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Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E

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Human eukaryotic translation initiation factor 4E (eIF4E) binds to the mRNA cap structure and interacts with eIF4G, which serves as a scaffold protein for the assembly of eIF4E and eIF4A to form the eIF4F complex. eIF4E is an important modulator of cell growth and proliferation. It is the least abundant component of the translation initiation machinery and its activity is modulated by phosphorylation and interaction with eIF4E-binding proteins (4E-BPs). One strong candidate for the eIF4E kinase is the recently cloned MAPK-activated protein kinase, Mnk1, which phosphorylates eIF4E on its physiological site Ser209 *in vitro*. Here we report that Mnk1 is associated with the eIF4F complex via its interaction with the C-terminal region of eIF4G. Moreover, the phosphorylation of an eIF4E mutant lacking eIF4G-binding capability is severely impaired in cells. We propose a model whereby, in addition to its role in eIF4F assembly, eIF4G provides a docking site for Mnk1 to phosphorylate eIF4E. We also show that Mnk1 interacts with the C-terminal region of the translational inhibitor p97, an eIF4G-related protein that does not bind eIF4E, raising the possibility that p97 can block phosphorylation of eIF4E by sequestering Mnk1.

Keywords: eIF4E phosphorylation/initiation factor/kinase specificity/Mnk1/translation regulation

Introduction

Translation is an important target for regulation of gene expression in response to a large array of extracellular stimuli. The initiation step is rate-limiting and represents a major target for translational control (reviewed in Mathews *et al*., 1996). In the most general case, initiation is facilitated by the 5′ cap structure, m^7GpppN (where N is any nucleotide). Cap function is mediated by the eukaryotic translation initiation factor 4F (eIF4F), which consists of eIF4E, the cap-binding subunit, eIF4A, an RNA helicase, and eIF4G which serves as a scaffold protein for the assembly of eIF4E and eIF4A. eIF4G also binds to ribosomes through another initiation factor, eIF3. There are two functional homologs of mammalian eIF4G, termed eIF4GI and eIF4GII, which share 46% identity and which exhibit similar biochemical activities (Gradi *et al*., 1998). The N-terminal third of eIF4G interacts with eIF4E, while the C-terminal two-thirds contains two separate binding sites for eIF4A and one binding site for eIF3. It is thought that, through its interaction with eIF4E, eIF4G functions by bringing the eIF4A helicase activity to the mRNA 5′ end to facilitate ribosome binding by unwinding mRNA 5′ secondary structure. eIF4E is the least abundant of all initiation factors (Hiremath *et al*., 1985; Duncan *et al*., 1987), and under most circumstances is considered to be the rate-limiting factor in the binding of ribosomes to the mRNA (Sonenberg, 1996). Consequently, eIF4E is a major target for regulation.

eIF4E is phosphorylated on Ser209 following treatment of cells with growth factors, hormones and mitogens (Flynn and Proud, 1995; Joshi *et al*., 1995; Makkinje *et al*., 1995). Phosphorylated eIF4E was reported to have higher binding affinity for the cap (Minich *et al*., 1994), and to form a more stable eIF4F complex (Bu *et al*., 1993), resulting in enhanced translation. The three-dimensional structure of eIF4E was determined by X-ray crystallography (Mareotrigiano *et al*., 1997) and NMR (Matsuo *et al*., 1997). The co-crystal X-ray structure of eIF4E bound to m^7GDP suggests a model to account for the enhanced cap affinity engendered by Ser209 phosphorylation (Marcotrigiano *et al*., 1997). According to the crystal structure, Lys159 could form a salt bridge with phosphorylated Ser209, creating a retractable clamp over the mRNA, thus stabilizing the interaction between the mRNA 5′ end and eIF4E. eIF4E function is also regulated by its reversible association with the 4E-binding proteins (4E-BPs or PHAS) (Lin *et al*., 1994; Pause *et al*., 1994; Poulin *et al*., 1998). Hypophosphorylated 4E-BPs associate strongly with eIF4E, while hyperphosphorylated 4E-BPs do not bind eIF4E (Pause *et al*., 1994; Sonenberg and Gingras, 1998). 4E-BPs have no effect on cap binding, but instead block eIF4F assembly because they compete with eIF4G for binding to eIF4E (Haghighat *et al*., 1995; Mader *et al*., 1995). Another protein likely to affect eIF4F function is the recently cloned p97/NAT-1/DAP-5 (Imataka *et al*., 1997; Levy-Strumpf *et al*., 1997; Shaughnessy *et al*., 1997; Yamanaka *et al*., 1997), which shares homology with the C-terminal two-thirds of eIF4G. p97 is postulated to act as an inhibitor of translation by forming inactive complexes that include eIF4A and eIF3, but which do not contain eIF4E (Imataka *et al*., 1997; Yamanaka *et al*., 1997).

Protein kinase C (PKC) has been considered as a potential eIF4E kinase. Indeed, treatment of cells with 12-*O*-tetradecanoyl-13-acetate (TPA) leads to phosphorylation on Ser209 (Morley and Traugh, 1990;

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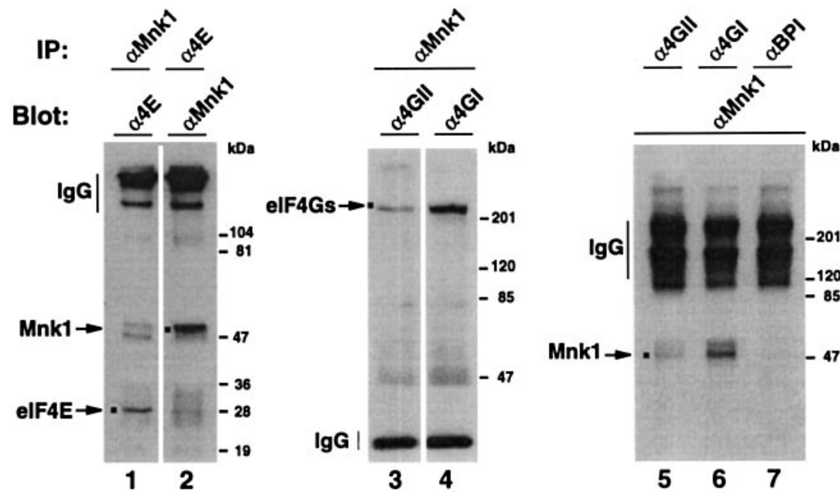

Fig. 1. Mnk1 is co-immunoprecipitated with eIF4E and eIF4G but not with 4E-BP1. Extracts from 293 cells (5×10^6 cells) were immunoprecipitated and analyzed by Western blotting with specific antibodies indicated at the top of the figure, as described in Materials and methods. Samples were dissolved in Laemmli buffer containing β-mercapthoethanol (lanes 3 and 4) or lacking β-mercapthoethanol (lanes 1, 2 and 5–7).

