

## MINIREVIEW

# Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation?

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Eukaryotic initiation factor 4E (eIF4E) plays an important role in mRNA translation by binding the 5'-cap structure of the mRNA and facilitating the recruitment to the mRNA of other translation factors and the 40S ribosomal subunit. eIF4E can interact either with the scaffold protein eIF4G or with repressor proteins termed eIF4E-binding proteins (4E-BPs). High levels of expression can disrupt cellular growth control and are associated with human cancers. A fraction of the cellular eIF4E is found in the nucleus where it may play a role in the transport of certain mRNAs to the cytoplasm. eIF4E undergoes regulated phosphorylation (at Ser209) by members of the Mnk group of kinases, which are activated by multiple MAP kinases (hence Mnk = MAP-kinase signal integrating kinase). The functional significance of its phosphorylation has been the subject of considerable interest. Recent genetic studies in *Drosophila* point to a key

role for phosphorylation of eIF4E in growth and viability. Initial structural data suggested that phosphorylation of Ser209 might allow formation of a salt bridge with a basic residue (Lys159) that would clamp eIF4E onto the mRNA and increase its affinity for ligand. However, more recent structural data place Ser209 too far away from Lys159 to form such an interaction, and biophysical studies indicate that phosphorylation actually decreases the affinity of eIF4E for cap or capped RNA. The implications of these studies are discussed in the light of other, *in vitro* and *in vivo*, investigations designed to address the role of eIF4E phosphorylation in mRNA translation or its control.

**Keywords:** eIF4E; phosphorylation; Mnk; mRNA; initiation complex.

## INTRODUCTION

mRNA translation is a site for the regulation of gene expression under a wide range of different conditions. These include, in animal cells, responses to hormones, growth factors, vasoactive agents and cytokines, as well as nutrients such as amino acids and sugars. These conditions generally activate translation. Conversely, under a range of stressful conditions such as oxidative or osmotic stress, DNA damage or nutrient withdrawal, the rate of protein synthesis is decreased. These effects happen within minutes and are considered to be due to changes in the activity, or other functions, of components of the translational machinery. Regulation appears to be achieved primarily by changes in their states of phosphorylation. Within the overall process of mRNA translation, control seems to be exerted mainly at the stage of translation initiation, during which the 40S subunit is recruited to the 5'-end of the mRNA, the start codon is located and the 60S subunit then joins, to create a complete ribosome capable of entering the elongation stage of the process.

Eukaryotic initiation factor (eIF) 4E is one of the most intensively studied components of the translational machin-

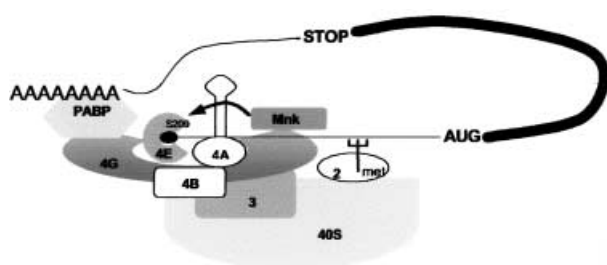
ery. This small ( $\approx 24$  kDa) protein binds to the 'cap' structure at the 5'-end of the mRNA and, by interacting with a scaffold protein, eIF4G, serves to recruit other components including the 40S ribosomal subunit to the 5'-end of the message (Fig. 1, see also accompanying review by Proud [1]). The cap contains a guanosine triphosphate moiety, methylated at position 7 of the base, and linked via a 5'-5' phosphodiester bond to the first nucleotide of the mRNA (see also Fig. 3). Base methylation also occurs on this and on the second nucleotide of the message.

## eIF4E INTERACTS WITH OTHER PROTEINS

eIF4E is frequently described as the least abundant translation factor although the evidence for this is not strong, being limited to examination of a very limited range of cell types at a time when tools were only available to study a small number of initiation factors. However, rather than abundance *per se*, it is likely that the availability of eIF4E is critically important for the activity of the initiation process. To function in cap-dependent translation initiation, eIF4E must form initiation complexes involving the scaffold protein eIF4G and its other binding partners, which include the RNA helicases eIF4A<sub>I</sub> and eIF4A<sub>II</sub> (indicated with '4A' in Fig. 1). One molecule of either eIF4A<sub>I</sub> or eIF4A<sub>II</sub> can bind to eIF4G [2], but a functional difference between the two forms has not been established. The complex of eIF4E, eIF4A, and eIF4G is known as the eIF4F complex. eIF4E interacts with eIF4G via a site through which it also binds inhibitory proteins termed 4E-BPs. Association of eIF4E with a 4E-BP prevents it from forming productive initiation

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**Fig. 1. Recruitment of initiation complexes to the 5'-cap structure.** eIF4E binds to the 5'-5' m<sup>7</sup>GpppG cap-structure (represented by a black dot) at the 5'-end of the messenger RNA. Binding of the scaffold protein eIF4G to the dorsal site of eIF4E allows recruitment of several other factors to the mRNA, e.g. eIF4A, the poly(A)-binding protein (PABP), which binds to the N-terminus of eIF4G, and the Mnk, which binds to the C-terminus of eIF4G. A central domain in eIF4G binds eIF3, which will bring in the 40S small ribosomal subunit and consequently eIF2 with the initiator methionyl tRNA (Met-tRNA<sub>i</sub><sup>Met</sup>). The helicase activity of eIF4A, which is enhanced by eIF4B, is thought to be required for unwinding of secondary structures in the 5'-UTR region, allowing subsequent movement of the whole complex along the 5'-UTR, until the initiation codon (AUG) of the open reading frame is recognized by the anticodon of the Met-tRNA (not shown). The interaction of the RNA with PABP through its poly(A)-tail and the binding of PABP to eIF4G circularizes the RNA, a process that is thought to be important for re-initiation of translation or may be required for verification that the mRNA is full-length, i.e. has a cap and a poly(A)-tail. The open reading frame of the mRNA is shown as a thick line. Initiation factors are abbreviated. The arrow indicates the phosphorylation of eIF4E at Ser209 by the Mnk. The trident structure represents the initiator tRNA<sub>i</sub><sup>Met</sup>.

