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Phosphorylation of eIF-4E positively regulates formation of the eIF-4F translation initiation complex following DNA damage

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Abstract

The eukaryotic translation initiation factor 4E (eIF-4E) is essential for cap-dependent protein translation. However, the role of eIF-4E phosphorylation in protein translation is still unclear. In this study, the function of eIF-4E phosphorylation in the formation of the translational initiation complex eIF-4F following DNA damage was investigated. Our results show that etoposide treatment caused a rapid increase in eIF-4E phosphorylation. The addition of CGP57380, a specific inhibitor of the eIF-4E kinase Mnk, not only inhibited eIF-4E phosphorylation but also resulted in reduced interaction between eIF-4E and eIF-4G. Furthermore, neither the p38 MAPK inhibitor nor the ERK inhibitor caused significant inhibition in eIF-4E phosphorylation induced by etoposide. However, a JNK-specific inhibitor, SP600125, strongly suppressed etoposide-induced eIF-4E phosphorylation. Our results provide the first evidence indicating that phosphorylation of eIF-4E by Mnk, possibly mediated by JNK or JNK-like kinases, is critical for formation of the translational initiation complex eIF-4F following DNA damage.

Keywords: Translation initiation; eIF-4E phosphorylation; DNA damage; eIF-4F complex formation

Eukaryotic mRNA translation initiation is a highly regulated process. In eukaryotic cells, almost all mRNAs possess a cap structure (m^7GpppX) at their 5′ ends. Translation initiation requires recognition and binding to the cap structure by eIF-4E [1]. After eIF-4E binds to the cap structure, a translation initiation complex, termed eIF-4F, forms in the vicinity of the cap structure.

Formation of eIF-4F is a major control point for regulating the mRNA translation initiation. eIF-4F consists of three subunits: eIF-4E, the cap-binding protein; eIF-4G, a large scaffolding protein that binds both eIF-4E and eIF-4A; and eIF-4A, an ATP-dependent RNA helicase that facilitates melting of the secondary structure of mRNA. The eIF-4F complex then recruits the 40S ribosomal subunit to the mRNA in order to initiate the eukaryotic protein translation [1].

Since recognition and binding of the cap structure by eIF-4E is considered a common rate-limiting step in protein translation initiation, eIF-4E plays a critical role in cap-dependent translation initiation [1]. It is known that the activity of eIF-4E can be regulated at two different levels [1,2]. One is via its association with a family of translational repressors, referred to as eIF-4E binding proteins (4E-BPs); the other is through the phosphorylation of eIF-4E by its upstream kinases.

4E-BPs modulate the availability of eIF-4E for participation in the eIF-4F complex formation. In their hypophosphorylated state, 4E-BPs compete with eIF-4G for a common binding site on eIF-4E, thus blocking eIF-4F assembly and inhibiting cap-dependent protein translation [1]. Hyperphosphorylation of the 4E-BPs in response to insulin and other growth factors causes the release of

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eIF-4E, thus allowing the association of eIF-4E with eIF-4G and the assembly of the eIF-4F complex [3].

In addition, eIF-4E itself can undergo phosphorylation. The major phosphorylation site of eIF-4E in mammalian cells is Ser-209, which is phosphorylated by the protein kinases, Mnk1 and Mnk2, *in vivo* [4,5]. Since phosphorylation of eIF-4E is up-regulated in response to growth factors and cytokines [6,7], it has been postulated that eIF-4E phosphorylation plays a positive role in cell growth by stimulating protein translation efficiency [5]. Previous results have also shown that Ser-209 of eIF-4E is phosphorylated in response to different types of cyto- or genotoxic stress [6–9]. It was reported that increased phosphorylation of eIF-4E may play a positive role in selectively stimulating mRNA translation of low efficiency proteins under stress conditions where general protein synthesis is inhibited [8,9].

