databases (U63323), is shorter by 24 nucleotides than that of the human clone (Figure 1). The mRNA is expressed in every tissue and cell line examined (Figure 2), implying a fundamental role for the protein in cells. The first ATG of the extended ORF is the same as that identified in clone A (nucleotide position 925) and it is preceded by in-frame stop codons at nucleotide positions 178 and 205. The sequence 4-1-A (12–3820 in Figure 1) was used for further studies.

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_r 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 of the 97 kDa protein starts at a non-AUG initiator 5' upstream of clone A. Although there is one ATG triplet in the 5' upstream region (nucleotide position 21 in Figure 1), it is predicted to encode a small polypeptide (16 amino acids), and is out-of-frame of clone A ORF. Furthermore, transfection of 6-4-A(HA), which does not contain this ATG (Figure 1), into cells produced a protein indistinguishable from p97-HA (Figure 3, lane 3).

We predicted that GTG at nucleotide positions 307–309 (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, gccgcc/gcc(nonAUG)ga/cu, for non-AUG initiators (Boeck and Kolakofsky, 1994; Grunert and Jackson, 1994). To test this possibility, we mutated the GTG into GGG or ATG in the 4-1-A(HA) construct, and transfected the DNA into HeLa cells. The ATG mutant yielded 4-fold more

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.

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

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 pre-absorbed with 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.

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 serum (which was raised against the C-terminal sequence of p97) pre-absorbed with GST or GST-C detects non-specific interacting polypeptides with M_r s between 95 and 100 kDa in extracts from primate cell lines including HeLa and COS-1, which obscure the p97 band (data not shown). Anti-GST-C-terminal peptide antiserum detected two bands with apparent M_r s of 97 (indicated by an arrow) and 65 kDa (Figure 5A, lane 2), both of which disappeared when the serum was absorbed with the antigen (lane 1).

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 (Lamphear *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. Lamphear *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 (Lamphear *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