complexes with eIF4G; the 4E-BPs thus act as inhibitors of cap-dependent translation [3,4]. The shuttling protein 4E-T also binds to eIF4E through a similar region [1,5]. 4E-BP1 is the best understood of the three 4E-BPs known in mammals. It undergoes phosphorylation at multiple sites, increased phosphorylation resulting in its release from eIF4E. Amino acids, insulin and growth factors are among the numerous stimuli known to increase the phosphorylation of 4E-BP1 and thus promote eIF4F complex formation [4,6,7]. Phosphorylation of 4E-BP1 is blocked by rapamycin, which inhibits the mTOR (mammalian target of rapamycin) signalling pathway (see accompanying review by Proud [1]). Rapamycin thus blocks the release of 4E-BP1 from eIF4E and prevents formation of complexes containing eIF4E and eIF4G.

## ROLES OF eIF4E IN CELLULAR REGULATION

eIF4E appears to play a critical role in cell regulation. Artificial overexpression of eIF4E causes loss of cellular growth control and this can be reversed by expression of either 4E-BP1 or 4E-BP2 [8–11]. Overexpression of eIF4G can also cause cell transformation, presumably by competing with endogenous 4E-BPs for eIF4E increasing its availability for eIF4F complex formation [12]. These effects may be associated with the role for eIF4E in enhancing the export of the mRNA for cyclin D1 from the nucleus to the cytoplasm. The shuttling protein 4E-T [5] is thought to

transfer eIF4E to the nucleus, where it appears to be important in transporting the cyclin D1 mRNA to the cytoplasm [13]. The increase in cyclin D1 expression is likely to promote G1 to S progression, and, consistent with this, injection of eIF4E promotes progression into S-phase [10]. Interestingly, a number of human tumours – especially those of neck, oesophagus and breast – show high levels of expression of eIF4E [14]. High levels appear to correlate with aggressive, metastatic, tumours and eIF4E levels may be of value in cancer diagnosis [15] or even in therapy [16]. Since 4E-T also binds to eIF4E via the site occluded by 4E-BPs, rapamycin is expected to interfere with nuclear transport of eIF4E. This may in part explain how rapamycin blocks cell cycle progression: cyclin D1 is required for G1 to S progression, the stage at which rapamycin blocks T-cell activation. Export of cyclin D1 mRNA from the nucleus by eIF4E is modulated by binding of the promyelocytic leukaemia protein PML to eIF4E [17]. PML seems to bind to the dorsal site to eIF4E, as the interaction is abrogated upon mutation of Trp73 in eIF4E to alanine, as shown before for the eIF4G-eIF4E and the eIF4E-4E-BP1 interactions. However, PML seems to lack the eIF4E-binding consensus sequence that is found in eIF4G and the 4E-BPs, e.g. YxxxxLϕ. The PML-eIF4E interaction appears to decrease the affinity of eIF4E for capped mRNA, an effect that may be important in the antitumorigenic effects of PML. For a recent detailed review on the possible roles of eIF4E in the nucleus see ref [18].

eIF4E is among a variety of translation initiation factors that are modified upon induction of apoptosis [19] and eIF4E appears to be important in modulating programmed cell death [20,21]. eIF4E is dephosphorylated during apoptosis [22] and eIF4E is inactivated (by increased binding to 4E-BP1) in response to DNA damage that leads to apoptosis, but at times well before commitment to cell death [23]. During apoptosis, 4E-BP1 is cleaved near its N-terminus to yield a fragment that binds to eIF4E but is not subject to normal regulation, thus acting as a dominant inhibitor of eIF4F formation and cap-dependent translation [24].

## eIF4E UNDERGOES REGULATED PHOSPHORYLATION

In higher animals – mammals and insects (at least in *Drosophila*) – eIF4E is a phosphoprotein. It is phosphorylated by the MAP-kinase signal-integrating kinases Mnk1 and Mnk2, at a single site *in vivo*, Ser209 in mammals, which lies near the C-terminus of the primary sequence [25,26]. There has been some confusion about the existence of other phosphorylation sites – Ser53 was initially identified as the site of phosphorylation, but this residue is now known not to be phosphorylated. Considerable excitement was generated when a Ser53→Ala mutant was found not to cause the cell transformation observed upon over-expression of the wildtype protein [9], apparently suggesting that phosphorylation was essential for its transforming function. Given what we now know, it is more likely that mutation of Ser53 to Ala interferes with the overall structure of eIF4E and thereby affects its function. This finding indicates the degree of caution that must be exercised when interpreting data obtained from presumptive phosphorylation site mutants without strong protein chemical data to support the identification of the site. In particular, it is crucial to show

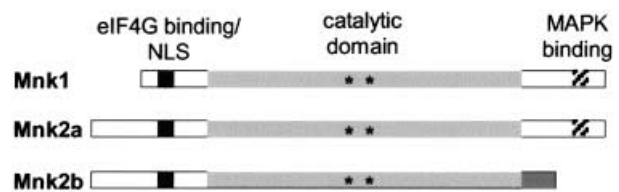
that *in vivo* phosphorylation of the protein is abolished by the mutation by radiolabelling cells expressing the relevant mutant protein. In the case of multiply phosphorylated proteins, the task is more complex, and analysis must be accompanied by appropriate phosphopeptide mapping [27].