DNA damage is known to cause decreased general protein synthesis [10]. However, synthesis of many proteins, such as those involved in cell cycle arrest and apoptosis, needs to be up-regulated after DNA damage. We therefore investigated if eIF-4E is phosphorylated in response to DNA damage and if the phosphorylation event plays a role in protein translation initiation in response to DNA damage.

Experimental procedures

Chemicals. 7-Methyl GTP–Sepharose beads were purchased from Amersham Bioscience. Anti-(P)-eIF-4E (Ser-209) was from Cell Signaling. Anti-eIF-4E antibody was from BD Transduction Laboratories. Antibodies against eIF-4G and 4E-BP1 were from Santa Cruz. PD98059, SB203580, rapamycin, SP60012, and etoposide were from Calbiochem. CGP57380 was a generous gift from Dr. Hermann Gram.

Cell cultures and preparation of cellular extracts. MCF-7 cells, a human breast cancer cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Following treatment with different reagents, cells were lysed with TGN buffer [3]. Protein concentrations of the cell lysates were determined by the Lowry Method.

Isolation of eIF-4E by M^7-GTP–Sepharose chromatography. The procedure was modified from the method used by Yang et al. [11]. Briefly, cell lysates containing equal amounts of protein were incubated with 35 μl of M^7-GTP–Sepharose beads overnight at 4 °C. Subsequently, the beads were washed with TGN buffer and PBS. After washing, SDS–PAGE sample loading buffer was added to the beads.

SDS–PAGE and immunoblotting. Samples isolated by M^7-GTP beads or cell lysates containing equal amounts of protein were subjected to SDS–PAGE. After SDS–PAGE, proteins were transferred to a nitrocellulose membrane. The membrane was then used for immunoblotting.

Results

eIF-4E is phosphorylated in response to etoposide in MCF-7 cells

We first treated MCF-7 cells with etoposide, a DNA damaging agent that mimics ionizing radiation (IR) and causes DNA double strand breaks [12]. Samples treated with etoposide had the same abundance of total eIF-4E as those from the control group, whereas the phosphorylation level of eIF-4E increased in a time-dependent manner following etoposide treatment (Fig. 1A).

To investigate whether the fraction of eIF-4E bound to the cap-structure is also phosphorylated in response to etoposide, the phosphorylation of eIF-4E isolated by Sepharose beads conjugated with M^7-GTP, which mimics the cap-structure of mRNA, was examined. The phosphorylation level of eIF-4E increased dramatically following etoposide treatment (Fig. 1B). The levels of total eIF-4E in the cap-bound fraction still remained constant and were used as a loading control to quantify the abundance of other translational factors associated with eIF-4E.