Frederickson and Sonenberg, 1992; Whalen *et al*., 1996) and PKC phosphorylates Ser209 *in vitro* (Whalen *et al*., 1996). However, a recently characterized MAP kinase-activated protein kinase (MAPKAPK), Mnk1 (Fukunaga and Hunter, 1997; Waskiewicz *et al*., 1997), was also shown to phosphorylate eIF4E on Ser209 *in vitro* (Waskiewicz *et al*., 1997). Mnk1 is phosphorylated and activated by Erk1, Erk2 and p38 MAP kinases *in vitro* and *in vivo* (Fukunaga and Hunter, 1997; Waskiewicz *et al*., 1997). Studies conducted *in vivo* argue in favor of Mnk1 as a physiological kinase of eIF4E (Morley and McKendrick, 1997; Wang *et al*., 1998). These studies demonstrated the mitigation of growth factor- and stress-induced eIF4E phosphorylation by pharmacological inhibitors of the Erks and p38. These inhibitors also blocked eIF4E phosphorylation induced by phorbol esters, suggesting that PKC might lie upstream of the Erks and p38. Moreover, expression of dominant-negative forms of Mnk1 in cells results in inhibition of Ser209 phosphorylation in response to TPA (Waskiewicz *et al*., 1999).

The basis of specific substrate recognition and phosphorylation by protein kinases is not completely understood. All protein kinases interact via their catalytic pocket with the phosphoacceptor hydroxyamino acid as well as with several flanking residues. While the residues surrounding the phosphoacceptor site provide a certain degree of specificity, this is unlikely to be sufficient to achieve specific recognition of a physiological substrate and to distinguish among substrates of closely related protein kinases. The observation that Ser209 and surrounding residues conform well to the PKC consensus phosphorylation site (Pearson and Kemp, 1991) could explain the ability of PKC to phosphorylate eIF4E *in vitro*. However, it is reasonable that additional sequences in eIF4E might specify kinase preference *in vivo*.

Here we show that Mnk1 does not interact with eIF4E directly, but is associated with the C-terminal region of eIF4G. Moreover, an eIF4E mutant defective in its ability to interact with eIF4G is poorly phosphorylated *in vivo*. These results strongly support the idea that eIF4G acts as a docking site to bring Mnk1 next to its substrate eIF4E. In addition, Mnk1 interacts with the C-terminal region of p97, suggesting a possible role for p97 in the regulation of eIF4E phosphorylation.

Results

Mnk1 associates with the eIF4F complex

To determine whether eIF4E and Mnk1 interact *in vivo*, co-immunoprecipitation experiments were performed in human 293T cells. Either rabbit anti-human Mnk1 or anti-human eIF4E antibodies were used for immunoprecipitation, which was followed by Western blotting with the reciprocal antibody. Under SDS–PAGE reducing conditions, the heavy chain of the rabbit immunoglobulins co-migrates with Mnk1 and the light chain co-migrates with eIF4E, rendering detection of these two immunoprecipitated proteins difficult. To circumvent this problem, immunoprecipitates were dissolved in Laemmli buffer lacking β-mercaptoethanol to maintain the disulfide bonds between the light and heavy chains. eIF4E co-immunoprecipitated with Mnk1 (Figure 1, lane 1) and, reciprocally, Mnk1 was co-immunoprecipitated with eIF4E (lane 2). The interaction between eIF4E and Mnk1 could be indirect through association with eIF4E-interacting proteins such as the eIF4Gs or 4E-BPs. To address this, we first determined whether the two isoforms of eIF4G, eIF4GI and its recently cloned homolog, eIF4GII (Gradi *et al*., 1998), co-immunoprecipitate with anti-Mnk1 antibodies. Both eIF4G forms were brought down with anti-Mnk1 antibodies (lanes 3 and 4). In the reciprocal experiments, the antibodies against both eIF4G isoforms precipitated Mnk1 (lanes 5 and 6). In contrast, an antibody against 4E-BP1 failed to co-precipitate Mnk1 (lane 7). These results demonstrate that Mnk1 interacts with the eIF4F complex but not with the eIF4E–4E-BP1 complex. However, these data do not specify which eIF4F subunit binds Mnk1.

Mnk1 binds eIF4G but not eIF4E

To study further the interaction between Mnk1 and the eIF4F complex *in vivo*, we used a series of wild-type and mutant proteins (schematically represented in Figure 2). The proteins were tagged with either the hemagglutinin

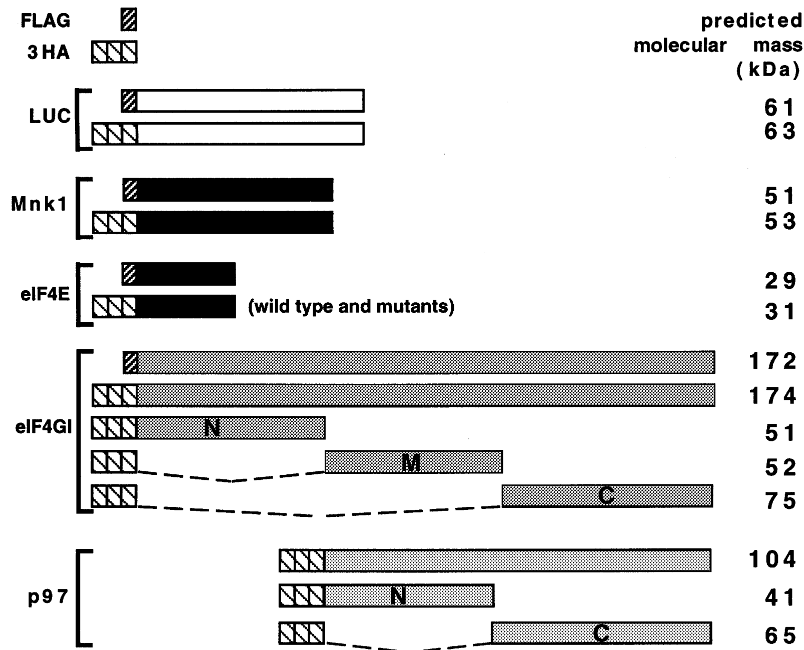

Fig. 2. Diagram illustrating the HA- and Flag-tagged proteins. The predicted molecular mass of each protein is shown (kDa).

(HA) or the Flag epitope to allow for their discrimination from endogenous proteins. To exclude the possibility that the HA or Flag sequences interfere with the interaction between Mnk1 and eIF4F, tagged Mnk1 and eIF4E were expressed in 293T cells and assayed for co-immuno-precipitation. All proteins were expressed, albeit with some small differences in amount (Figure 3A). The presence of HA or Flag epitope did not prevent Mnk1 interaction with eIF4F (Figure 3B, lanes 1 and 3). Furthermore, their presence did not engender adventitious interactions, as the tagged luciferase protein (Luc) failed to interact with either tagged Mnk1 or tagged eIF4E (lanes 2 and 4).