The structures of both yeast and mammalian eIF4E have been determined – the former by NMR methods, the latter crystallographically. They show a similar overall fold, which has been likened to a baseball glove, in which the methylated base is sandwiched between two highly conserved tryptophan residues [28,29]. The binding site for the 4E-BPs and eIF4G is a hydrophobic region located on the concave dorsal surface of the protein. Binding of 4E-BP1 or eIF4G to this region induces conformational changes that greatly increase the affinity of eIF4E for capped nucleotide. It is therefore puzzling that the binding of PML to eIF4E, which also involves the dorsal surface of eIF4E, actually decreases the affinity of eIF4E for capped mRNA [17]. Ser209 is located close to the putative channel through which the capped RNA enters the cap-binding site of the eIF4E molecule [28]. This channel is putative as the crystal structure involved only 7-methylGDP and not a capped oligonucleotide.

Phosphorylation of eIF4E is increased in response to a variety of conditions [30]. These include serum treatment of cells, growth factors, phorbol esters, and in some cell types, insulin [31]. Where tested, these effects appear to be mediated via the MEK/Erk pathway, as they are blocked by inhibitors of MEK [31–34]. Certain cytokines and stressful conditions also increase the phosphorylation of eIF4E [32,34,35], and, where studied, the effects appear to be due to the p38 MAP kinase pathway [32,35]. Strictly, since the available inhibitors act on the  $\alpha$  and  $\beta$  isoforms of p38 MAP kinase [36,37], it should be made clear that it is these forms that are responsible for the increases in eIF4E phosphorylation rather than the more-distantly related  $\gamma$  or  $\delta$  enzymes. It is the  $\alpha$  and  $\beta$  isoforms that can phosphorylate and activate the Mnks [34,38,39]. Although certain other stresses (e.g. heat shock, oxidative or osmotic stress) also activate the p38 MAP kinase pathway, they do not cause increased phosphorylation of eIF4E [32]. This probably reflects the fact that they cause loss of eIF4F complexes (due to dephosphorylation of 4E-BP1, which then sequesters eIF4E [32,40], separating it from the Mnks bound to eIF4G). Similarly, the dephosphorylation of eIF4E induced by infection of cells with adenovirus [41] appears to be due to the displacement of Mnks from eIF4F complexes by an adenovirus-encoded 100 kDa protein that competes with Mnks for binding to eIF4G [42]. In contrast, infection of cells with murine coronavirus activates Mnks and increases eIF4E phosphorylation in a p38 MAP kinase-dependent manner [43].

## THE eIF4E KINASES, THE Mnks

The Mnks were identified independently by the work of Fukunaga and Hunter [38] and Waskiewicz and Cooper [39], using screens for substrates or binding partners, respectively, of Erk and p38 MAP kinases. Each group identified two related kinases, now termed Mnk1 and Mnk2. They share substantial similarity (88%) in their catalytic domains, and their N- and C-termini also share



**Fig. 2. Features of the Mnks.** The N-termini of Mnk1 and the Mnk2s are similar (except that they are extended in human Mnk2s; no specific function has yet been reported for this extension.). The N-termini contain a polybasic region which may be involved both in binding to eIF4G [33] and may also function as a nuclear localization sequence (NLS). The catalytic domains of Mnk1 and the Mnk2s are also strongly similar. Phosphorylation of two threonine residues (\*) within the catalytic domain has been shown to be essential for activation, although additional phosphorylation sites also exist (not shown [46]). The sequences of the C-termini of Mnk1 and Mnk2a are similar and contain sequences for MAPK binding. Small differences within these sequences play a role in determining the specificity for ERK and p38MAPK. The last 29 amino acids of Mnk2b are quite different from the C-terminal sequences of Mnk1 or Mnk2a and Mnk2b thus lacks the MAPK binding site.

quite high levels of similarity (respectively 77% and 65%) (Fig. 2). It is now clear that there is a second form of human Mnk2, generated by alternative use of coding exons during splicing, resulting in proteins with quite different C-termini (Fig. 2). The final 80 amino acids of human Mnk2 (which are very similar to the C-terminus of Mnk1) are replaced by an entirely different C-terminal tail of 29 amino acids in the Mnk2b protein [44]. All three Mnk species can interact with eIF4F complexes *in vivo* [33,45,46]. The first, and so far only, report concerning Mnk2b showed that it interacts with the oestrogen receptor in two-hybrid studies. This interaction is specific for Mnk2b (it is not observed for Mnk1 or Mnk2a) and for the  $\beta$ -isoform of the oestrogen receptor [44]. The possible functional significance of this interaction remains unclear. Interestingly, the other isoform of the oestrogen receptor ( $\alpha$ ) is phosphorylated by another kinase that lies downstream of the Erk signalling, p90<sup>RSK</sup> [47]. It is so far unclear whether Mnk2b phosphorylates the  $\beta$  form of the oestrogen receptor.

Mnk1 and murine Mnk2 (which will be called Mnk2a below as it is the homologue of the human Mnk2a form) can be activated by phosphorylation *in vitro* by Erk or by p38 MAP kinase [38,39,46] although there are important differences in their *in vivo* activities. *In vivo*, Mnk1 displays a low level of activity, which is greatly enhanced by treatment of cells with agents that activate either the Erk or the p38 MAP kinase  $\alpha/\beta$  pathway [32,38,39]. As indicated above, the effects of such treatments are blocked by inhibitors of these pathways. In contrast, Mnk2a has high basal activity, which is not enhanced further by agents that activate Erk/p38 MAP kinase [46]. Since the high basal activity is reduced by inhibitors of these pathways, it seems to be due to the low basal levels of activity of the pathways that exist in unstimulated cells. This suggests that Mnk2a may be unusually readily phosphorylated and activated by Erk/p38 MAP kinase, and experiments performed *in vitro* bear this out [46]. Preliminary data suggest that Mnk2b also has relatively high basal activity.