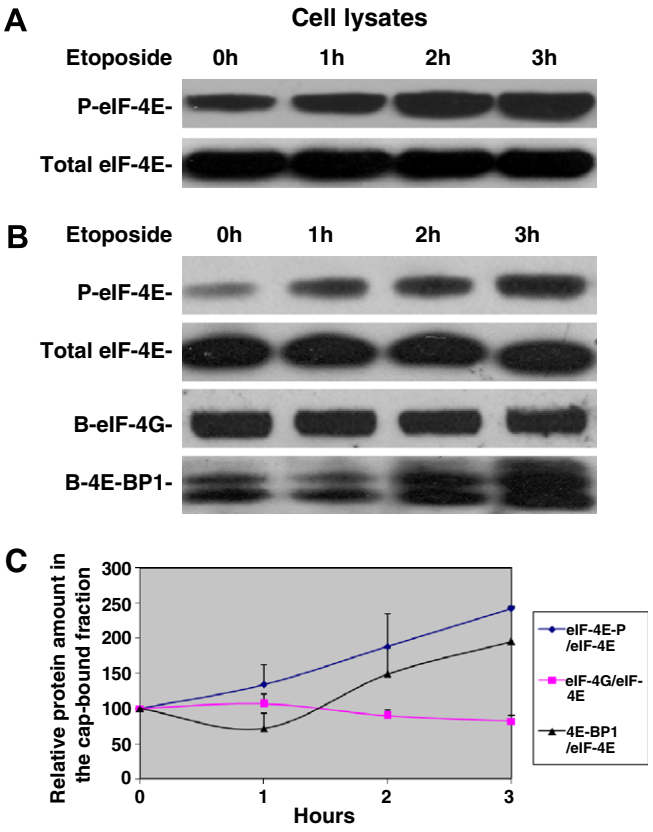

Fig. 1. (A) eIF-4E phosphorylation in cell lysates increased following etoposide treatment. MCF-7 cells were treated with 10 μM etoposide at the indicated times. Cell lysates were then prepared and resolved by SDS–PAGE. The phosphorylation of eIF-4E (P-eIF-4E or eIF-4E-P) and total eIF-4E were analyzed by immunoblotting. The results presented are representative of three separate experiments. (B) Phosphorylation of the cap-bound eIF-4E is also elevated in response to etoposide. Cell lysates prepared above were incubated with M^7-GTP–Sepharose beads. The isolated samples were then resolved by SDS–PAGE. eIF-4E and its associated eIF-4G (B-eIF-4G) and 4E-BP1 (B-4E-BP1), as well as P-eIF-4E, were visualized by immunoblotting using their respective antibodies. (C) Quantification of the levels of eIF-4G, 4E-BP1, and eIF-4E-P in the cap-bound fraction. The abundance of eIF-4G and 4E-BP1 bound to eIF-4E as well as eIF-4E-P was quantified by densitometry using cap-bound eIF-4E as a loading control. The ratios of eIF-4E-P, eIF-4G, and 4E-BP1 versus total eIF-4E in the control sample (0 h) were designated as 100. The presented data are the means ± SEM of three separate experiments.

Etoposide-induced phosphorylation of eIF-4E does not lead to a substantial decrease in eIF-4E and eIF-4G binding

The assembly of eIF-4F translation initiation complex directly represents the efficiency of translation initiation. Since eIF-4G is the major component of eIF-4F that recruits other initiation factors to the translation initiation machinery, the abundance of eIF-4G in the cap-bound fraction is defined as an indicator of eIF-4F activity [1]. Therefore, we further investigated the effect of etoposide on the formation of the eIF-4F complex.

The level of 4E-BP1 bound to eIF-4E was substantially increased following etoposide treatment (Fig. 1B). This result is consistent with a previous finding that IR causes increased interaction between 4E-BP1 and eIF-4E [10]. Theoretically, the dramatically increased interaction between 4E-BP1 and eIF-4E should result in a substantially reduced amount of eIF-4G available for cap-dependent translation, because eIF-4G and 4E-BP1 compete for the same binding site on eIF-4E. However, to our surprise, the amount of eIF-4G associated with eIF-4E remained at a relatively similar level following etoposide treatment (Fig. 1B). After correcting with the amount of total eIF-4E, the slight decrease (~20% decrease versus control after 3h of etoposide treatment) in the association of eIF-4G with eIF-4E following etoposide treatment was disproportionate to the substantially increased interactions (~100% increase after 3h of etoposide treatment) between 4E-BP1 and eIF-4E (Fig. 1C). This result suggests that phosphorylation of eIF-4E might be the reason why etoposide treatment did not lead to a remarkable decrease in eIF-4E and eIF-4G binding despite the dramatically increased association between 4E-BP1 and eIF-4E.

CGP57380 prevents the etoposide-induced phosphorylation of eIF-4E and decreases the abundance of eIF-4G associated with eIF-4E

CGP57380 is a cell-permeable, specific inhibitor of the eIF-4E kinases, Mnk1 and Mnk2 [13]. It was reported that CGP57380 can efficiently suppress the phosphorylation of eIF-4E induced by many environmental stimuli, and it does not inhibit the activities of other kinases, such as p38 MAPK, JNK1, and ERK1/2 [13].