While eIF4G and 4E-BP1 interact with eIF4E through a common binding site (Haghighat *et al.*, 1995; Mader *et al.*, 1995), Mnk1 co-immunoprecipitated with eIF4G, but not with 4E-BP1 (Figure 1). This observation raised the possibility that Mnk1 interacts directly with eIF4G rather than with eIF4E. This is also consistent with the finding that in a yeast two-hybrid assay using Mnk1 as bait, eIF4GII was identified as an interacting protein (Waskiewicz *et al.*, 1999). To identify the subunit of eIF4F interacting with Mnk1, we used a mutant form of murine eIF4E, eIF4E-W73A. Trp73 is located on the dorsal surface of eIF4E (Marcotrigiano *et al.*, 1997) and, when mutated into alanine, eIF4E is incapable of interacting with eIF4GI *in vitro* (A.-C.Gingras, unpublished observations). This was also demonstrated recently for yeast eIF4E (Ptushkina *et al.*, 1998). To confirm that the mutation affects eIF4E binding to eIF4GI *in vivo*, HA-tagged eIF4E and W73A mutant were expressed in 293T cells (Figure 4A, right panel) together with Flag-eIF4GI (left panel, lanes 3 and 4) and assayed for co-immunoprecipitation. As expected from the *in vitro* studies, the W73A mutant failed to interact with eIF4GI *in vivo* (Figure 4B, compare lane 4 with 3). HA-tagged eIF4E and W73A mutant proteins were also expressed together with Flag-Mnk1 (Figure 4A, lanes 1 and 2). While wild-type eIF4E

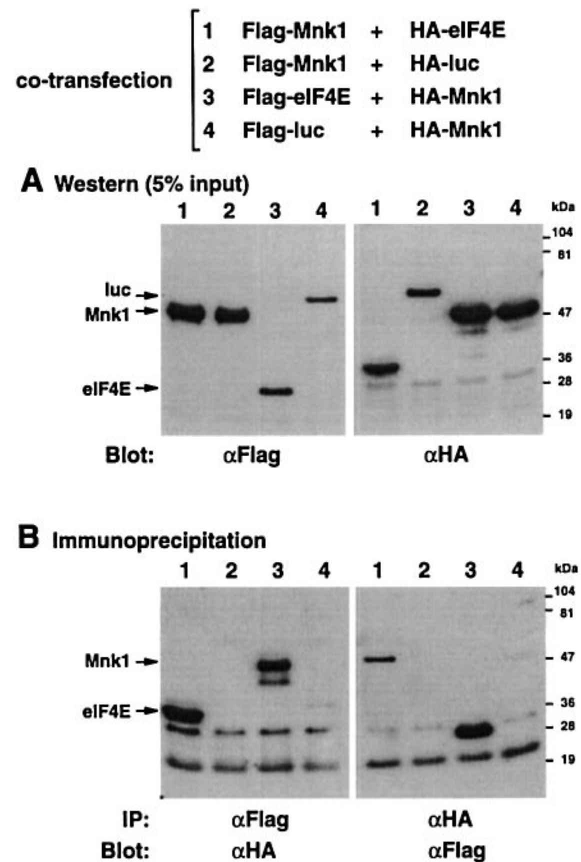

Fig. 3. The interaction between eIF4E and Mnk1 is not affected by the presence of Flag and HA epitopes. 293T cells (2×10^6 cells) were transfected with 2 μg of plasmid expressing the protein indicated at the top of the figure. After 36 h, cells were harvested in lysis buffer, and a fraction (5% input) of the lysate (**A**) and the total immuno-precipitate (**B**) were analyzed by immunoblotting as described in Materials and methods. Approximately 25 and 15% of the total Flag- and HA-tagged proteins were immunoprecipitated, using anti-Flag and anti-HA antibodies, respectively.

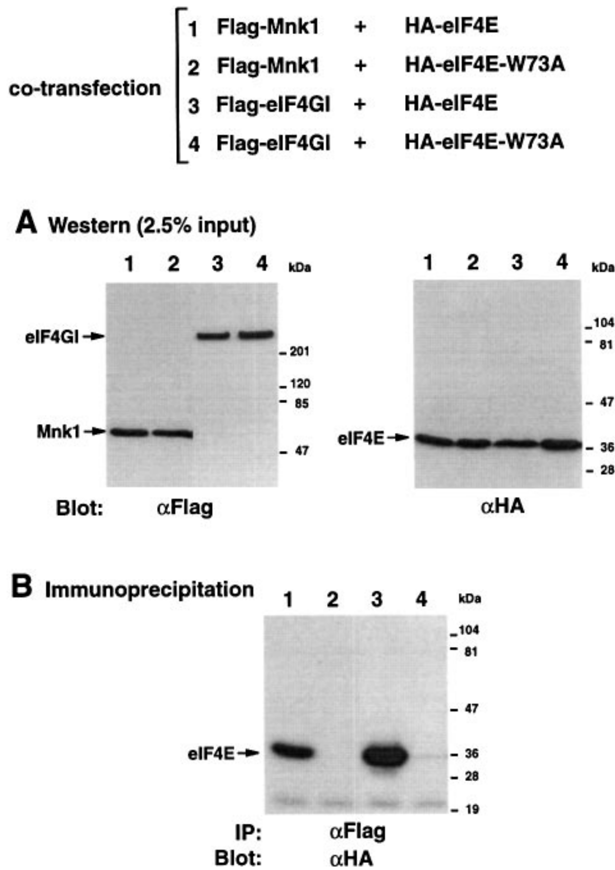

Fig. 4. The interaction between eIF4E and Mnk1 occurs through eIF4G *in vivo*. 293T cells (10^6 cells) were co-transfected with 2 µg of plasmid expressing the protein indicated at the top of the figure. After 36 h, cells were harvested in lysis buffer, and a fraction (2.5% input) of the lysate (**A**) and the total immunoprecipitate (**B**) were analyzed by immunoblotting using M2 anti-Flag and HA11 anti HA-antibodies as described in Materials and methods.

associated with the kinase, the eIF4E-W73A mutant failed to interact with Mnk1 (Figure 4B, compare lane 2 with 1). These findings indicate that, *in vivo*, Mnk1 interacts with eIF4E through its binding to eIF4G.

To delineate further the role of eIF4G as a docking site for Mnk1, recombinant proteins were used. GST–Mnk1 was incubated with either Flag-eIF4E or His-eIF4GI or both, and the interaction was assayed by co-immunoprecipitation. A weak association between Mnk1 and eIF4E was detected in the absence of eIF4GI (Figure 5, lanes 4 and 8). However, the interaction between Mnk1 and eIF4E was greatly enhanced in the presence of eIF4GI (compare lane 4 with 1, and lane 8 with 5), which strongly interacts with eIF4E (lanes 3 and 6). No adventitious protein binding to the antibody-coupled protein A–agarose beads was observed (lanes 2 and 7). Taken together, the *in vivo* and *in vitro* data indicate that Mnk1 associates with eIF4F through its interaction with eIF4G rather than eIF4E.