eIF4G	1	MSGARTASTPTPPQTGGSLEPQANGETPQVAVIVRPDDRSQGAIIADRPGLPGP
p97		
eIF4G	55	EHSPPSESQPSSTPTSPSPVLEPGSEPNLAVLSIPGDTMTTIQMSVEESTPIS
p97		
eIF4G	109	RETGPEYRLSPEPTPLAEPILEVEVTLSKPVPESEFSSSPLQAAPTPLASHTVEI
p97		
eIF4G	163	HEPNGMVPSSEDLPEVESSPELAPPPACPSSESPVPIAPTAQPEELLNGAPSPPA
p97		
eIF4G	217	VDLSPVSEPEEQAKEVTASVAPPTIPSATPATAPSATSPAQEEEMEEEEDEEEG
p97		
eIF4G	271	EVGEAGEGESEKRGEEELLPESTPIPANLSQNLEAAAATQVAVSVPKRRRIKE
p97		
eIF4G	325	LNKKEAVGDLDAFKEANPAVPEVENQPPAGSNPGPSESGSVPPRPEEADETW
p97		
eIF4G	379	DSKEDKIHNAENIQPGEQKYEYKSDQWKPPNLEEKRYDRELLGFQFIFCQMQ
p97		
eIF4G	433	KPEGLPHISDVVLDKANKTPLRLDPTRLQGINCSPDFTSPFANLGRRTTLSTRG
p97	1	MESA
eIF4G	487	PPRGGP GGELARGAQAQGLGPRHSQQGPRKEPRKIATVLMTEDIKLNKAQKAWK
p97	5	IAEGG-ASRF--SAISSGGGGSRG--APQHYPKTAGNSEFLGKTPGQN-AQK-MWI
eIF4G	541	PSKRRTAADKDRGEEADAGSKTQD-LFRWRHSILNKLTTPQMFFQQLMKQVTLAII
p97	52	PARSTRRDDNSAANNSANEKERHDAIFRKVRGILNKLTPEKFDKLCLELLNVGV
eIF4G	594	DTEDASKGLSFLRRPFQSP--TSLWP IQHVP LPHGA--ESAHYQKPT--V
p97	106	ESKILLKGVILLIVDKALEPKYSSLYAQLCLRLLAEDAPNFDGPAEAGQPGQK
eIF4G	640	TVNTRKLLLNRCQKFEFKDDKDDDEVFERKQKQEMDEAATAEERERKKEE-EEAR
p97	160	STTFRRLIISKLODFEFENRTRNVQVYDKRENPL--LPEE-EEQRA
eIF4G	694	IARRCSLGNKFFIGELFKLKM L TEA I L M H D C V V K L L K N H D E - - - - - E S L E C L C
p97	202	LAKIKMLGNKFFIGELGKLLDLIHESILHKCIKTLLKKKRVQLKDMGLEDLECLC
eIF4G	741	RLLTIVGKDLDFEKAKPRMDDQYFNQMEKIIKEKKTSSTHFM LQDVLDLRGSNW
p97	256	QIMRTVGPRLDHERAKSLMDQYFARMCSLMLSKELEPARIRFLQDVTVELEHWH
eIF4G	795	VPRRG--DQGPRLTRSLRRLRWKTSRAHQSAIAAHARAVTSVGAVTQA--LPISA
p97	310	VPRKAFLDNGPKTINQ-IRQDAVK-DLGVFIPAPMAQGMRS-DFFLEGGFMPPR
eIF4G	845	VDF--PMMVAD--TVPISKSGSRPTDTSRLTKITKPGSIDSNQQLFAPG
p97	361	MKMDRDLGGIADMFQGMPSGIGTGPGVQDRFSPTMGR--HRSNQLF--
eIF4G	891	GRLSWGKSSSGSGGAQPSDAASEAARPATSTLIRFSAIQQCAVPTESITDNRVVQ
p97	408	---NGHGHIMPTQ---SQFGEMG-GKFMKSQGLSGLYHNQS--QGLLSQ
eIF4G	945	RSSLRERGEKAGDRGDRLEVRNIGESTVGTGLIVSRTPATKRTFSKEVEERSIT
p97	450	LQQSKMDMPFRFSKKG-QLNADIEISLRPAQSLM--NKNQVPKLELQPSITM
eIF4G	999	RPSQPEGLR-KAASLTEDRDRGRDAVKREAAAL--PPVSP LKAA LSEETLEKK
p97	497	IPPSAQPPRTQTTPPGQ--TPQLGLKTNPPIIQEKPAKTSKKPPPSKEETLEKL
eIF4G	1048	SKAIIETYLHLNDMKEAVQCVRLEMAPSPSLIFIFVRHGVESTLEERSA IAREHMG
p97	548	TETVTEYLNSGNANEAVNGREMRAPKHFPEMLSKVILSLDRLSDKEDKAKAS
eIF4G	1101	QLHLQQLCAHGLSTAQYYQGLYEIELEAEDMEIDTPHVWLYLAE LVT-PTLQEG
p97	602	SLISLLKQEGIA TSDNFMQAFLNVLDQCPKLEVDIPLVKS YLAQFAARAIISE-
eIF4G	1154	GVP MGEIFREITKPTPLRGKAASTIFELIQLG LCKSMGPKKVGTLWREAGLSWKE
p97	655	LVSISEI--AQPLSE-GLTHFPLFLQLQQLAKLQDREWLTEFQQSKVNMOK
eIF4G	1208	FLPEGGQDIGAFVAEQVEYTLGEESEA PGQRATIPSEETNRQTEKL LKEGSSNQ
p97	704	MLPEI--IDQNKDR--MLEILEGKGLSFLFPL LKLEKELEKLQIKLDPSPQ
eIF4G	1261	RVFDWIEANLISEQI-VSNTLVRAIMTAV--CYSAIIFETPLRVDA--SSAPSKQEL
p97	749	TIYKWI KDNISP K L H V D K G F V N I L M T S F L Q Y I S S E V N P P S D E T D S S A P S K E Q L
eIF4G	1304	---V L K G D I V L K Y I C D E A E G A T G A I - R L Q A L V V T L E Q P - P N L H M F F D A L
p97	803	E Q E K Q L L L S F K P V M Q R F L I D H V D L Q V S A I Y A L Q V H C Y N S N F P K G M L L R F E V H - F
eIF4G	1350	YDE D V Y K E D A F Y S W E S S K D P A E Q - G G K G V A L K S V T A F F K W I R E V R G G V - - - - -
p97	858	Y D M E I I E E A F L A M K - E D I T Q E F P G K G K A L F Q V N Q W L T W I E T A E E E S E E E A D
eIF4G	1396	
p97	907	

