Biochemical Pharmacology, Vol. 60, pp. 1237–1243, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00429-9

Phosphorylation of the Cap-Binding Protein eIF4E by the MAPK-activated protein kinase Mnk1

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ABSTRACT. The purpose of this review is to summarize recent experimental data describing the regulation of the phosphorylation of eIF4E, the cap-binding protein, by the MAPK-activated protein kinase Mnk1. Mnk1 does not interact directly with eIF4E, but uses a docking site in eIF4G, a partner of eIF4E. Consequently, control of eIF4E phosphorylation may not strictly depend on changes in Mnk1 activity. The possibility that integrity of the eIF4E/eIF4G/Mnk1 complex also impinges upon eIF4E phosphorylation is discussed. BIOCHEM PHARMACOL **60**;8:1237–1243, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. translation initiation; cap binding protein; Mnk1; phosphorylation; cell cycle

The initiation step of translation is rate-limiting and represents a major target for translational control [1]. Through a complex modulation of protein–protein and protein–RNA interactions, translation initiation is under the control of diverse signal transduction pathways. Specifically, the rate of translation initiation is correlated with cell growth and is influenced by mitogens which activate the MAPK† cascade. The formation of the cap-binding complex eIF4F is a key regulatory step sensitive to pharmacological inhibitors of the MAPK cascade. eIF4F is a multiprotein factor responsible for the recruitment of the 40S ribosomal subunit to the mRNA 5′ end. Until recently, the protein kinases that directly impinged upon phosphorylation of eIF4F components were not clearly identified. However, the newly cloned MAPK-activated protein kinase Mnk1 has been shown to be physically associated with eIF4F and to directly phosphorylate eIF4E, a component of the eIF4F complex. These new features and their impact on translational control are presented in the following review.

THE eIF4F COMPLEX

Translation initiation of all nuclear-encoded mRNAs is facilitated by the 5′ cap structure, m^7GpppN (where N is any nucleotide), which is the target of the eIF4F complex. eIF4F consists of three subunits (Fig. 1A): (1) eIF4E, the cap-binding subunit; (2) eIF4A, an RNA helicase; and (3) eIF4G, which serves as an adaptator protein for the assembly of eIF4E and eIF4A. eIF4G also binds to the ribosome-associated initiation factor eIF3, thus providing a link between mRNA and ribosome. Recently, a new functional homologue of mammalian eIF4G which shares 46% identity with eIF4GI has been cloned and termed eIF4GII [2]. It is thought that, through its interaction with eIF4E, eIF4G functions by bringing the eIF4A helicase activity to the mRNA 5′ end, thus facilitating ribosome binding by unwinding the mRNA 5′ secondary structure. However, in addition to eIF4E, eIF4A, and eIF3, eIF4G binds several other proteins involved in translation initiation. The N-terminal segment interacts with PABP, while the C-terminal fragment contains an additional binding site for eIF4A and one binding site for the eIF4E kinase, Mnk1 (see below).

eIF4E is the least abundant of all initiation factors [3, 4]. Under most conditions, it is considered to be the rate-limiting factor in the binding of ribosomes to the mRNA [5] and is a major target for regulation. eIF4E is phosphorylated on Ser209 following treatment of cells with growth factors, hormones, and mitogens [6–8]. The best candidate for eIF4E phosphorylation is the recently characterized MAPK-activated protein kinase Mnk1 (see below).

eIF4E function is also regulated by its reversible association with the 4E-BPs (or PHAS-I; [9–11]). 4E-BPs become hyperphosphorylated in response to a large number of extracellular stimuli [12] and consequently dissociate from eIF4E. In contrast, hypophosphorylated 4E-BPs associate strongly with eIF4E and inhibit cap-dependent translation (Fig. 1B; [9, 13]). 4E-BPs have no effect on cap binding. Instead, they block eIF4F assembly because they compete with eIF4G for a common binding site on eIF4E [14, 15].

Another protein suspected to affect eIF4F function is p97/NAT-1 (novel APOBEC-1 Target1)/DAP-5 (hereafter called p97), a member of the eIF4G family [16–19]. p97 is homologous only to the C-terminal two-thirds of eIF4G

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† *Abbreviations:* MAPK, mitogen-activated protein kinases; eIF, eukaryotic translation initiation factor; PABP, polyadenylate-binding protein; 4E-BP, eIF4E-binding protein; Erk, extracellular signal-regulated protein kinase; and TPA, tumor-promoting agent.