The C-terminal region of eIF4G contains a Mnk1-binding site

Three fragments of the human eIF4GI, N-terminus (amino acids 1–457), middle (amino acids 478–883) and C-terminus (amino acids 884–1404) (Imataka *et al.,* 1997) (Figure 2), were used to localize the Mnk1-binding site

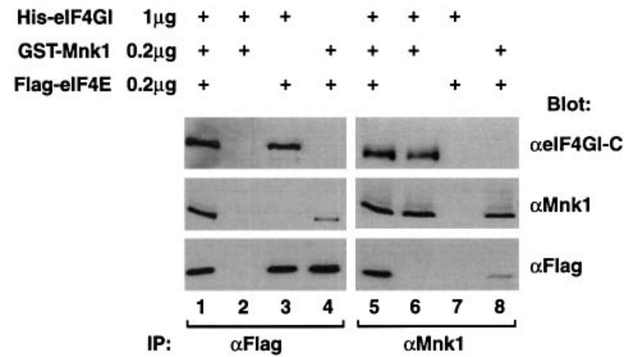

Fig. 5. eIF4G facilitates the interaction between eIF4E and Mnk1 *in vitro*. Recombinant proteins were mixed as indicated at the top of the figure and incubated in the presence of protein A–agarose beads coupled to either anti-Flag (left) or anti-Mnk1 (right) antibodies. Bound proteins were analyzed by immunoblotting using the antibodies indicated on the right of the figure, as described in Materials and methods.

in eIF4GI. HA-tagged fragments were co-expressed with Flag-tagged Mnk1 to comparable levels (Figure 6A, lanes 1–4). The interaction between Mnk1 and eIF4GI fragments was assayed by co-immunoprecipitation. Mnk1 precipitated neither the N-terminal region nor the middle fragment of eIF4GI (Figure 6B, lanes 2 and 3). In contrast, Mnk1 was co-immunoprecipitated with the C-terminal region as efficiently as with full-length eIF4GI (compare lane 4 with 1). All eIF4GI fragments were functional as HA-tagged eIF4E co-expressed with the N- and the C-terminal regions of eIF4GI (Figure 6A, lanes 5 and 6) only interacted with the N-terminal region (Figure 6B, compare lane 5 with 6). In addition, endogenous eIF4A was co-immunoprecipitated with full-length eIF4GI as well as with the middle and the C-terminal regions, but not with the N-terminal region (compare lanes 1, 3, 4 and 6 with lanes 2 and 5). To rule out the possibility that Mnk1 interacts with the newly identified N-terminal region of eIF4GI (Imataka *et al.*, 1998), co-immunoprecipitation experiments with tagged Mnk1 and the eIF4GI fragment containing the new sequence were performed. No interaction was detected (data not shown). Thus, Mnk1 binds to eIF4F exclusively via the C-terminal region of eIF4G.

Because the C-terminal region of eIF4G harbors a binding domain for eIF4A, we wished to test whether Mnk1 and eIF4A can bind simultaneously to eIF4GI. Recombinant proteins were mixed and their interactions assayed by co-immunoprecipitation. While Mnk1 bound to the C-terminal fragment of eIF4GI (Figure 7, lane 2), no direct interaction was detected between Mnk1 and eIF4A (lane 4). However, in the presence of both Mnk1 and the C-terminal region of eIF4GI, eIF4A was co-immunoprecipitated by Mnk1 antibodies (lane 1). These data demonstrate that the binding of Mnk1 and eIF4A to the C-terminal region of eIF4GI is not mutually exclusive.

Abrogation of eIF4E binding to eIF4G dramatically decreases eIF4E phosphorylation in vivo

One important prediction from our findings is that phosphorylation of the eIF4E-W73A mutant, which cannot interact with eIF4G, should be impaired in intact cells. To examine this, the HA-tagged eIF4E wild-type and W73A mutant were transfected into 293T cells, followed

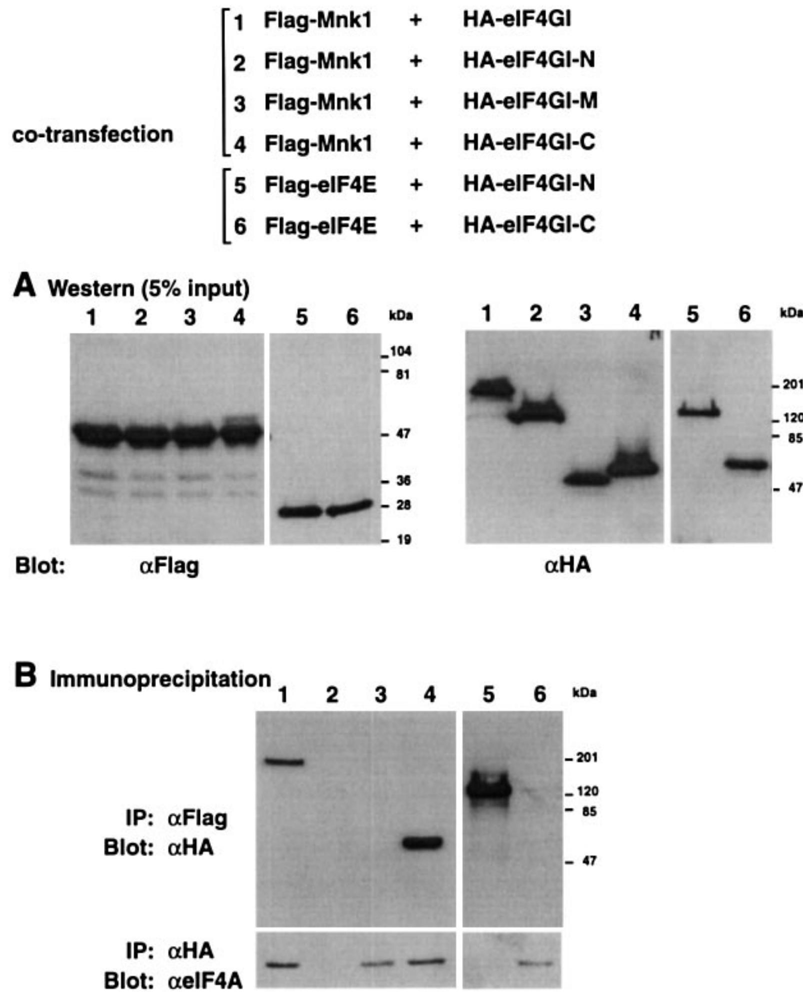

Fig. 6. Mnk1 associates with the C-terminus of eIF4G. 293T cells (2×10^6 cells) were transfected with 2 μg of plasmid expressing the protein indicated at the top of the figure. After 36 h, cells were harvested in lysis buffer, and a fraction (5% input) of the lysate (**A**) and the total immunoprecipitate (**B**) were analyzed by immunoblotting using M2 anti-Flag, HA11 anti-HA and anti-eIF4A antibodies as described in Materials and methods.

by metabolic labeling with [^{32}P]orthophosphate. The specificity of phosphorylation was determined using the HA-tagged S209A/T210A double mutant. (Although Ser209 is the physiological phosphorylation site, when it is mutated to alanine, Thr210 becomes phosphorylated to a significant extent *in vivo*; Whalen *et al.*, 1996.) Incorporation of ^{32}P into the eIF4E-W73A mutant was reduced to $<$20% of the level obtained for wild-type eIF4E (Figure 8A and B, compare lane 2 with 1). As expected, no radioactivity was detected in the immunoprecipitates obtained from cells transfected with the double mutant S209A/T210A (lane 3). The decrease in incorporation of radioactivity was not due to reduced protein expression, as W73A and S209A/T210A mutants were expressed to levels similar to that of eIF4E wild-type (Figure 8C, compare lanes 2 and 3 with lane 1). To confirm that in this particular experiment, eIF4E-W73A failed to interact with eIF4G, the membrane that had been first blotted with anti-HA antibodies (Figure 8C) was reprobed with anti-eIF4GI antibodies (Figure 8D). In agreement with the results obtained in the *in vivo* binding assay (Figure 4), the W73A mutation severely impaired eIF4E binding to eIF4G (Figure 8D, compare lane 2 with 1). In contrast, the double mutation S209A/T210A only modestly affected