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 (Arg486–Gly487) (Lamphear *et al.*, 1993) and by the L protease of foot-and-mouth disease virus (Gly479–Arg480) (Kirchweiger *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 Theiler'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, 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(DRI)[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

Fig. 9. Functional analysis of p97 and eIF4G by transient transfection. (A) p97 and eIF4G expression vectors, pcDNA3-Bam-ATG-p97 and pcDNA3-eIF4G (2 µg each) were transfected into vTF7-3-infected HeLa cells together with pGEM-EMCV-IRES-CAT (1 µg) as indicated. The total amount of DNA was adjusted to 5 µg with an empty vector, pcDNA3. Cells were processed for Western blotting for p97 and eIF4G (lower panel). The level of CAT protein synthesized in cells transfected with the empty vector was assigned a value of 100. The columns and bars represent the means and standard errors of four independent transfections. (B) pcDNA3-CAT (1 µg) was used as a reporter plasmid. To express p97 and eIF4G in a cap-independent manner, poliovirus IRES-dependent expression vectors pSP72IRES-p97 and pSP72IRES-eIF4G (2 µg each) were used. The total amount of DNA was adjusted to 5 µg with pSP72IRES.

Fig. 10. p97 inhibits translation. (A) S2-6 cells transformed with pRep-9-CMVt-p97 (lanes 1 and 2) or control vector pRep-9-CMV (lanes 3 and 4) were cultured with (lanes 1 and 3) or without (lanes 2 and 4) tetracycline (tet) (1 µg/ml) for 40 h. Cell extracts (50 µg protein) were resolved by SDS-PAGE, and Western blotting was performed for p97, eIF4G, eIF4A and eIF4E. (B) Cells were labeled with [3,4,5-³H]leucine for the indicated times. Incorporation of radioactivity into the trichloroacetic acid (5%) insoluble fraction of cells (20 µg protein) was measured. p97 vector, tet+, Δ; p97 vector, tet-, ○; control vector, tet+, □; control vector, tet-, ■. Each point represents the average obtained from two dishes.

(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 eIF4F complexes. One is a complex of two polypeptides, p220 and p26, which are homologs of mammalian eIF4G and eIF4E. The other complex, called eIF(iso)4F, consists of p28, another homolog of mammalian eIF4E, and p82 (Browning *et al.*, 1990; Allen *et al.*, 1992). p82 exhibits significant sequence similarity to human eIF4G (Allen *et al.*, 1992), and the binding site for eIF4E is conserved in the N-terminus of p82 (Mader *et al.*, 1995). eIF(iso)4F, like eIF4F, stimulates translation *in vitro* (Abramson *et al.*, 1988). Yeast also has two genes encoding eIF4G homologs, *TIF4631* and *TIF4632* (Goyer *et al.*, 1993). Although both contain an eIF4E 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 eIF4G to become a repressor by losing the binding site for eIF4E.

p97 mRNA has no initiator AUG and translation starts exclusively at a GUG codon. The nucleotide sequence surrounding the initiator GUG is GCCAAAGUGGAG, which matches the consensus rule that purins 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 GAU, where G at +4 and A at +5 are more important than U at +6 (Boeck and Kolakofsky, 1994; Grunert 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* (Bruening 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 localizations 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*, eIF4F 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- γ -induced programmed 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 eIF4E 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 embryo brain cDNA library by random cloning. A human placenta cDNA library in λ gt11 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 *Xho*I site was used for PCR with a sense primer (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. pcDNA3-4-1-A(HA) and pcDNA3-6-4-A(HA) contain the corresponding p97 cDNA sequences downstream of nucleotide positions 12 and 30 in Figure 1, respectively. pcDNA3-ATG-A(HA) contains the sequence downstream of nucleotide 473 and a part of the sequence of transcription factor BTEB (-15 to 10, 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 *Sac*I site (nucleotide 600) was ligated to a fragment from *Sac*I to the 3' terminus of #20881 to construct pcDNA3-HA-p97. An *Eco*RI fragment of the human eIF4G cDNA (kindly provided by Dr Rhoads; Yan *et al.*, 1992) was used to construct pcDNA3-HA-eIF4G. 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 *Bam*HI (nucleotide 172) to the 3' terminus, in which the initiator GTG had been mutated into ATG, was inserted into pcDNA3 to generate pcDNA3-*Bam*-ATGp97. A point mutation (GTG→ATG or GGG) was introduced using a commercial kit (Amersham). To construct pcDNA3-eIF4G, the *Eco*RI fragment of eIF4G 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 eIF4G in a cap-independent manner, the *Bam*-ATGp97 and the *Eco*RI fragment of eIF4G cDNA were inserted downstream of the IRES to construct pSP72IRES-p97 and pSP72IRES-eIF4G.