Next, we pre-treated MCF-7 cells with CGP57380 prior to etoposide treatment. The phosphorylation of eIF-4E induced by etoposide was remarkably suppressed by CGP57380, indicating that the Mnk kinases are the major upstream kinases that lead to eIF-4E phosphorylation in response to etoposide (Fig. 2A). The abundance of eIF-4G and 4E-BP1 associated with eIF-4E in the absence and presence of CGP57380 was further compared. Our results again show that etoposide treatment only resulted in a slight decrease in the association of eIF-4G with eIF-4E. However, treatment with both etoposide and CGP57380 resulted in a further reduction in the interaction between eIF-4G and eIF-4E (Fig. 2A and B). Although,

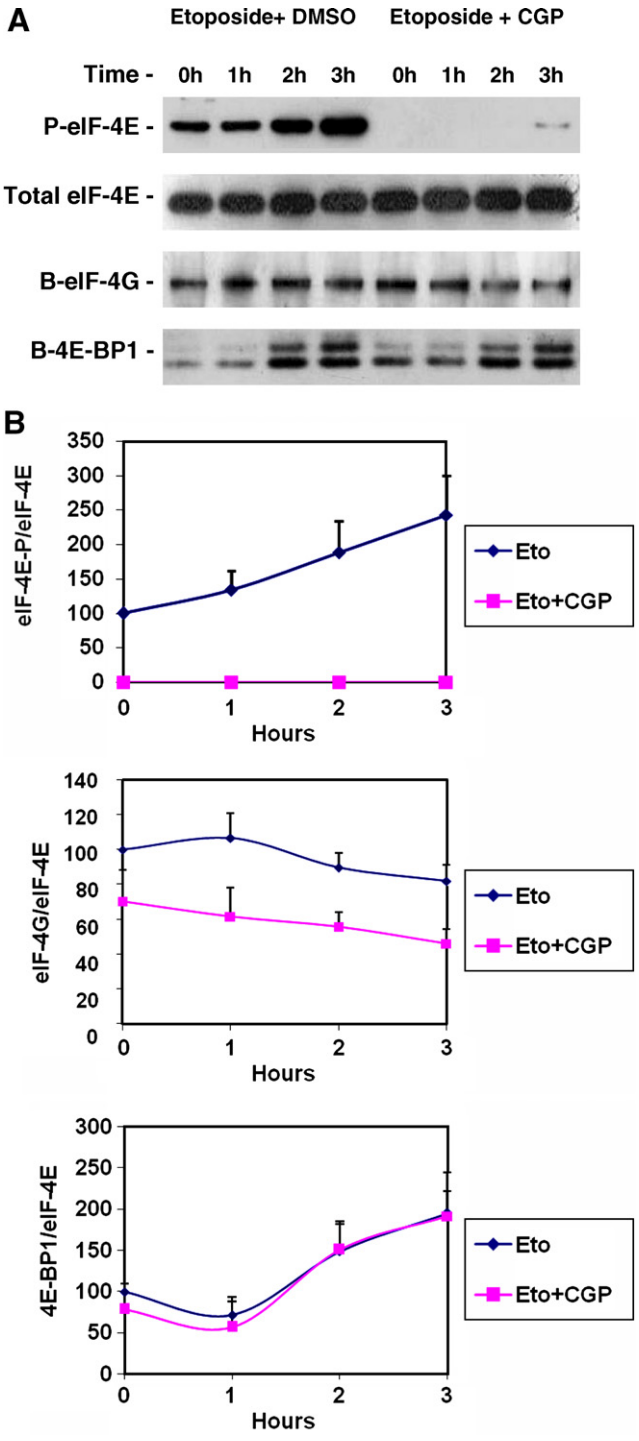

Fig. 2. The assembly of eIF-4F is affected by the phosphorylation of eIF-4E induced by etoposide. (A) MCF-7 cells were pre-treated with or without 10 μM CGP57380 for 60 min and then with 10 μM etoposide for the indicated times. After cell lysis, eIF-4E was isolated by M^7-GTP–Sepharose chromatography as described in Fig. 1B. P-eIF-4E, eIF-4E and its associated proteins were visualized. (B) The abundance of eIF-4E-P, eIF-4G and 4E-BP1 in the cap-bound fraction was quantified as described in Fig. 1C. The presented data are the means ± SEM of three separate experiments.