Received 3 May 2000; accepted 9 June 2000.

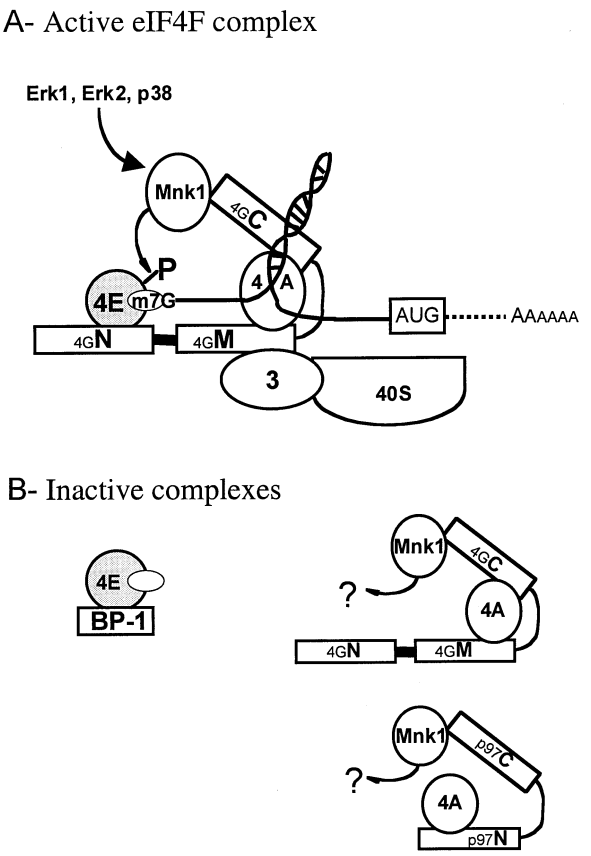

FIG. 1. Regulation of cap-dependent translation (adapted from [20]). (A) Cap function is mediated by the eIF4F complex, which is composed of three subunits. eIF4E is the cap-binding protein, eIF4A an RNA helicase, and eIF4G serves as a scaffold protein. eIF4G can be dissected into three segments: the N-terminus fragment (4GN) binds eIF4E; the middle portion (4GM) associates with eIF4A and eIF3; and the C-terminus segment (4GC) recruits another molecule of eIF4A and the kinase Mnk1. Through eIF3, eIF4G also binds to the 40S subunit of ribosomes. The model shows that phosphorylation of eIF4E occurs in the eIF4F complex, suggesting that eIF4F assembles prior to eIF4E phosphorylation. The association of eIF4E with eIF4G brings Mnk1 in the vicinity of eIF4E. The resulting phosphorylation of eIF4E might enhance its affinity for eIF4G and stabilize its interaction with the mRNA-5′ end. (B) When eIF4E is sequestered by the underphosphorylated forms of 4E-BPs, the eIF4G/Mnk1 complex is released and cap-dependent translation is inhibited. p97 is thought to inhibit cap-dependent translation by sequestering both eIF4A (associated to the N-terminus of p97) and Mnk1 (bound to the C-terminus of p97).

and contains the binding sites for eIF4A, eIF3 [16], and Mnk1 [20], but does not interact with eIF4E or PABP (Fig. 1B). The biological role of p97 is unclear. It is thought to act as a translational modulator by forming complexes that include eIF4A, eIF3, and Mnk1, but exclude eIF4E and PABP. p97 expression is enhanced in apoptotic cells [17, 21]. Furthermore, p97 mRNA is extensively edited in tissues containing tumors caused by the transgene expression of the apolipoprotein B mRNA-editing enzyme [19]. It is then likely that, under certain circumstances, p97 possesses other functions due to changes in its amino acid sequence after mRNA editing.