Transient transfection

HeLa cells were infected with recombinant vaccinia virus vTF7-3 (Fuerst *et al.*, 1986), and then transfected with plasmids (5 μ g) using Lipofectin (Gibco-BRL). For expression in COS-1 cells, plasmids (10 μ g) were transfected by electroporation (Bio-Rad Gene PulserII, 1200 V, 25 μ F).

Immunoprecipitation

After transfection, HeLa and COS-1 cells were cultured for 16 and 48 h, respectively, and then labeled with [³⁵S]methionine (100 μ Ci/ml) for 1 h in 3.5 cm dishes. Cells were lysed in 0.5 ml of buffer A [150 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 2 μ g of anti-HA antibody (12CA5, a kind gift from M.Tremblay) for 6 h in the cold room. Protein G-Sepharose (30 μ l of 50% slurry) was added and the mixture was incubated for 2 h. After washing with buffer A (1 ml, three times), immunoprecipitates were collected by centrifugation and proteins were eluted with Laemmli buffer for SDS-10% PAGE. For co-immunoprecipitation experiments, transfected HeLa cells (6 cm dish) were lysed in 1 ml of buffer B (100 mM KCl, 0.5 mM EDTA, 20 mM HEPES-KOH pH 7.6, 0.4% NP-40, 20% glycerol, 1 mM DTT, 1 mM PMSF, 5 μ g/ml pepstatin, 5 μ g/ml leupeptin). After centrifugation, an aliquot (0.5 ml) was mixed with anti-HA antibody (2 μ g). Immunoprecipitates were washed with buffer B (1 ml, three times), and resolved by SDS-10%

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 SDETDSSAPSKEQ (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 ETAESEEESEAD (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-eIF4A, anti-eIF3 and anti-eIF4G antibodies were kind gifts from Drs.H.Trachsel, J.Hershey and L.Carrasco, respectively. Anti-eIF4E antibody was as described (Lazaris-Karatzas *et al.*, 1992).

CAT assay and RNase protection assay

The CAT assay was performed as described (Gorman *et al.*, 1982). 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. Translation activity was calculated by dividing CAT activity by the normalized CAT mRNA amount.

In vitro transcription and translation

Capped RNA was synthesized by T7 RNA polymerase in the presence of the cap analog, m⁷GpppG. Rabbit reticulocyte lysate (25 µl, final volume) (Promega) was programmed 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] (Blanan 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 (Beauparlant *et al.*, 1996) to construct pRep9-CMVt-p97. An NIH3T3-derived cell line, S2-6 (Shockett *et al.*, 1995), was transformed with pRep9-CMVt (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.

Acknowledgements

We thank John Shaughnessy, Adi Kimchi and Thomas Innerarity for sharing unpublished information, John Hiscott for pRep9-CMVt, David Schatz for S2-6 cells, Colin Lister for sequence alignments, Francis Poulin for conjugation of peptide(INT), Ashkan Haghighat for purification of anti-HA-antibody, Alessandra Gradi for probing the Northern blot with actin, and Eran Rom and Andrew Craig for critically reading the manuscript. H.I. was supported by Defi Corporatif Canderel Fellowship

and by the Naito Foundation (Japan). This work was supported by a grant from the Medical Research Council of Canada to N.S.