the inhibition of eIF-4E phosphorylation by CGP57380 did not affect the association of 4E-BP1 with eIF-4E, decreased phosphorylation of eIF-4E caused by

CGP57380 was concomitant with a reduction in the abundance of eIF-4G associated with eIF-4E (Fig. 2B). This result suggests that increased eIF-4E phosphorylation following DNA damage may indeed help retain eIF-4G associated with eIF-4E, thereby relieving the inhibitory effect of 4E-BP1 on the interaction between eIF-4E and eIF-4G.

Neither ERK nor p38 MAPK is the major upstream kinase mediating the activation of Mnks in response to etoposide

It is widely accepted that Mnks are activated by either ERK1/2 or p38 MAPK, depending on environmental stimuli. p38 MAPK is known as the kinase that phosphorylates Mnk1 when cells are exposed to different types of cytotoxic stress, while ERK phosphorylates Mnk1 in response to growth factors and cytokines [14]. We next used the ERK1/2 inhibitor, PD98059, and the p38 MAPK inhibitor, SB203580, to pre-treat MCF-7 cells prior to etoposide treatment. The phosphorylation levels of cap-bound eIF-4E were examined. Contrary to previous reports, when compared to the cells treated with etoposide alone, no substantial decrease in eIF-4E phosphorylation was observed when SB203580, PD98059, or both were used to treat the cells (Fig. 3A and B). These results indicate that neither p38 MAPK nor ERK is the major kinase responsible for eIF-4E phosphorylation following etoposide treatment.

JNK/SAPK is a candidate upstream kinase that activates Mnk in response to etoposide

To search for the upstream kinase that mediates the activation of Mnk1 in response to etoposide, MCF-7 cells were pre-treated with inhibitors of several other kinases, including PI-3K and c-jun N-terminal kinase/stress activated kinase (JNK/SAPK). The only inhibitor that could effectively suppress etoposide-induced eIF-4E phosphorylation was the JNK-specific inhibitor, SP600125 (Fig. 4A). The activation of JNK following etoposide treatment with or without SP600125 was next investigated. The phosphorylation of JNK was found to be largely elevated by etoposide, whereas SP600125 strongly inhibited the activation of JNK (Fig. 4B). These results suggest that in response to etoposide, JNK or a JNK-like kinase may be a candidate upstream kinase leading to eIF-4E phosphorylation in MCF-7 cells.

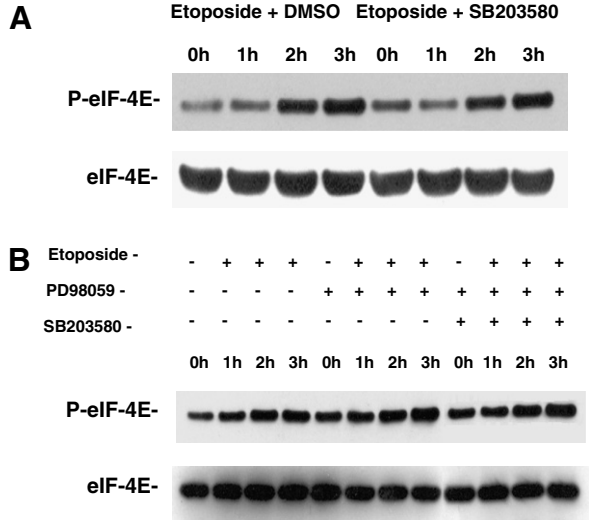

Fig. 3. Neither ERK nor p38 MAPK is responsible for eIF-4E phosphorylation induced by etoposide. MCF-7 cells were pre-treated with 25 μM SB203580 (A), 10 μM PD98059, or both (B) for 60 min and then incubated with 10 μM etoposide for the indicated times. eIF-4E was then isolated by M^7-GTP–Sepharose chromatography as described in Fig. 1B. P-eIF-4E, eIF-4E and its associated proteins were visualized by immunoblotting. The results presented in (A and B) are representative of three separate experiments.