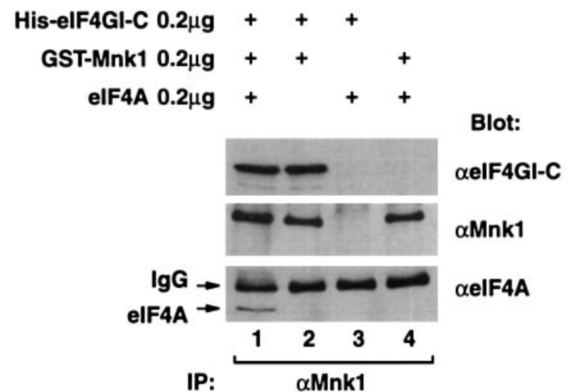

Fig. 7. eIF4A and Mnk1 can associate simultaneously with the C-terminal region of eIF4GI. Recombinant proteins were mixed as indicated at the top of the figure and incubated in the presence of protein A–agarose beads coupled to anti-Mnk1 antibodies. Bound proteins were analyzed by immunoblotting using the antibodies indicated on the right of the figure, as described in Materials and methods.

the interaction between eIF4E and eIF4GI (compare lane 3 with 1). The signal detected in the upper region of the autoradiogram (Figure 8A, denoted by an asterisk) co-

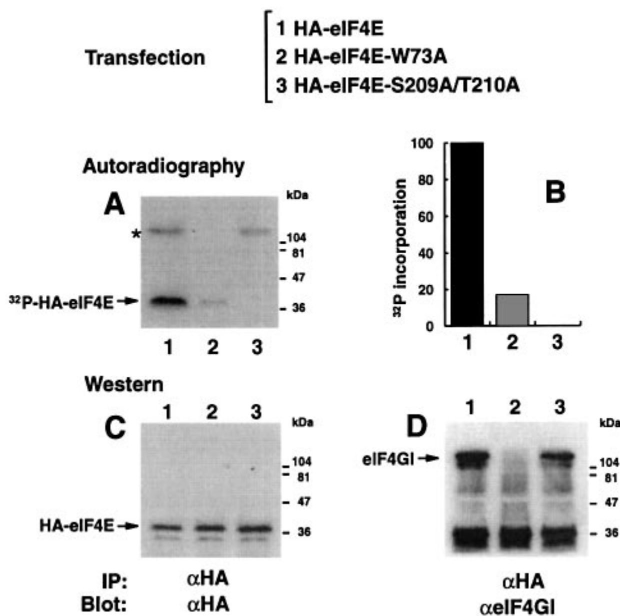

Fig. 8. Efficient eIF4E phosphorylation *in vivo* requires binding to eIF4G. (**A**) HA-tagged eIF4E wild-type, W73A and S209A/T210A mutants were expressed in 293T cells (5×10^6 cells per transfection). Following labeling of the cells with [^{32}P]orthophosphate, HA-tagged proteins were immunoprecipitated using anti-HA antibodies, resolved by 12% SDS–PAGE, transferred onto a nitrocellulose membrane and autoradiographed as described in Materials and methods. The asterisk denotes the position of eIF4GI. (**B**) Signals obtained for ^{32}P-labeled HA-eIF4E proteins were quantified on a PhosphorImager (Fuji Corp.) and expressed as a percentage of the intensity measured for HA-eIF4E wild-type. (**C**) Expression of mutant proteins. The membrane utilized in (A) was probed with anti-HA antibodies. (**D**) Interaction of eIF4GI with eIF4E wild-type and mutants. The membrane utilized in (A) and probed with anti-HA antibodies (C) was reprobed with anti-eIF4GI antibodies.

migrated with eIF4G. This signal most probably represents phosphorylated eIF4G co-immunoprecipitated with HA-eIF4E and the S209A/T210A mutant (lanes 1 and 3). This is supported further by the finding that the signal was not detected after immunoprecipitation with the eIF4E-W73A mutant (lane 2).

One possible complication in the interpretation of our data is that the W73A mutation might alter the folding of eIF4E, rendering it defective as a kinase substrate. To ensure that such misfolding was not responsible for the observed decrease in eIF4E-W73A phosphorylation, we have tested the capacity of wild-type and mutant eIF4E proteins to be phosphorylated by Mnk1 *in vitro*. HA-tagged eIF4E wild-type, W73A and S209A/T210A mutants were expressed in 293T cells. After 36 h, cell lysates were subjected to immunoprecipitation using anti-HA antibodies. Equal amounts of HA-tagged proteins bound to agarose beads were phosphorylated by purified, activated Mnk1. Both the wild-type and W73A mutant proteins were phosphorylated, albeit with minor differences in intensity (Figure 9A and B, compare lanes 1–3 with lanes 4–6). In contrast, the S209A/T210A mutant was not phosphorylated (lanes 7–9), in agreement with the finding that Mnk1 phosphorylates eIF4E on its physiological site, Ser209 (Waskiewicz *et al*., 1997). The slightly enhanced Mnk1 phosphorylation of wild-type eIF4E, as compared with that of the W73A mutant, may be attributed to the presence of a co-immunoprecipitated kinase. Indeed, immunoprecipitates obtained with wild-type HA-eIF4E incubated in the absence of exogenous Mnk1 revealed a significant intrinsic protein kinase activity, whereas the HA-eIF4E-W73A mutant failed to co-immunoprecipitate a kinase activity capable of phosphorylating eIF4E (Figure 9C). As Mnk1 interacts with eIF4E through eIF4G, it is conceivable that the kinase activity detected in the HA-eIF4E immunoprecipitates is that of Mnk1.

In cases of direct docking, it is generally observed that the phosphorylation reaction engenders a loss of affinity between the kinase and the substrate. Thus, if eIF4GI were solely to provide specificity to Mnk1 phosphorylation for eIF4E, it is anticipated that eIF4E mutated at Ser209 should retain the ability to interact with Mnk1. To test this, HA-tagged wild-type eIF4E and S209A/T210A, S209D or S209E mutants were expressed together with Flag-tagged Mnk1, and the interaction assayed by co-immunoprecipitation. No significant difference was detected in the ability of Mnk1 to interact with Ser209 mutants as compared with wild-type eIF4E (data not shown). Taken together, these data demonstrate that eIF4E bound to eIF4G is a much better substrate for phosphorylation as a consequence of Mnk1 interaction with the C-terminus of eIF4G.