References

- Abramson,R.D., Browning,K.S., Dever,T.E., Lawson,G.T., Thach,R.E., Ravel,J.M. and Merrick,W.C. (1988) Initiation factors that bind mRNA: a comparison of mammalian factors with wheat germ factors. *J. Biol. Chem.*, **263**, 5462–5467.
- Acland,P., Dixon,M., Peter,G. and Dickson,C. (1990) Subcellular fate of the int-2 oncoprotein is determined by choice of initiation codon. *Nature*, **343**, 662–665.
- Allen,M., Metz,A.M., Timmer,R.T., Rhoads,R.E. and Browning,K.S. (1992) Isolation and sequence of the cDNAs encoding the subunits of the isozyme form of wheat protein synthesis initiation factor 4F. *J. Biol. Chem.*, **267**, 23232–23236.
- Beauparlant,P., Lin,R. and Hiscott,J. (1996) The role of the C-terminal domain of the IκBα in protein degradation and stabilization. *J. Biol. Chem.*, **271**, 10690–10696.
- Belsham,G.J. and Sonenberg,N. (1996) RNA–protein interactions in regulation of picornavirus RNA translation. *Microbiol. Rev.*, **60**, 499–511.
- Beretta,L., Gingras,A.-C., Svitkin,Y.V., Hall,M.N. and Sonenberg,N. (1996) Rapamycin blocks the phosphorylation of 4E-BP-1 and inhibits cap-dependent initiation of translation. *EMBO J.*, **15**, 658–664.
- Blanan,M. and Rutter,W. (1992) Interaction cloning: identification of a helix–loop–helix zipper protein that interacts with c-Fos. *Science*, **256**, 1014–1018.
- Boeck,R. and Kolakofsky,D. (1994) Positions +5 and +6 can be major determinants of the efficiency of non-AUG initiation codons for protein synthesis. *EMBO J.*, **13**, 3608–3617.
- Browning,K.S., Humphreys,J., Hobbs,W., Smith,G.B. and Ravel,J.M. (1990) Determination of the amounts of the protein synthesis initiation and elongation factors in wheat germ. *J. Biol. Chem.*, **265**, 17967–17973.
- Bruening,W. and Pelletier,J. (1996) A non-AUG translational initiation event generates novel WT1 isoforms. *J. Biol. Chem.*, **271**, 8446–8454.
- Bugler,B., Amalric,F. and Prats,H. (1991) Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. *Mol. Cell. Biol.*, **11**, 573–577.
- Chambers,J.C., Kenan,D., Martin,B.J. and Keene,J.D. (1988) Genomic structure and amino acid sequence domains of the human La autoantigen. *J. Biol. Chem.*, **263**, 18043–18051.
- Duncan,R., Milburn,S.C. and Hershey,J.W.B. (1987) Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translation control: heat shock effects on eIF4F. *J. Biol. Chem.*, **262**, 380–388.
- Etchison,D.E., Milburn,S.C., Edery,L., Sonenberg,N. and Hershey,J.W.B. (1982) Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eukaryotic initiation factor 3 and a cap binding protein complex. *J. Biol. Chem.*, **257**, 14806–14810.
- Florkiewicz,R.Z. and Sommer,A. (1989) Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. *Proc. Natl Acad. Sci. USA*, **86**, 3978–3981.
- Fuerst,T.R., Niles,E.G., Studier,F.W. and Moss,B. (1986) Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl Acad. Sci. USA*, **83**, 8122–8126.
- Gingras,A.-C., Svitkin,Y., Belsham,G.J., Pause,A. and Sonenberg,N. (1996) Activation of the translational suppressor 4E-BP-1 following infection with encephalomyocarditis virus and poliovirus. *Proc. Natl Acad. Sci. USA*, **93**, 5578–5583.
- Gorman,C.M., Moffat,L. and Howard,B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.*, **2**, 1044–1051.
- Goyer,C., Altmann,M., Lee,H.S., Blanc,A., Deshmukh,M., Woolford,J.L., Jr, Trachsel,H. and Sonenberg,N. (1993) *TIF4631* and *TIF4632*: two yeast genes encoding the high-molecular-weight subunits of the cap-binding protein complex (eukaryotic initiation factor 4F) contain an RNA recognition motif-like sequence and carry out an essential function. *Mol. Cell. Biol.*, **13**, 4860–4874.
- Grunert,S. and Jackson,R. (1994) The immediate downstream codon strongly influences the efficiency of utilization of eukaryotic translation initiation codons. *EMBO J.*, **13**, 3618–3630.