The suppression of rapamycin-induced eIF-4E phosphorylation by SP600125 causes a reduction in eIF-4F assembly as well

Phosphorylation of 4E-BPs is controlled largely by the mammalian target of rapamycin (mTOR) kinase [15]. A specific inhibitor of the mTOR kinase, rapamycin, prevents phosphorylation of 4E-BPs and blocks the release of 4E-BPs from eIF-4E. Therefore, rapamycin mimics a variety

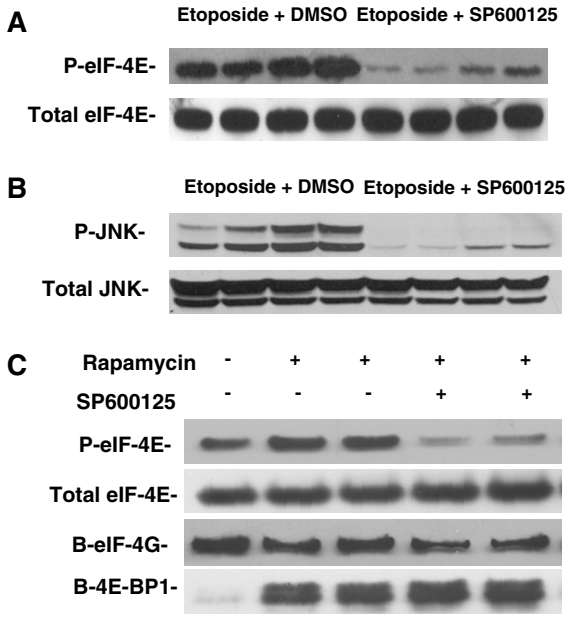

Fig. 4. (A) eIF-4E phosphorylation is inhibited by SP600125. Subconfluent MCF-7 cells were pre-treated with or without 10 μM SP600125 for 60 min and then with 10 μM etoposide for the times indicated. Protein samples in the cell lysates were resolved by SDS–PAGE, and total eIF-4E and P-eIF-4E were visualized by immunoblotting. (B) SP600125 inhibits the activation of JNK following etoposide treatment. MCF-7 cells were treated with SP600125 and etoposide as described above. Protein samples in the cell lysates were resolved by SDS–PAGE. Phosphorylation of JNK (P-JNK) and total JNK were visualized by immunoblotting. (C) Inhibition of rapamycin-induced eIF-4E phosphorylation also causes decreased association between eIF-4G with eIF-4E. MCF-7 cells were pre-treated with 20 nM rapamycin for 60 min and then with 10 μM SP600125 for the times indicated. After cell lysis, eIF-4E was pulled down with M^7-GTP–Sepharose beads as described in Fig. 1B. P-eIF-4E, eIF-4E and its associated proteins were visualized by immunoblotting. The results presented in (A–C) are representative of three separate experiments.

of cytotoxic stress and strongly inhibits cap-dependent translation via impairment of eIF-4F complex assembly. Interestingly, recent findings indicate that rapamycin treatment also leads to increased eIF-4E phosphorylation and that rapamycin-induced phosphorylation is also mediated by Mnk protein kinases [16].

We treated the MCF-7 cells with rapamycin and found that the level of 4E-BP1 associated with eIF-4E was dramatically enhanced (Fig. 4C). As expected, eIF-4E phosphorylation also increased. Subsequently, we treated MCF-7 cells with rapamycin in the absence or presence of SP600125. We found that SP600125 also suppressed eIF-4E phosphorylation induced by rapamycin (Fig. 4C). While rapamycin treatment resulted in a reduced interaction (~30% decrease versus control after 3 h of etoposide treatment) between eIF-4E and eIF-4G, reduced eIF-4E phosphorylation caused by SP600125 was associated with an additional decrease (~50% after 3 h of etoposide treatment) in the level of eIF-4G associated with eIF-4E (Fig. 4C). This result demonstrates that rapamycin-induced eIF-4E phosphorylation is also a positive factor in the formation of the eIF-4F complex, which may attempt to relieve the inhibitory effect caused by enhanced binding between 4E-BP1 and eIF-4E during cellular stress.