The differences in basal activity or regulation of Mnk2a as compared to Mnk1 have potentially important implications for the control of the phosphorylation of eIF4E. In cells that only, or mainly, contain Mnk1, the level of eIF4E phosphorylation will be determined by two factors. The first is the state of activation of the Erk or p38 MAP kinase pathways, which regulate the activity of Mnk1. The second is the level of eIF4F complexes which bring together eIF4E and the Mnks through their common binding partner, eIF4G. The level of eIF4F complexes is determined by factors such as amino acid availability and other stimuli, including growth factors and insulin. Thus, in HEK293 cells, agents such as the phorbol ester TPA, which activates Erk and increases eIF4F formation, increase the level of phosphorylation of eIF4E, while insulin, which increases eIF4F formation but does not activate Erk, does not increase eIF4E phosphorylation above its low basal level in these cells [48].

The high basal levels of activity of Mnk2 are likely to have two important consequences for cellular levels of eIF4E phosphorylation. Firstly, this is likely to be relatively high in cells possessing significant levels of these kinases (provided they contain eIF4F complexes under a given condition). Secondly, the primary determinant of eIF4E phosphorylation in cells mainly expressing an Mnk2 isoform will be the level of eIF4F complexes, rather than increases in Erk/p38 MAP kinase activity. The requirement for eIF4F complex formation for efficient phosphorylation of eIF4E provides an input for amino acids/glucose (which enhance formation of such complexes [1]) and well as for insulin, which in some cell types does not activate Erk, but does generally enhance formation of eIF4F complexes. Analysis of expression of RNAs encoding the different Mnks suggests that all three forms are expressed in all tissues tested, although expression levels of Mnk2a seem to be lower in brain, heart and ovary [33,44]. However, such a tissue-specific analysis of protein levels has not yet been carried out.

### **IN VITRO ANALYSIS OF THE EFFECTS OF eIF4E PHOSPHORYLATION ON ITS PROPERTIES**

The effect of phosphorylation on the properties of eIF4E has been the subject of substantial interest. Given that stimuli that increase the rate of protein synthesis generally increase the state of phosphorylation of eIF4E, it was generally thought likely that phosphorylation would somehow activate eIF4E, e.g. perhaps increase its affinity for cap or capped mRNA. Minich *et al.* [49] were the first to try to address this important issue. Their work was performed before the identification of the Mnks, and they used chromatography on RNA-cellulose to separate phosphorylated from unphosphorylated eIF4E. The fraction of eIF4E that was not retained on this resin was found to consist only of the phosphorylated form, while the bound material was unphosphorylated. Using fluorescence methods, it was found that the fraction containing the phosphorylated eIF4E showed a three to four times higher affinity for m<sup>7</sup>GTP and for capped (globin) RNA. Two important caveats with this approach are that the basis of the resolution of these forms on RNA-Sepharose is quite unclear and that it is possible that one or other fraction was

contaminated with other proteins that influence the affinity of eIF4E for RNA. For example, the 4E-BPs, which greatly increase the binding of eIF4E to cap [50], were not known at this time and would in any case not have been detected by the methods used in their study.

With the discovery of the Mnks, it became possible to phosphorylate eIF4E *in vitro*, to defined extents, and use this material to explore the effect of phosphorylation on its function. Scheper *et al.* [51] employed this approach. When the binding of eIF4E to cap analogue (m<sup>7</sup>GTP) was examined by fluorescence quenching, it was clear that phosphorylated eIF4E bound with lower affinity (2.5-fold difference) than the unphosphorylated protein [51]. Replacement of Ser209 by either of the two acidic amino acids, Glu or Asp, has almost no effect on the binding of eIF4E to m<sup>7</sup>GTP, indicating that a carboxylate group is a very poor mimic of phosphoserine in this case. Scheper *et al.* [51] also employed the surface plasmon resonance approach first described by von der Haar *et al.* [52] to examine binding of eIF4E to a capped oligonucleotide (i.e. one carrying m<sup>7</sup>GTP at its 5'-end). This ligand is immobilized by virtue of a biotin group at its 3'-end, which allows very tight binding to the streptavidin chip surface. Arguably, this capped oligonucleotide more accurately resembles the physiological ligand of eIF4E, capped mRNA. In this case, phosphorylation of eIF4E again reduced its affinity for the ligand (by about fivefold). Acidic mutations at Ser209 also decreased the affinity of eIF4E for capped oligonucleotide, although to a lesser extent than phosphorylation. In contrast, Shibata *et al.* [53] have reported that replacement of Ser209 by an acidic residue decreases release of eIF4E from cap, i.e. increases the affinity of eIF4E for this ligand.

The analysis of Scheper *et al.* [51] indicated that the phosphorylation of eIF4E does not affect its binding to 4E-BP1. Since 4E-BP1 binds to the same (dorsal) surface of eIF4E as eIF4G, it is likely that phosphorylation of eIF4E also has no effect on the binding of eIF4E to eIF4G. However, for technical reasons, Scheper *et al.* [51] were unable to test this directly. This finding can be explained in terms of the structure of eIF4E, since the region that binds 4E-BP1/eIF4G is remote from Ser209 in the 3D structure [28,29,50,54]. Binding of 4E-BP1/eIF4G to eIF4E does greatly increase its affinity for capped RNA [50]. This effect is maintained for phosphorylated eIF4E, the difference in binding affinity between free or 4E-BP1-bound eIF4E between the phosphorylated and unphosphorylated forms of eIF4E being similar (although the absolute binding affinity is about 100-fold less for the free eIF4E in each case).