RECRUITMENT OF Mnk1 AND INITIATION FACTORS BY THE ADAPTATOR PROTEIN eIF4G

The Central Core Domain

Two recent papers have identified the central region of eIF4G as an active "ribosome recruitment core". Using an elegant *in vivo* system, the first report shows that the central part of eIF4G, fused to the iron-responsive element-binding protein 1 (IRE-BP1), suffices to recruit ribosomes to the 5′ end of mRNAs possessing iron-responsive elements (IRE) upstream of the open reading frame [22]. Refined deletion analysis demonstrated that, once attached to the mRNA 5′ end (via IRE-BP1/IRE interaction), the fused eIF4G domain functions only when it contains the binding sites for eIF4A and eIF3. It was therefore suggested that the central region of eIF4G behaves as a "ribosome recruitment core", which only requires a means to bind upstream of an open reading frame. The second report demonstrates that, in the cap-dependent translation initiation process, this means is provided by the very well-conserved eIF4E binding site located next to the core domain, in the N-terminal portion of eIF4G [23]. These studies support the view that the eIF4F complex, defined as the complex composed of the eIF4G core domain bound to eIF4E and eIF4A, represents an essential component of the recruitment of ribosomes to the mRNA 5′ end. Therefore, both the N- and C-terminal fragments of eIF4G, which interact with other proteins, are thought to play a regulatory role.

The N- and C-terminal Regulatory Fragments

The PABP-binding site in the N-terminal portion of the two functional homologues of eIF4G was first identified in yeast [24]. Later, the interaction between mammalian PABP and eIF4G was discovered in rotavirus-infected cells, in which the virus-encoded non-structural protein 3 (NSP3) competed with PABP for eIF4G binding [25]. Rotavirus mRNAs do not possess a poly(A) tail, but contain a specific NSP3-binding site at their 3′ end. The competition between NSP3 and endogenous PABP for eIF4G binding is thought to mediate the shut-off of host translation and to facilitate preferential translation of viral mRNAs [25]. Subsequently, and with the help of the recently cloned eIF4GII, a new N-terminal fragment of eIF4GI has been identified, and the interaction between eIF4G and PABP was shown to occur in mammalian cells through this new N-terminus [26]. Due to the simultaneous binding of eIF4G to eIF4E and PABP, one could predict a physical link between the 5′ cap structure and the 3′ poly(A) tail. Consistent with this, a reconstituted eIF4E/eIF4G/PABP complex has been shown to mediate the circularization of capped, polyadenylated RNAs *in vitro* [27]. The interaction between PABP and eIF4G may

account for the synergistic effect of the 5′ cap and the 3′ poly(A). However, a direct illustration of mRNA circularization has yet not been shown *in vivo*. Also, how poly(A) tail stimulates translation initiation is not known. It is possible that circularization facilitates the recycling of ribosomes from the 3′ to the 5′ end. Another attractive explanation is that circularization would allow preferential translation of intact and correctly processed mRNAs, since only mRNAs with both a 5′ cap and a 3′ poly(A) tail would circularize.

The C-terminal fragment of eIF4G contains an additional binding site for eIF4A [28]. As the core domain defined above functions autonomously to recruit the ribosome and since inclusion of the additional eIF4A-binding site only slightly enhances this process both *in vivo* and *in vitro*, the second eIF4A-binding domain in the C-terminal third of eIF4G is thought to act as a modulator cassette. Independently, the extreme C-terminus of eIF4G forms a docking site for the eIF4E kinase Mnk1 [20].

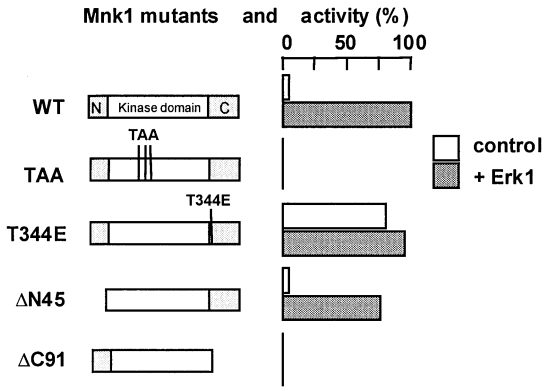

FIG. 2. Characterization of human Mnk1 mutants (adapted from [29]). As compared to wild-type Mnk1 (WT), mutation of threonine residues in the T-loop domain (TAA mutant) renders Mnk1 inactive, while mutation of the Thr344 into glutamic acid in the C-terminus (T344E mutant) activates Mnk1, independently of Erk1. Deletion of the N-terminus (ΔN45) has little effect, while deletion of the C-terminus (ΔC91), including Thr344, prevents Mnk1 activation by Erk1.