- Haghighat, A., Mader, S., Pause, A. and Sonenberg, N. (1995) Repression of cap-dependent translation by 4E-binding protein-1: competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J.*, **14**, 5701–5709.
- Hann, S.R., King, M.W., Bentley, D.L., Anderson, C.W. and Eisenman, R.N. (1988) A non-AUG translational initiation in c-myc exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphomas. *Cell*, **52**, 185–195.
- Hann, S.R., Sloan-Brown, K. and Spotts, G.D. (1992) Translational activation of the non-AUG-initiated c-myc 1 protein at high cell densities due to methionine deprivation. *Genes Dev.*, **6**, 1229–1240.
- Imataka, H., Sogawa, K., Yasumoto, K., Kikuchi, Y., Sasano, K., Kobayashi, A., Hayami, M. and Fujii-kuriyama, Y. (1992) Two regulatory proteins that bind to the basic transcription element (BTE), a GC box sequence in the promoter region of the rat p-4501A1 gene. *EMBO J.*, **11**, 3633–3671.
- Imataka, H., Nakayama, K., Yasumoto, K., Mizuno, A., Fujii-kuriyama, Y. and Hayami, M. (1994) Cell-specific translational control of transcription factor BTEB expression: the role of an upstream AUG in the 5'-untranslated region. *J. Biol. Chem.*, **269**, 20668–20673.
- Kevil, C., Carter, P., Hu, B. and De Benedetti, A. (1996) Translational enhancement of FGF-2 by eIF4 factors, and alternate utilization of CUG and AUG codons for translation initiation. *Oncogene*, **11**, 2339–2348.
- Kirchweiger, R. et al. (1994) Foot-and mouth disease virus leader proteinase: purification of the Lb form and determination of its cleavage site on eIF-4 γ . *J. Virol.*, **68**, 5677–5684.
- Kozak, M. (1989) The scanning model for translation: an update. *J. Cell Biol.*, **108**, 229–241.
- Lamphear, B., Yan, R., Yang, F., Waters, D., Liebig, H., Klump, H., Kuechler, E., Skern, T. and Rhoads, R. (1993) Mapping the cleavage site in protein synthesis initiation factor eIF-4 γ of the 2A proteases from human coxsackievirus and rhinovirus. *J. Biol. Chem.*, **268**, 19200–19203.
- Lamphear, B., Kirchweiger, R., Skern, T. and Rhoads, R. (1995) Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases: implications for cap-dependent and cap-independent translational initiation. *J. Biol. Chem.*, **270**, 21975–21983.
- Lazaris-Karatzas, A., Smith, M.R., Jaramillo, M.L., Liu, Y.L., Kung, H.-F. and Sonenberg, N. (1992) Ras mediates translation initiation factor 4E-induced malignant transformation. *Genes Dev.*, **6**, 1631–1642.
- Ledda-Columbano, G.M., Coni, P., Faa, G., Manenti, G. and Columbano, A. (1992) Rapid induction of apoptosis in rat liver by cycloheximide. *Am. J. Pathol.*, **140**, 545–549.
- Levy-Strumpf, N., Deiss, L.P., Berissi, H. and Kimchi, A. (1997) DAP-5, a novel homologue of the translation initiation factor 4G, isolated as a putative modulator of interferon- γ -induced programmed cell death. *Mol. Cell Biol.*, in press.
- Liebig, H.-D. et al. (1993) Purification of two picornaviral 2A proteinases: interaction with eIF-4 γ and influence on *in vitro* translation. *Biochemistry*, **32**, 7581–7588.
- Lin, T.-A., Kong, X., Haystead, T.A., Pause, A., Belsham, G., Sonenberg, N. and Lawrence, J.C., Jr (1994) PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science*, **266**, 653–656.
- Mader, S., Lee, H., Pause, A. and Sonenberg, N. (1995) The translation initiation factor eIF4E binds to a common motif shared by the translation factor eIF-4 γ and the translational repressors 4E-binding proteins. *Mol. Cell Biol.*, **15**, 4990–4997.
- Martin, D.P., Schmidt, R.E., DiStefano, P.S., Lowry, O.H., Carter, J.G. and Johnson, E.M., Jr (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.*, **106**, 829–844.