### **HOW DOES PHOSPHORYLATION INFLUENCE THE STRUCTURE OF eIF4E?**

To date, there is no direct structural information for the phosphorylated form of eIF4E. Based on the crystal structure of the mammalian protein, Marcotrigiano *et al.* [28] speculated that, when phosphorylated, Ser209 might form a salt bridge with Lys159, and that this might clamp eIF4E onto the capped mRNA. This would be consistent with the increase in affinity for capped ligand reported by Minich *et al.* [49] but is hard to reconcile with the more recent data of Scheper *et al.* [51], which show the opposite effect. It is important to note that the original cocystal structure of the unphosphorylated protein involved m<sup>7</sup>GDP

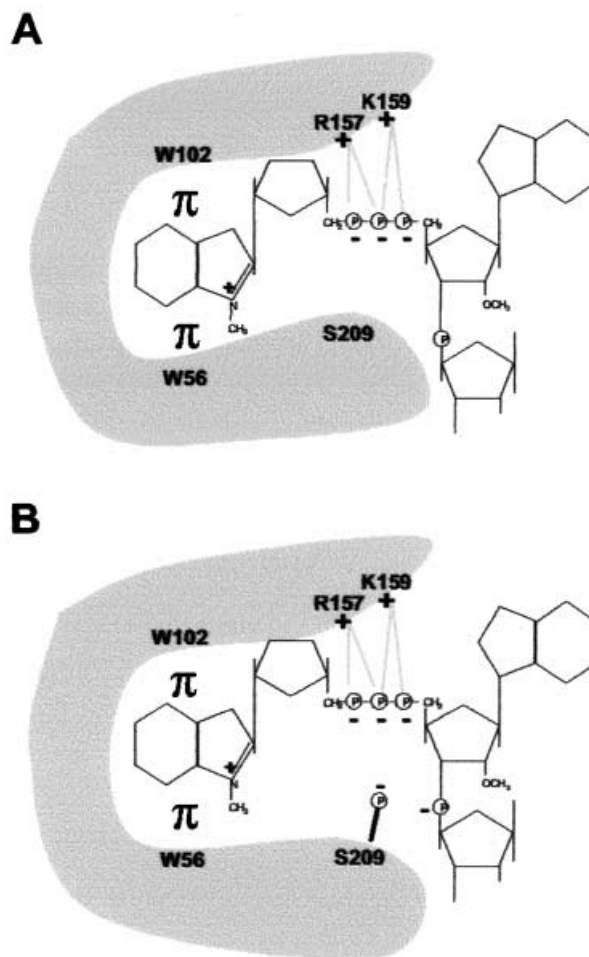
rather than the complete cap structure or a capped oligonucleotide as ligand. Most significantly, the electron density around Lys159 was poorly defined and the actual structure around this residue was therefore unclear. More recent crystallographic studies by Tomoo *et al.* [55] and Niedzwiecka *et al.* [56] did include larger ligands (respectively  $m^7\text{GpppA}$  and  $m^7\text{GpppG}$ ) and yielded better data for the structure around Lys159. This reveals that Lys159 is 12–19 Å away from Ser209, too far for formation of a salt bridge between Ser209(P) and Lys159, even given the likely flexibility of this region of the eIF4E molecule. The C-terminal loop containing Ser209 lies close to the second nucleoside (A in the structure of Tomoo *et al.*), with hydrogen bonds and van der Waals contacts between these residues and the ligand. The distance between Ser209 and the third phosphate group is substantially shorter than the distance between Ser209 and Lys159 (as determined by using PROTEIN EXPLORER Software (E. Martz, available at <http://proteineexplorer.org> and the PDB structure file deposited by Niedzwiecka *et al.* [56] available at <http://www.rcsb.org/pdb/>). It may be that, by introducing additional negative charge in this region, phosphorylation at Ser209 creates electrostatic repulsion between the protein and the negatively charged nucleotide ligand, or the negatively charged third phosphate group of the cap-structure, resulting in the weakened interaction observed in the biophysical studies of Scheper *et al.* [51]. It is notable that phosphorylation had a greater effect on binding to oligonucleotide than to  $m^7\text{GTP}$  alone: this could suggest that the phosphate group at Ser209 weakens interactions between eIF4E and phosphate groups in the body of the RNA as well as those within the cap-structure. However, no structural information is available for complexes of eIF4E with oligonucleotides larger than the  $m^7\text{GpppA/G}$  ligand.

Mutation of Lys159 to an uncharged residue weakens the binding of eIF4E to capped oligonucleotide [51], suggesting that positive charge here is important for ligand binding. The neighbouring arginyl residue at 157 is already known from structural studies to have important interactions with the phosphate groups of the cap-structure [28,29,55]. Indeed, even conservative replacement of this residue by lysine greatly decreases the binding of eIF4E to RNA [51].

It thus appears that phosphorylation of eIF4E does not result in closure of the RNA-binding cleft (clamping) – this would be inconsistent both with the recent biophysical data [51] and the new structural information [55]. Indeed, the finding that phosphorylation of eIF4E actually increases its on-rate for binding capped oligonucleotide [51] is inconsistent with cleft closure, which would be expected to have the opposite effect on the rate of ligand binding. Figure 3 depicts a simple model of the structure of eIF4E and the possible consequences of phosphorylation of Ser209.

### OTHER APPROACHES TO ASSESSING THE ROLE OF eIF4E PHOSPHORYLATION

At first sight, it seems hard to reconcile the recent finding that phosphorylation of eIF4E actually decreases its affinity for capped RNA with the fact that eIF4E phosphorylation is increased by conditions that activate protein synthesis. This will be discussed in the light of models described below.



**Fig. 3. Reduced affinity of eIF4E for the cap-structure upon phosphorylation at residue 209.** (A) Schematic structure of eIF4E (in grey) bound to  $m^7\text{GpppG}$ . The 7-methylated base lies deep within the cap-binding slot and is intercalated between Trp56 and Trp102, the interaction being favoured by  $\pi$ - $\pi$  stacking as indicated and the delocalized positive charge on the methylated guanine. Several other interactions stabilize the binding of the cap-structure, e.g. hydrogen bonds between the  $m^7\text{G}$  moiety and Trp102, Glu103, and Trp166 and a direct interaction between the ribose and Trp56 (not indicated). Other direct interactions involve the phosphate groups of the ligand and Lys162 (not indicated), Arg157 and Lys159 (as indicated by grey lines). Trp166 and Arg112 make further contacts with the phosphates through water molecules (not indicated). (B) Reduced affinity of phosphorylated eIF4E upon phosphorylation. Introduction of negative charge at Ser209 by phosphorylation of this residue may cause electrostatic repulsion between this phosphate group and the phosphates of the cap structure, or of the backbone of the RNA, thereby reducing the affinity of eIF4E for its ligand and potentially opening up the cap-binding cleft.