The C-terminal region of p97 interacts with Mnk1

The protein p97/NAT-1/DAP-5 is homologous to the C-terminal two-thirds of eIF4G. It was shown to act as a general translational repressor, presumably by forming dead-end complexes that include eIF4A and eIF3, but exclude eIF4E (Imataka *et al*., 1997). Since Mnk1 associates with the C-terminus of eIF4GI, it was of interest to determine whether the corresponding fragment in p97 could also interact with Mnk1. To examine this, HA-tagged versions of full-length p97, N-terminal fragment (amino acids 1–406), which is homologous to the middle fragment of eIF4G, and C-terminal fragment (amino acids 407–907) were co-expressed with Flag-Mnk1 to similar levels (Figure 10A). Immunoprecipitates obtained with anti-Flag antibodies were resolved by SDS–PAGE and interactions between the proteins were revealed by Western blotting with anti-HA antibodies. The C-terminal fragment as well as the full-length p97 protein bound to Mnk1 (Figure 10B, lanes 1 and 3). In contrast, the N-terminal fragment failed to interact with Mnk1 (lane 2). From the intensities of the signals, it appears that the interaction between Mnk1 and p97 is as efficient as Mnk1 binding to eIF4GI (compare lane 1 with 4). To compare further the binding of Mnk1 to eIF4GI, eIF4GII and p97, we tested its ability to co-immunoprecipitate each of these proteins. The amount of endogenous Mnk1 associated with the three proteins reflected the differences in their expression (Figure 11, compare lanes 1–3 with lanes 4–6). This observation suggests that the related proteins bind Mnk1 with similar affinities. While eIF4GI and eIF4GII share a high degree of homology in the C-terminal region (Gradi *et al*., 1998), the corresponding region in p97 shares only 25% identity (Imataka and Sonenberg, 1997). However, some amino acid stretches are conserved between eIF4GI, eIF4GII and p97, suggesting that they could be involved in Mnk1 binding.

Discussion

Here we provide strong evidence that phosphorylation of eIF4E *in vivo* is dependent on its interaction with eIF4G,

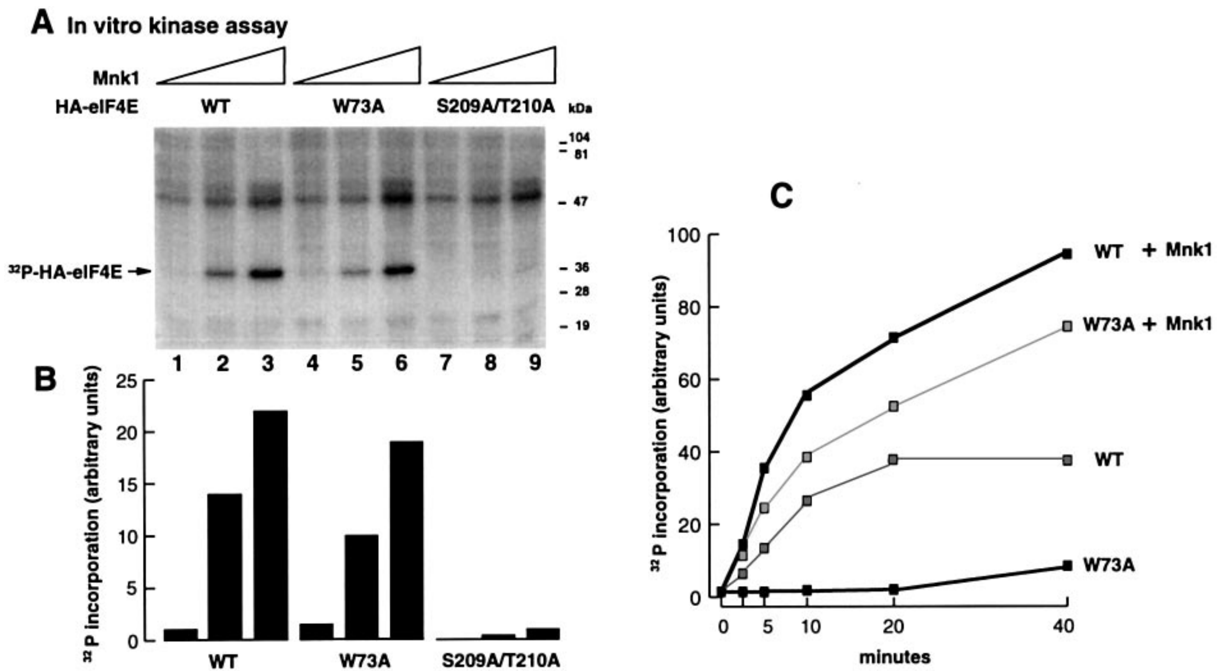

Fig. 9. Mnk1 phosphorylates the HA-eIF4E-W73A mutant *in vitro* as efficiently as wild-type HA-eIF4E. 293T cells (5×10^6 cells) were transfected with 2 μg of pcDNA3 vector expressing HA-eIF4E wild-type, HA-eIF4E-W73A and HA-eIF4E-S209A/T210A. After 36 h, cell extracts were prepared in lysis buffer and HA-tagged eIF4E protein was immunoprecipitated using anti-HA antibodies. (**A**) Equal amounts of agarose bead-coupled eIF4E proteins were subjected to *in vitro* Mnk1 kinase assay in the presence of increasing amounts (0.1, 0.4 and 1 μg) of activated Mnk1 for 15 min at 30°C, resolved by SDS–PAGE and visualized by autoradiography as described in Materials and methods. (**B**) Phosphorylation of HA-eIF4E proteins was quantified using a PhosphorImager (Fuji Corp.). (**C**) Kinetics of the phosphorylation of HA-eIF4E wild-type and W73A mutant performed in the presence (0.4 μg) or absence of Mnk1, as indicated on the right side of the figure. Samples were resolved by SDS–PAGE, visualized by autoradiography, and phosphorylation of HA-eIF4E proteins was quantified using a PhosphorImager (Fuji Corp.).

which possesses a binding site for Mnk1. The use of a docking site in a separate polypeptide to increase specificity and efficiency of phosphorylation of a given protein is already established for several other highly specific serine/threonine kinases. (i) Specific phosphorylation of E2F-1 by CDK–cyclin A requires a cyclin A-binding site (Krek *et al*., 1994). (ii) The binding of βARK to G-βγ facilitates the phosphorylation of the β-adrenergic receptor by βARK (Inglese *et al*., 1995). (iii) In the case of JunD, the absence of an effective JNK docking site renders binding of JunD to JNK very inefficient *in vitro*. However, the phosphorylation reaction occurs *in vivo* as the heterodimerization of JunD with a third partner capable of recruiting JNK via its own docking site, such as c-Jun, facilitates the binding of JunD to JNK (Kallunki *et al*., 1996).

What is the purpose of the requirement for eIF4G for eIF4E phosphorylation? The physiological entity that binds to the mRNA 5′ cap structure is the eIF4F complex rather than eIF4E alone (Haghighat and Sonenberg, 1997; Muckenthaler *et al*., 1998; Ptushkina *et al*., 1998). Thus, the requirement for eIF4G for eIF4E phosphorylation would ensure that eIF4E will be regulated by phosphorylation only as part of eIF4F. This is consistent with the finding that when complexed with 4E-BP1, which competes with eIF4G for a common binding site on eIF4E, eIF4E is not a substrate for Mnk1 *in vitro* (Wang *et al*., 1998). Another possible explanation for the restriction of eIF4E phosphorylation to the eIF4F complex is based on the three-dimensional structure of eIF4E. As described earlier, Lys159 is juxtaposed next to the flexible loop containing Ser209 (7.5 Å distance when the flexible loop is extended, according to computer modeling). It was proposed that the trajectory of the mRNA would lie just below Lys159 and Ser209. Thus, the phosphorylated Ser209 could form a salt bridge with Lys159, generating a clamp over the mRNA and enhancing its interaction with eIF4E (Marcotrigiano *et al.,* 1997). This scenario raises the intriguing possibility that phosphorylation of eIF4E might occur *in vivo* only when bound to the cap structure as part of the eIF4F complex to ensure the clamping of the mRNA. The finding that the phosphorylation of eIF4E stabilizes its interaction with the cap structure further supports this hypothesis (Minich *et al*., 1994). Together with the results already published and discussed above, the data presented here suggest a model whereby Mnk1, as part of the eIF4F complex, regulates the cap-binding activity of eIF4F (Figure 12).