- Mengod, G. and Trachsel, H. (1985) Eukaryotic protein initiation factor eIF-3: determination of concentration and association with ribosomes in rabbit reticulocyte and HeLa cell lysates. *Biochim. Biophys. Acta*, **825**, 169–174.
- Merrick, W.C. and Hershey, J.W.B. (1996) The pathway and mechanism of eukaryotic protein synthesis. In Hershey, J.W.B., Mathews, M.B. and Sonenberg, N. (eds), *Translational Control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 31–70.
- Meyer, L.J., Milburn, S.C. and Hershey, W.B. (1982) Immunochemical characterization of mammalian protein synthesis initiation factors. *Biochemistry*, **21**, 4206–4212.
- Ohlmann, T., Rau, M., Morley, S.J. and Pain, V.M. (1995) Proteolytic cleavage of initiation factor eIF-4 γ in the reticulocyte lysate inhibits translation of capped mRNAs but enhances that of uncapped mRNAs. *Nucleic Acids Res.*, **23**, 334–340.
- Ohlmann, T., Rau, M., Pain, V.M. and Morley, S.J. (1996) The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF)4G is sufficient to support cap-independent translation in the absence of eIF4E. *EMBO J.*, **15**, 1371–1382.
- Pain, V.M. (1996) Initiation of protein synthesis in eukaryotic cells. *Eur. J. Biochem.*, **236**, 747–771.
- Pause, A., Belsham, G.J., Gingras, A.-C., Donze, O., Lin, T.-A., Lawrence, J.C., Jr and Sonenberg, N. (1994a) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature*, **371**, 762–767.
- Pause, A., Methot, N., Svitkin, Y., Merrick, W.C. and Sonenberg, N. (1994b) Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation. *EMBO J.*, **13**, 1205–1215.
- Polunovsky, V.A., Wendt, C.H., Ingbar, D.H., Peterson, M.S. and Bitterman, P.B. (1994) Induction of endothelial cell apoptosis by TNF α : modulation by inhibitors of protein synthesis. *Exp. Cell Res.*, **214**, 584–594.
- Polunovsky, V.A., Rosenwald, I.B., White, L.C., Sonenberg, N. and Bitterman, P.B. (1996) Translational control of programmed cell death: eukaryotic translation initiation factor 4E blocks apoptosis in growth-factor-restricted fibroblasts with physiologically expressed or deregulated Myc. *Mol. Cell Biol.*, **16**, 6573–6581.
- Rau, M., Ohlmann, T., Morley, S.J. and Pain, V.M. (1996) A reevaluation of the cap-binding protein, eIF4E, as a rate-limiting factor for initiation of translation in reticulocyte lysate. *J. Biol. Chem.*, **271**, 8983–8990.
- Rozen, F., Ederly, I., Meerovitch, K., Dever, T.E., Merrick, W.C. and Sonenberg, N. (1990) Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol. Cell Biol.*, **10**, 1134–1144.
- Saris, C.I.M., Domen, J. and Berns, A. (1991) The *pim-1* oncogene encodes two related protein-serine/threonine kinases by alternative initiation at AUG and CUG. *EMBO J.*, **10**, 655–664.
- Shockett, P., Difilippantonio, M., Hellman, N. and Schatz, D. (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc. Natl Acad. Sci. USA*, **92**, 6522–6526.
- Smith, R. and Smith, T. (1992) Pattern-induced multi-sequence alignment (PIMA) algorithm employing secondary structure-dependent gap penalties for comparative protein modelling. *Protein Engng*, **5**, 35–41.
- Sonenberg, N. (1996) mRNA 5' cap-binding protein eIF4E and control of cell growth. In Hershey, J.W.B., Mathews, M.B. and Sonenberg, N. (eds), *Translational Control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 271–294.
- Tarun, S.Z. and Sachs, A.B. (1996) Association of the yeast poly(A) tail binding protein with translation initiation factor eIF4G. *EMBO J.*, **15**, 7168–7177.
- Yan, R., Rychlik, W., Etchison, D. and Rhoads, R. (1992) Amino acid sequence of the human protein synthesis initiation factor eIF-4 γ . *J. Biol. Chem.*, **267**, 23226–23231.

Received on September 5, 1996; revised on October 16, 1996