However we will first consider other recent data that bear on the role of eIF4E phosphorylation in mRNA translation, bearing in mind that the main mechanism governing the actual availability of eIF4E for translation is not its phosphorylation (which probably does not affect its binding to eIF4G [51,57,58]) but rather its binding to, and release from, the 4E-BPs described above (see also the accompanying review by Proud [1] and other articles [3,4,6]).

One approach to studying the role of the phosphorylation of eIF4E is to study the effects of expression of eIF4E kinases, or of eIF4E variants with mutations at the phosphorylation site, on protein synthesis or cell/organismal biology. Data from two such studies do indeed demonstrate that phosphorylation of eIF4E cannot, by itself, drive formation of eIF4E/eIF4G complexes. Over-expression of Mnk1 in HEK293 cells [48,57] or in cardiomyocytes [59] increased the phosphorylation of eIF4E without any rise in formation of eIF4E/eIF4G complexes. Forced increases in eIF4E phosphorylation also did not increase the overall rate of protein synthesis in these studies. These data are consistent with the notion that phosphorylation of eIF4E does not affect its interaction with eIF4G and indicates that it is also insufficient on its own to activate the translational machinery. Furthermore, insulin activates protein synthesis in HEK 293 cells without any effect on eIF4E phosphorylation, which remains very low [48]. This presumably reflects the fact that insulin does not significantly activate Erk in HEK293 cells. Thus, eIF4E phosphorylation does not seem to be essential for activation of protein synthesis at least by insulin (which, after all, does switch on a range of other translation factors [60]). Although, changes in eIF4E phosphorylation and protein synthesis do correlate under a variety of conditions, there are a growing number of exceptions [62,63] (reviewed by Kleijn *et al.* [61]).

Using an eIF4E-dependent *in vitro* translation system, McKendrick and coworkers [64], showed that the non-phosphorylatable Ser209Ala mutant of eIF4E was as efficient as the wildtype protein in supporting protein synthesis. eIF4E phosphorylation does not therefore seem to be required for mRNA translation here, one caveat being that eIF4E does not undergo regulated phosphorylation in this system. The Ser209→Ala mutant was also as effective as wildtype human eIF4E in complementing the disruption of the endogenous eIF4E gene in budding yeast. It is unclear how to interpret this result as eIF4E from *Saccharomyces cerevisiae* lacks an equivalent of Ser209 and as there is no homologue of Mnk1/2 in yeast, but it could indicate that phosphorylation of eIF4E adds an extra regulatory input to the initiation process in higher eukaryotes.

Knauf *et al.* [57] used two complementary approaches to examine the roles of the Mnks in controlling translation. They found that expression of active mutants of Mnk1 or Mnk2 (2a), which markedly raised the level of eIF4E phosphorylation, actually led to impairment of cap-dependent translation compared to cap-independent translation (driven by a viral internal ribosome entry sequence). Furthermore, they employed a specific inhibitor of the Mnks (CGP 57380) to block eIF4E phosphorylation and found no effect of this compound on cell proliferation, initiation factor complex formation or the ability of agents that activate the Erk pathway to stimulate protein synthesis. The authors argue that the inhibitory effect of Mnk activity on cap-dependent translation may act to limit translation under certain physiological conditions, although it is not clear how, and why, this would come about. The observation that high levels of eIF4E phosphorylation impair cap-dependent translation is certainly in accordance with the observation that phosphorylation of eIF4E decreases its affinity for capped RNA [51].

Probably the best way to examine the overall biological importance of a given phosphorylation event is to use