The binding affinity of Mnk1 to eIF4G is not known, but is likely to be high. Indeed, even after stringent nickel-chelate affinity column purification, we detected a significant intrinsic kinase activity in the preparations of His-tagged eIF4GI from insect cells (S.Pyronnet, unpublished observations). Although we could not identify the kinase associated with eIF4GI preparations (probably because our anti-Mnk1 antibody was raised against human Mnk1), it is very likely that the kinase activity detected is that of the insect ortholog of Mnk1. This is supported by the observation that the incubation of HPLC-purified eIF4E together with His-tagged eIF4GI in the presence of [γ-^{32}P]ATP led to *in vitro* phosphorylation of eIF4E (S.Pyronnet, unpublished data). Finally, anti-HA immunoprecipitates obtained from HA-eIF4E-expressing 293T cells contained a kinase activity capable of phosphorylating

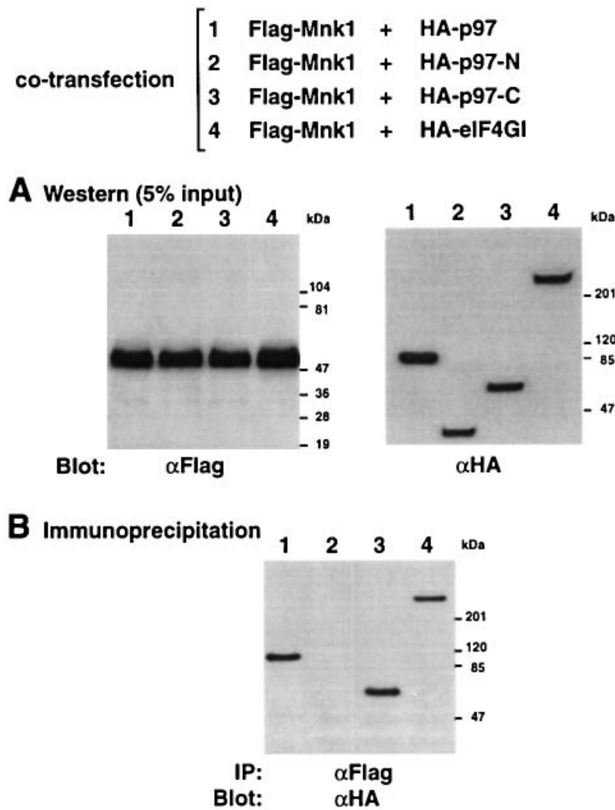

Fig. 10. The C-terminus of p97 contains a Mnk1-binding site. 293T cells (2×10^6 cells) were transfected with 2 μg of plasmid expressing the protein indicated at the top of the figure. After 36 h, cells were harvested in lysis buffer, and a fraction (5% input) of the lysate (**A**) and the total immunoprecipitate (**B**) were analyzed by immunoblotting using anti-Flag and anti-HA antibodies as described in Materials and methods.

eIF4E *in vitro*, whereas in the HA-eIF4E-W73A precipitates no kinase activity was detected (Figure 9).

While PKC initially was described as a possible eIF4E kinase, as it phosphorylates Ser209 *in vitro*, Mnk1 is the best candidate for eIF4E phosphorylation *in vivo*. However, Mnk1 shares significant homology with other serine/threonine kinases such as human p90^{RSK}, MAPKAPK-2 (2pK) and MAPKAPK-3 (3pK) whose kinase domains exhibit 36, 34 and 33% amino acid identity to that of Mnk1, respectively (Fukunaga and Hunter, 1997). No obvious similarity is found in the N-terminal regions of these kinases beyond the catalytic domain, while the C-terminal regions show significant homologies. Neither p90^{RSK} nor 2pK can phosphorylate eIF4E *in vitro*, whereas it was suggested that 3pK immunoprecipitated from human cells could phosphorylate eIF4E *in vitro* (Morley and McKendrick, 1997). Nevertheless, the interaction between eIF4G and murine Mnk1 seems to occur through the N-terminal region of Mnk1 (J.Cooper, personal communication), a region which shares no homology with the N-terminal region of 3pK.

eIF4G is a phosphoprotein and its phosphorylation is enhanced in response to several extracellular stimuli, including growth factors and hormones. For example, phosphorylation of eIF4G occurs concomitantly with that of eIF4E in response to TPA (Morley and Traugh, 1989, 1990). It is therefore anticipated that common signaling pathways impinge upon phosphorylation of both eIF4E

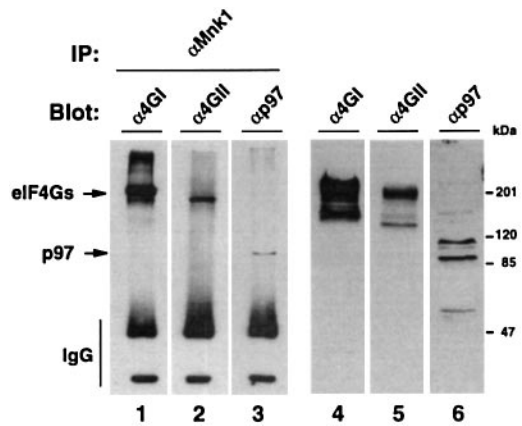

Fig. 11. The amount of Mnk1 associated with eIF4GI, eIF4GII or p97 reflects the expression levels of the proteins. Extracts from 293 cells (10^7 cells) were immunoprecipitated and analyzed by Western blotting with the specific antibodies indicated at the top of the figure, as described in Materials and methods.