THE Mnk FAMILY OF PROTEIN KINASES

The Mnk family of protein kinases was discovered simultaneously in two separate laboratories. Human Mnk1 was cloned with the help of a new expression screening method developed to identify protein kinase substrates [29]. Screening an HeLa cDNA library by *in situ*, solid-phase phosphorylation using activated Erk1 MAP kinase yielded the cDNA encoding Mnk1. Further analysis showed that Mnk1 belongs to a novel class of protein kinases that are activated through both the Erk and p38 MAP kinases, but not by the JNK/SAPK (c-jun N-terminal kinase/stress-activated protein kinase) pathways. Mouse Mnk1 and Mnk2 were cloned using a two-hybrid screen designed to isolate novel Erk2-binding partners [30]. Clones which possessed homology with the C-terminus of the known Erk substrate Rsk (ribosomal protein 56 kinase) were selected and used to screen a mouse cDNA library. This study yielded two related cDNA clones, Mnk1 and Mnk2. As for human Mnk1, *in vitro* and *in vivo* experiments showed that mouse Mnk1 is a target for both Erk and p38 MAP kinases. Additional data demonstrated that Mnk1 can efficiently phosphorylate eIF4E on its physiological site Ser209 *in vitro* [31]. Also, dominant negative or activated Mnk1 mutants (see below) have revealed that Mnk1 directly modulates eIF4E phosphorylation in the cell. Mnk2, which is not activated by p38, also phosphorylates eIF4E, but to a lesser extent.

All the sites phosphorylated in human Mnk1 by Erk and p38 MAP kinases have not been identified. However, a number of sites match the consensus sequence ψX[S/T]P recognized and phosphorylated by the MAPKs. Preliminary results show that among these sites, Thr344 is one of the major sites phosphorylated by Erk1 *in vitro* and upon TPA treatment *in vivo* [29]. Supporting a critical role of phosphorylated Thr344 in Mnk1 activation, replacement of Thr344 with a glutamic acid suffices for kinase activation (Fig. 2, T344E mutant). Conversely, deletion of the C-terminal portion of Mnk1 containing Thr344 prevents its activation by MAPKs (Fig. 2, ΔC91 mutant). This residue is conserved in the mouse Mnk1 sequence at position 332 and its replacement with acidic residues also activates kinase activity [30]. Furthermore, substitution of Thr197 and Thr202 in the T-loop by alanines (T2A2 mutant) renders mouse Mnk1 defective [31]. Similarly, when corresponding sites in the T-loop of human Mnk1 are mutated into alanines (Fig. 2, TAA mutant), Mnk1 is no longer activated by MAPKs (R. Fukunaga, personal communication).

Mnk1 BINDING PARTNERS

To examine the roles of the N- and C-terminal regions beyond the catalytic domain, diverse Mnk1 mutants were constructed and tested for eIF4G (this paper) and for MAPK [29] binding. Deletion of the N-terminal 45 amino acids completely prevented Mnk1 binding to eIF4GI (Fig. 3, ΔN45 mutant), but did not significantly affect phosphorylation or activation by Erk1 (Fig. 2). Refined deletions delineated a minimal sequence, i.e. amino acids 25–44, as a motif necessary (Fig. 3 and Fig. 4A) but not sufficient (data not shown) for the interaction with eIF4GI. This motif contains a stretch of 8 basic amino acids (RRRKKKRR) also found in the mouse Mnk1 and Mnk2 sequences (Fig. 4B). A basic stretch at the N-terminus of a protein is often considered as a potential nuclear import signal. Intriguingly, another putative binding partner of Mnk1 is importin α [31]. importin α binding also requires the N-terminal basic region, suggesting that eIF4G and importin α may interact with Mnk1 in a mutually exclusive manner. To date, immunofluorescence studies have not revealed Mnk1 in the nucleus ([31]; J. Dostie, personal communication). It remains possible, however, that Mnk1