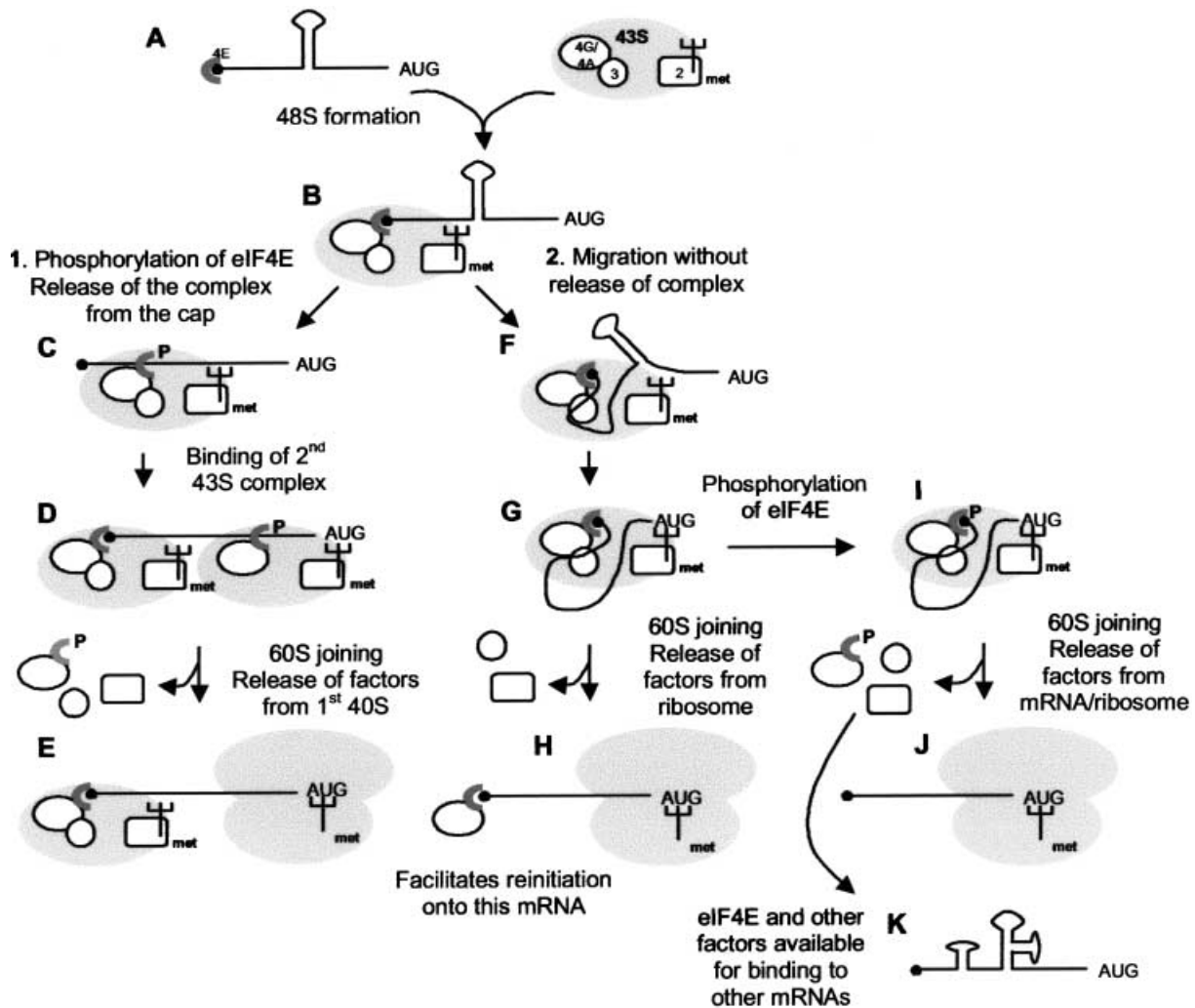
genetic approaches. LaChance *et al.* [65] have achieved this in *Drosophila* by mutating the equivalent of Ser209 in the fruitfly eIF4E (Ser251) to alanine. Two main phenotypic consequences were observed. The first is a retardation of development and the second is reduced size of the adult animals. Body parts of Ser251→Ala flies are appropriately proportioned, and studies on the ommatidia of the compound eye suggest that major defect is in cell size rather than cell number. These findings convincingly indicate a role for phosphorylation of eIF4E in cell and organismal physiology. Interestingly, reduction in cell and animal size is also associated with mutations designed to interfere with phosphorylation of another component of the translational machinery, ribosomal protein S6 [66,67].

## MODELS FOR THE PHYSIOLOGICAL ROLE OF eIF4E PHOSPHORYLATION

In view of data showing that phosphorylation of eIF4E decreases its affinity for capped ligand, why is it appropriate in a physiological context that growth factors or cytokines that activate Erk or p38 MAP kinase signalling should cause increased phosphorylation of eIF4E? What role might phosphorylation of eIF4E play in the initiation process?

Two possibilities are depicted in Fig. 4. In both cases eIF4E initially binds to the 5'-cap of the mRNA, an interaction that is stabilized by binding of 4E-BPs (not indicated) or eIF4G (diagram A). The order in which eIF4G, eIF4A and the 43S ribosomal complex [consisting of the 40S subunit with other initiation factors, e.g. eIF2 and eIF3, and the initiator methionyl-tRNA (Met-tRNA<sub>i</sub><sup>Met</sup>)] bind to form the 48S (diagram B) complex has not yet been elucidated. The eIF4G-eIF3 interaction makes it possible that eIF4G interacts with the 43S complex prior to engagement with the mRNA (as shown) although other scenarios are possible. The two models shown differ with respect to the point in the initiation process at which eIF4E phosphorylation occurs and whether eIF4E phosphorylation is required to enhance initiation on the same mRNA or on another message. In both models, the release of eIF4E from the cap is not essential for scanning, as suggested by the observations (a) that insulin activates protein synthesis in the absence of an increase in eIF4E phosphorylation [48] and (b) that the S209A mutant can support protein synthesis [64].

In the model depicted on the left (model 1), phosphorylation of eIF4E occurs immediately after the assembly of the initiation complex in which eIF4F is formed, thereby recruiting the Mnks to act on eIF4E (diagram C). Given that phosphorylation of eIF4E weakens its affinity for capped mRNA, it would be important for phosphorylation of eIF4E to occur only after the 48S complex had formed on the mRNA. This is achieved by the requirement for both the Mnk and eIF4E to be part of complexes with eIF4G in order for efficient phosphorylation of eIF4E to occur [45]. Reduced affinity of eIF4E for the cap-structure by phosphorylation of eIF4E prior to 48S formation would result in its release from the cap, i.e. too early in the initiation process to allow productive complex formation. It is also possible that eIF4G binds to eIF4E before it interacts with eIF3, but that the structure of eIF4F complexes that form prior to engagement of eIF3 does not favour eIF4E phosphorylation, and that binding of eIF4G to the 48S complex (via



**Fig. 4. Possible roles for phosphorylation of eIF4E in the initiation process.** Models for the possible role of eIF4E phosphorylation in translation initiation are depicted. See the text for details. Only the 5'-UTR and start codon of the mRNA are shown; 'P' denotes the phosphorylation of Ser209 in eIF4E; the trident represents the initiator tRNA<sup>Met</sup>. Letters identify complexes referred to in the text, to which the reader is referred for detailed discussion of these models.

eIF3) triggers a change in the structure allowing phosphorylation of eIF4E. Phosphorylation of eIF4E, of course, requires that the Mnk associated with the ribosome to be active: in the case of Mnk1, for example, activity would be enhanced by triggering of the Erk or p38 MAP kinase pathways.