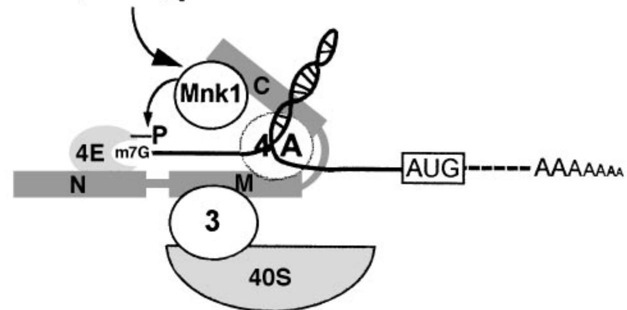

Fig. 12. Mnk1 phosphorylates eIF4E as a component of the eIF4F complex. The model shows that phosphorylation of eIF4E occurs in the eIF4F complex, suggesting that eIF4F assembles prior to eIF4E phosphorylation. eIF4E can interact in a mutually exclusive manner with either eIF4Gs or 4E-BPs. The association of eIF4E with eIF4Gs enhances binding of eIF4E to the 5′ cap structure (Haghighat and Sonenberg, 1997; Ptushkina *et al.*, 1998) and brings Mnk1 into the vicinity of eIF4E. The resulting phosphorylation of eIF4E might enhance its affinity for eIF4Gs (Bu *et al.*, 1993) and stabilize its interaction with the mRNA 5′ end (Minich *et al.*, 1994).

and eIF4G. In preliminary experiments, it was found that Mnk1 phosphorylates eIF4GI, eIF4GII and p97 *in vitro* (A.-C.Gingras and B.Raught, unpublished observations). Thus, Mnk1 is likely to play an important role in the regulation of cell growth and proliferation not only through the phosphorylation of eIF4E, but also via the phosphorylation of eIF4G and p97. Since the C-terminal fragment of p97 interacts with Mnk1 as efficiently as the corresponding region in the eIF4G amino acid sequence, but fails to interact with eIF4E, it is also conceivable that the interaction between p97 and Mnk1 may decrease eIF4E phosphorylation via Mnk1 sequestration.

Materials and methods

Plasmids and antibodies

Point mutations in murine eIF4E were made as follows: mutants S209A/T210A, S209D, S209E and W73A were generated by PCR mutagenesis. Coding sequences were then ligated in-frame in pcDNA3HA to express a fusion protein with three HA tags. pcDNA3 plasmids containing HA-tagged eIF4GI and p97 and their deletion mutants were described previously (Imataka and Sonenberg, 1997). HA-tagged human Mnk1

inserted into the pcMX expression vector (Umesono *et al*., 1991) was described previously (Fukunaga and Hunter, 1997). pcMX containing the Flag-tagged Mnk1 sequence was generated by PCR using HA-tagged Mnk1 as a template.

Monoclonal HA11 anti-HA and M2 anti-Flag antibodies were obtained from Sigma. Rabbit antisera were raised against mouse eIF4E (Frederickson *et al*., 1992) and human 4E-BP1 (Gingras *et al*., 1996), eIFG4II (Gradi *et al*., 1998), p97 (Imataka *et al*., 1997) and Mnk1 (Fukunaga and Hunter, 1997). Anti-eIF4GI antibody was a kind gift from Dr L.Carrasco. Anti-eIF4GI-C antiserum directed against the C-terminal region of eIF4GI (A.Gradi, unpublished) and anti-eIF4A antiserum (N.Methot, unpublished) were raised in rabbits.

Transient transfections

Transient transfections were conducted using Lipofectin (Gibco-BRL). For co-immunoprecipitations, human embryonic kidney (HEK) 293 or 293T cells were plated at 2×10^6/10 cm dish (except where indicated otherwise) in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS). At 80% confluency, cells were rinsed once with optiMEM (Gibco-BRL) and placed for 5 h at 37°C in 5 ml of optiMEM containing the DNA–Lipofectin complex prepared as specified by the manufacturer (Gibco-BRL). The amount of DNA used for each transfection is indicated in the figure legends. The medium was then replaced by DMEM/10% FCS, and cell extract was prepared after 36 h and assayed for immunoprecipitation followed by Western blotting. To prepare HA-eIF4E wild-type and mutant proteins used in the *in vitro* Mnk1 kinase assay, transfections were performed essentially as described above, except that cells were plated in 6 cm dishes. Total extract from each dish was immunoprecipitated to obtain HA-eIF4E wild-type or mutant proteins, and the proteins bound to agarose beads were used as substrates for Mnk1 kinase assays.

Metabolic labeling

293 cells were split into 100 mm dishes and grown to 80% confluency. Transient transfections with plasmids encoding wild-type and mutant HA-eIF4E were carried out as described above. At 36 h after transfection, cells were starved for 24 h and incubated for 5 h at 37°C in phosphate-free DMEM containing 0.5 mCi/ml [^{32}P]orthophosphate (Dupont NEN; 3000 mCi/mmol), then serum (10%) was added for 3 h. The medium was removed and the cells were rinsed twice in ice-cold phosphate-buffered saline (PBS). Cells were incubated for 30 min at 4°C in lysis buffer [50 mM Tris–HCl pH 7.4, 150 mM KCl, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM okadaic acid and ProteaseIN™ (Boehringer Mannheim)] and lysates were collected by scraping. Cell debris was removed by centrifugation at 10 000 *g* for 10 min at 4°C. Total radioactivity was monitored by spotting 1, 2, 5 and 10 µl of extract onto a phosphocellulose (P81) paper, which was washed extensively with 75 mM phosphoric acid and dried. Bound radioactivity was measured by scintillation counting.

Immunoprecipitation and immunoblotting

After transient transfection, the medium was removed and the cells were rinsed twice in cold PBS, incubated for 30 min at 4°C in lysis buffer and collected by scraping. Cell debris was removed by centrifugation at 10 000 *g* for 10 min at 4°C. Extracts were pre-cleared with protein A–agarose beads for 1 h at 4°C. The primary antibody was added for 1 h at 4°C, and immunoglobulin complexes were collected on protein A–agarose beads for 1 h at 4°C. The beads were washed five times with 1 ml of lysis buffer each. Protein complexes were generally recovered by boiling in Laemmli sample buffer and were analyzed by SDS–PAGE. Under conditions where the protein of interest co-migrated with either the heavy or light chain of immunoglobulins, proteins were heated at 75°C in Laemmli sample buffer devoid of β-mercaptoethanol. Western blotting was done using standard techniques.

In vitro kinase assay

HEK 293T cells were transfected with plasmids encoding wild-type and mutant HA-eIF4E using Lipofectin as described above. Extracts were prepared in lysis buffer and subjected to immunoprecipitation using anti-HA antibodies and protein A–agarose beads as described above, except that the washes were as follows: beads were rinsed once with lysis buffer, twice with lysis buffer containing 0.5 M KCl, once with lysis buffer and finally with 20 mM HEPES–KOH (pH 7.4). Erk1-activated GST–Mnk1 (Fukunaga and Hunter, 1997) was used to phosphorylate similar amounts of protein A–agarose bead-coupled HA-eIF4E wild-type and mutant proteins *in vitro*. Beads were incubated with activated GST–Mnk1 in kinase buffer (20 mM HEPES–KOH pH 7.4, 20 mM β-glycerophosphate, 10 mM $MgCl_2$, 1 mM DTT, 25 µM ATP, 10 µCi of [γ-^{32}P]ATP) at 30°C for different times. Kinase reactions were stopped by mixing with Laemmli buffer, subjected to SDS–PAGE and proteins were visualized by autoradiography. HA-eIF4E phosphorylation was quantified using a BAS-2000 PhosphorImager (Fuji Corp.).

In vitro association

Recombinant proteins were mixed in buffer A (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40) for 1 h on ice, then antibodies and protein A-coupled agarose beads were added. Following an additional incubation for 1 h on ice, the mixture was washed five times with 1 ml of buffer A. Bound proteins were subjected to SDS–PAGE followed by Western blotting using specific antibodies.

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