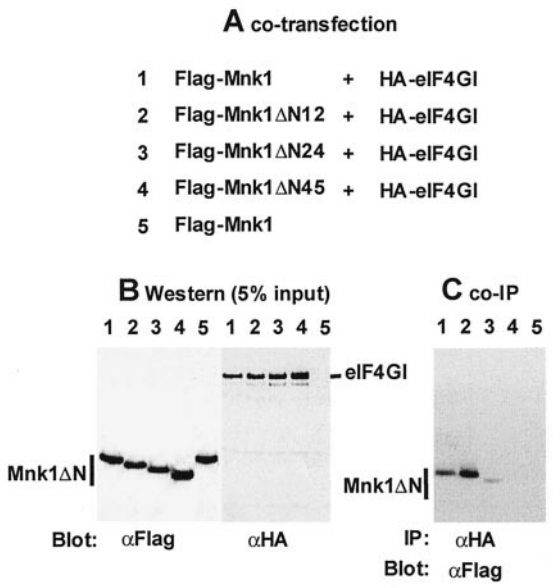

FIG. 3. The N-terminus of Mnk1 is necessary for binding to eIF4G. (A) 293T cells were co-transfected with vectors encoding for Flag-tagged Mnk1 wild-type (lane 1) or N-terminus deletion mutants (lanes 2–4) and HA-tagged wild-type eIF4GI proteins, as indicated. Cells were also transfected with Flag-Mnk1 vector alone (lane 5). (B) Thirty-six hours after transfection, expression of tagged proteins was verified by anti-Flag (left) and anti-HA (right) Western blotting. (C) Interaction between Flag-Mnk1 and Ha-eIF4GI was assayed by co-immunoprecipitation. Immunoprecipitation (IP) was performed using anti-HA antibodies and co-immunoprecipitated proteins were detected by Western blotting (Blot) using anti-Flag antibodies. Lane 5 shows that in the absence of HA-eIF4GI, anti-HA antibodies do not unspecifically immunoprecipitate full-length Flag-Mnk1.

enters the nucleus only under certain circumstances and/or perhaps shuttles rapidly out of the nucleus once it enters.

It is noteworthy that immunoblot analysis of cell lysates with an Mnk1 antiserum revealed two Mnk1 proteins of 51 and 52 kDa [20, 29]. The 51-kDa band is likely to be a molecular species whose translation is initiated at an internal AUG triplet (Fig. 4A, hMnk1a initiated at the AUG13). In the 52 kDa-species (hMnk1b), inclusion of the N-terminal 12 amino acids due to initiation at the first in-frame AUG reproducibly decreases Mnk1 binding to eIF4GI *in vitro* (~5-fold; Fig 3C, compare lanes 1 and 2). This is consistent with *in vivo* data showing that the major species co-immunoprecipitated with eIF4G antibodies is the 51-kDa band [20]. This feature is of unclear biological significance, but translation at alternative AUGs may represent a means to modulate Mnk1 binding to eIF4G and/or importin α.

Mnk1, THE BEST CANDIDATE FOR eIF4E PHOSPHORYLATION

A number of kinases have been considered as potential eIF4E kinases. One obvious candidate was protein kinase C (PKC), which phosphorylates Ser209 *in vitro* [32]. Also, following treatment of cells with phorbol esters, eIF4E is phosphorylated on Ser209 [33, 34]. Instead of a direct phosphorylation, recent data argue in favor of a role of PKC upstream of the Erk and p38 MAPKs, which in turn phosphorylate Mnk1 [31, 35, 36]. These studies demonstrated the mitigation of TPA-induced eIF4E phosphorylation by pharmacological inhibitors of the Erk and p38 MAPKs. However, the kinase domain of Mnk1 shares significant homology with the catalytic region of other MAPK-activated kinases (MAPKAP) such as MAPKAP-2 (2pK), MAPKAP-3 (3pK), and $p90^{RSK}$, whose kinase domains exhibit 34, 33, and 36% amino acid identity to that of Mnk1, respectively [29]. Intriguingly, it was suggested that 3pK immunoprecipitated from human cells could phosphorylate eIF4E *in vitro*. Nevertheless, since robust eIF4E phosphorylation *in vivo* requires its interaction with eIF4G [20] and since eIF4G is associated with the N-terminus of Mnk1 (Fig. 3), a fragment which shares no homology with the N-terminal region of 3pK, it is unlikely that 3pK efficiently phosphorylates eIF4E *in vivo*. Thus, eIF4G appears to serve as a docking site for the Mnk1 kinase to bring it close to its substrate eIF4E. These data are consistent with earlier observations that eIF4E as a part of the eIF4F complex (i.e. bound to eIF4G) is phosphorylated to a greater extent than unbound eIF4E [37]. In support of this model, expression of a kinase-dead Mnk1 mutant protein, such as the mouse T2A2 mutant, inhibits the phosphorylation of eIF4E in response to TPA [31]. To account for this dominant negative effect, it has been