Phosphorylation of eIF4E subsequent to formation of initiation complexes (diagram C) facilitates the release of eIF4E and associated factors, including the 40S subunit, from the cap structure, but these factors remain attached to the 48S initiation complex, during the scanning (an idea that has been suggested before by Morley [68]). The unwinding of any secondary structure is carried out by eIF4A. The binding constant for the eIF4E-eIF4G interaction is about three orders of magnitude higher than for cap-binding by eIF4E [54] indicating that the eIF4E complex will likely stay intact (note that the binding of either 4E-BP1 or eIF4G to eIF4E appears to be the determining factor in stabilizing the eIF4E:cap interaction [50], and could be regarded as causing the 'clamping' of eIF4E to the cap).

Several initiation factors have RNA-binding properties (e.g. eIF4G, eIF4B, subunits of eIF3) and, together with possible mRNA-ribosomal RNA interactions, this would probably suffice to ensure that stable binding of the initiation complex to the mRNA does not depend on the eIF4E:cap interaction alone.

In model 1, the release of the eIF4E exposes the cap and allows the recruitment of a second eIF4E molecule and associated proteins, plus the 40S subunit (diagrams D, E). This facilitates the rapid loading of the next initiation complex and ultimately the next ribosome onto the mRNA even before the first initiation complex has proceeded into elongation. This mechanism would serve to expedite ribosome loading and thus contribute to activation of translation initiation. Operation of such a model would be consistent with the observations of Morley and Naegele [58] that inhibition of eIF4E phosphorylation by the Mnk inhibitor CGP 57380 resulted in impaired polysome assembly, indicative of decreased recruitment of ribosomes onto the mRNA. The fact that this was not accompanied by

impaired rates of protein synthesis suggests that in their system such a regulation of ribosomal loading does not limit the overall rate of translation.

In model 2, phosphorylation of eIF4E occurs later in the initiation process, e.g. around the time that the start codon is recognized (as depicted in Fig. 4). Phosphorylation of eIF4E could function to enhance the release of factors from the cap-structure after 60S joining, rendering the cap-binding factors available for the translation of different mRNAs. eIF4E was shown to be highly phosphorylated in 48S complexes [69] indicating that phosphorylation most likely occurs prior to 60S joining. Without eIF4E phosphorylation, eIF4E more likely remains at the cap during initiation. The mRNA must loop through the initiation complex (as depicted in diagrams F–H), further ribosomes are prevented from binding during scanning by the first 40S subunit. Binding of further ribosomes requires the completion of scanning by the first 43S complex, and this may impose a limit on the rate of translation initiation, especially for mRNAs with long 5'-UTRs or ones rich in secondary structure that has to be unwound to allow scanning. eIF4F complexes could remain attached to the RNA, maybe by stabilization mediated by binding of PABP, obviating the requirement to reassemble eIF4F. Studies using tethered eIF4E or eIF4G has shown that eIF4F complexes that are fixed in their position on the RNA, do allow initiation, but the authors could not address the question as to whether this process was as efficient as the noncovalent binding of eIF4F complexes as it occurs naturally [70,71].

In the absence of an active Mnk, phosphorylation of eIF4E cannot occur and eIF4E is more likely to remain associated with the cap (diagram H). This may allow reinitiation of translation onto this mRNA, as indicated below diagram H. With an active Mnk within the complex, phosphorylation of eIF4E can occur but is proposed to be triggered late in the initiation process (perhaps due to conformational changes in the initiation factor complex), perhaps around the point where the anticodon of the Met-tRNA<sub>i</sub><sup>Met</sup> locates the start codon (diagram I). The eIF4E would now be less likely to remain associated with the cap, and would become available to bind other mRNAs and facilitate the initiation of their translation (diagram K). The released eIF4F is thus now free to bind to other mRNAs. Some of these RNAs may be activated for translation by other mechanisms, thus enabling them to be efficiently translated. The mechanisms by which such mRNAs would be activated may include changes in the binding to modulatory proteins that either repress or facilitate their translation. There are many precedents for roles for proteins binding to the 5'- or 3'-UTRs of specific mRNAs in modulating their translation. One could postulate here that such mRNA-binding proteins might themselves also be regulated by phosphorylation. The p38 MAP kinase pathway (which leads to eIF4E phosphorylation) is known to regulate mRNA binding proteins involved in modulating the stability or translation of, e.g. cytokine mRNAs [72]. This kind of mechanism would be important in situations where some reprogramming of translation is required – i.e. a qualitative shift to allow the translation of previously inactive or poorly active mRNAs. Such reprogramming may be required for responses to proliferative stimuli or to cytokines, the types of stimuli that activate the Erk and/or p38 MAP kinase pathways. On the other hand, insulin, as

an anabolic stimulus, may largely induce increased translation across the board of mRNAs that are already actively being translated.

How could increased phosphorylation of eIF4E actually inhibit cap-dependent translation, as observed by Knauf *et al.* [57]? The answer may lie in their experimental protocol, which led to a forced increase in the phosphorylation of a high proportion of the cellular eIF4E, decreasing its affinity for capped mRNA. Under such conditions, the reduced affinity of phosphorylated eIF4E for capped mRNA will exert a negative influence on translation initiation without any positive input from increased availability of eIF4F complexes (which is not enhanced by eIF4E phosphorylation [57,58]). This could account for the observed impairment of cap-dependent translation initiation, and for the absence of an effect on cap-independent translation.

These hypotheses require experimental evaluation. For both models, work using a reconstituted translation system, in the presence and absence of active Mnks, may help us to understand when in the initiation process eIF4E is phosphorylated and how it affects scanning and recruitment of further 40S subunits. For model 2, this could in part be achieved by microarray analysis to identify mRNAs that are translated or remain untranslated under different conditions in a given cell type. Microarray analyses have already been valuable in exploring translational control in several different systems [73,74]. The availability of vertebrate cells or animals with knock-in mutations that eliminate the site of phosphorylation in eIF4E (S209A) or with knock-outs of the Mnk1 or Mnk2 genes will also be a very valuable tool in studying the functional effects of eIF4E phosphorylation. The Mnks may of course have other roles in cellular physiology, and knock-outs of these enzymes (single or double) will again be crucial in identifying their physiological functions.

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