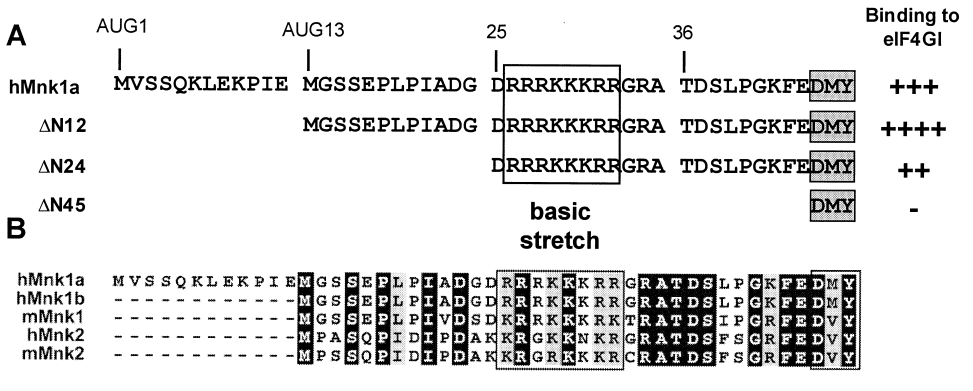

FIG. 4. The eIF4G-binding site is conserved in both human and mouse Mnk sequences. (A) Amino acids necessary for Mnk1 binding to eIF4GI contain a stretch of 8 basic residues (open box). Deletion until the kinase domain (filled box) completely abolished the interaction between Mnk1 and eIF4GI. (B) Alignment between human and mouse Mnk1 and Mnk2 sequences. The putative human Mnk2 sequence was obtained from the EMBL data base (accession number AC007136).

suggested that the T2A2 mutant kinase may displace the physiological eIF4E kinase and therefore inhibit eIF4E phosphorylation. Conversely, expression of activated Mnk1 increases eIF4E phosphorylation in the cell [31]. Taken together, these data argue in favor of Mnk1 as the physiological eIF4E kinase.

Mnk1 can phosphorylate eIF4GI *in vitro* [20]. However, deletion of the Mnk1-binding site in eIF4GI does not negatively impact upon the phosphorylation of the serum- and MAPK-activated eIF4GI phosphorylation sites *in vivo* [38]. Thus, it appears unlikely that Mnk1 mediates eIF4GI phosphorylation in response to MAPK activation. Independently, Mnk1 binds p97 [20], which is also a phosphoprotein. An important question remaining to be answered, therefore, is whether Mnk1 phosphorylates p97 and, if so, what the biological role of phosphorylated p97 may be. That p97 does not share a binding site for eIF4E may suggest the existence of other yet-unknown substrates for Mnk1 when the kinase is bound to p97 (Fig. 1B). In addition, since the carboxy-terminal fragment of p97 interacts with Mnk1 as efficiently as the corresponding region in the eIF4G protein [20], it is conceivable that, besides p97 phosphorylation, the interaction between p97 and Mnk1 may decrease eIF4E phosphorylation via Mnk1 sequestration. Similar questions can be raised for cleaved eIF4G. What are the substrates for Mnk1 when the kinase is bound to the carboxy-terminus of eIF4G, whose cleavage after viral infection or during apoptosis separates Mnk1 from its substrate eIF4E?

SIGNIFICANCE OF eIF4E PHOSPHORYLATION

The biological significance of eIF4E phosphorylation is not completely understood. Through an unknown mechanism, phosphorylated eIF4E was reported to form a more stable complex with eIF4G [39]. In addition, phosphorylated eIF4E possesses higher-binding affinity for the cap [40]. This latter observation is supported by recent predictions made from the co-crystal structure of eIF4E bound to m^7GDP [41]. According to the crystal structure, Lys159, which is juxtaposed to the flexible loop containing Ser209, could form a salt bridge with phosphorylated Ser209, thus covering the mRNA and stabilizing the interaction between the mRNA 5′ end and eIF4E. However, this computer prediction awaits experimental demonstration. The identification of Mnk1 as the most probable eIF4E kinase and future work conducted to analyze its role in translational control will likely help clarify the function of eIF4E phosphorylation.

The correlation between phosphorylation on Ser209 and the cellular translation rate is not observed in every situation. For instance, following exposure to anisomycin or arsenite, the translation rate diminishes, while eIF4E phosphorylation is augmented [36]. The increase in eIF4E phosphorylation can be attributed to activation of the stress-activated p38 protein kinase, as both events are abolished by the specific p38 inhibitor, SB203580. Independently, arsenite has been shown to inhibit translation initiation through eIF2α phosphorylation [42]. Thus, the discrepancy between a reduced translation rate and increased eIF4E phosphorylation upon arnsenite (and perhaps anisomycin) treatment might be explained by the induction of eIF2α kinases. In contrast, other types of cellular stress, including heat shock [3] or infection with encephalomyocarditis virus (EMCV, [43]), are accompanied by a decrease in eIF4E phosphorylation. In the case of heat shock, this is surprising since p38 MAPKs are activated under heat shock. One attractive explanation to account for the diminution of eIF4E phosphorylation in spite of p38 activation results from the observation that heat shock increases the binding of eIF4E to 4E-BP1. Similarly, 4E-BP1 is underphosphorylated upon EMCV infection [44], suggesting that eIF4E binding to eIF4G is impaired. Since eIF4G serves as a docking site for Mnk1 to phosphorylate eIF4E, phosphorylation of eIF4E cannot occur, as it is separated from the eIF4G/Mnk1 complex (Fig. 1B).

CELL CYCLE-DEPENDENT PHOSPHORYLATION OF eIF4E

eIF4E activity plays a role in cell cycle progression. For instance, mutants of the *CDC33* gene, which is the *Saccharomyces cerevisiae* homologue of the *eIF4E* gene, arrests yeast cells at G1 [45]. Consistent with this, expression of an antisense eIF4E RNA dramatically slackened cell cycle transit [46], whereas ectopic expression of eIF4E in HeLa cells was reported to accelerate cell cycle progression [47]. Furthermore, since protein synthesis is required for entry into and progression through the cell cycle, and since microinjection of eIF4E into quiescent NIH 3T3 fibroblasts induces DNA synthesis [48], eIF4E is thought to play a role, particularly in the G1 and S phases. In addition, eIF4E phosphorylation is strongly diminished at mitosis [49, 50], a cell cycle phase during which cap-dependent, but not internal ribosome entry site (IRES)-mediated translation initiation is severely impaired [51]. Paradoxically, Erk1 and Erk2 MAP kinases [52] as well as Mnk1* are activated at mitosis. Thus, how eIF4E dephosphorylation is stimulated at mitosis is an important question that remains to be addressed. It is possible that due to eIF4F complex disruption, eIF4E is no longer a substrate for Mnk1. If so, what are the mitotic substrates for activated Mnk1? Another even more intriguing question is whether preventing eIF4E dephosphorylation would also prevent the switch from cap-dependent to IRES-mediated translation initiation that occurs at the onset of mitosis [51]. The answers to these questions will likely lead to a greater comprehension of how the translation rate is controlled by eIF4E and Mnk1 activities during the cell cycle.

* Pyronnet S and Sonenberg N, unpublished data.

CONCLUSION

Thus, besides the regulation of Mnk1 activity, the requirement of eIF4G to recruit Mnk1 for eIF4E phosphorylation provides another way to control eIF4E phosphorylation. Since the physiological entity that binds to the mRNA 5′ cap structure is the eIF4F complex rather than eIF4E alone [53, 54], the simultaneous binding of eIF4E and its kinase Mnk1 to eIF4G implies that eIF4E phosphorylation might occur only when eIF4E is attached to the cap structure as part of the eIF4F complex. Conversely, when eIF4E is bound to the translational repressor 4E-BP1, it is no longer a substrate for Mnk1. This scenario exemplifies an elegant system evolved by the cell to avoid inappropriate phosphorylation of a protein when it is not needed for a physiological purpose.

I thank J. Dostie and R. Fukunuga for sharing unpublished data, and N. Sonenberg and A. Kahwajian for critical reading and English proof-reading of the manuscript.

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