Discussion

In this report, we show for the first time that eIF-4E is phosphorylated in response to etoposide-induced DNA damage. Using CGP57380, we demonstrate that Mnk protein kinases are activated and mediate phosphorylation of eIF-4E induced by etoposide. Moreover, treatment of cells with either etoposide or rapamycin resulted in both increased 4E-BP1 associated with eIF-4E and enhanced eIF-4E phosphorylation, suggesting that the two regulatory mechanisms of protein translation initiation (eIF-4E phosphorylation and the association of 4E-BP1 with eIF-4E) may cross-talk with each other. Our results also demonstrate that phosphorylation of eIF-4E plays a positive role in the formation of the large translation initiation complex eIF-4F following either etoposide or rapamycin treatment. Taken together, our data suggest that eIF-4E phosphorylation may be a general feedback mechanism that maintains the basic level of protein translation when cap-dependent translation is impaired by different types of cyto- or genotoxic stress.

Earlier studies have shown that phosphorylated eIF-4E has increased binding affinity for capped mRNA and that the phosphorylation of eIF-4E by Mnks is aided by eIF-4G [5]. Although it was thought that the phosphorylation of eIF-4E was directly related to increased translational efficiency of the cell, recent evidence suggests that the relationship between phosphorylation of eIF-4E and translation initiation is more complicated. A recent study reported that Mnk1 and/or Mnk2 knockout mice are viable, fertile, and develop normally, which suggests that eIF-4E phosphorylation at Ser209 is not essential for cell growth during development [17]. In another report, Knauf et al. [13] showed that the overexpression of Mnk-1 and Mnk-2 limits cap-dependent translation under normal growth conditions. Therefore, it appears that phosphorylation of eIF-4E may not be involved in protein translation regulated by growth factors and/or cytokines under normal growth conditions.

However, several lines of recent evidence demonstrate that eIF-4E is also phosphorylated in response to a variety of cyto- or genotoxic stress and may facilitate protein translation under these circumstances. An earlier report by Fraser et al. [9] found that cellular stress such as arsenite, anisomycin, and heat shock not only causes an increase in phosphorylation of eIF-4E, but also lead to increased eIF-4F complex formation. Oxidative stress has also been shown to cause increased phosphorylation of eIF-4E, which is paralleled by the increased presence of eIF-4E with high molecular protein complexes [8]. A report from another study examining the effect of CGP57380 on the *de novo* synthesis of protein during the recovery of human 293 cells from hypertonic stress indicated that a change of polysome distribution was observed after the addition of CGP57380. This suggests that during the recovery process, a subset of proteins is selectively translated through a mechanism involving phosphorylation of eIF-4E [18]. Together, these results are consistent with our findings suggesting that phosphorylation of eIF-4E may enhance translation of a subset of proteins by maintaining a stable interaction between eIF-4E and eIF-4G following etoposide tretatment.

As stated earlier, in response to DNA damage, synthesis of many proteins, such as those involved in cell cycle arrest, DNA damage repair, and apoptosis, needs to be up-regulated. It will be a difficult task to identify these proteins whose translation is directly up-regulated by eIF-4E phosphorylation following DNA damage, especially since the contribution of eIF-4E phosphorylation to the enhanced synthesis of each individual protein may be too small to measure. Our results from BrdU labeling experiments did however show that while etoposide treatment led to a substantially decreased cell proliferation rate (a consequence of both cell cycle arrest and cell apoptosis), the significant decrease in the BrdU labeling rate following etoposide treatment was partially reversed by the presence of CGP57380 (data not shown).

Because etoposide treatment and subsequent eIF-4E phosphorylation do not lead to substantially decreased association between eIF-4G and eIF-4E, we speculate that phosphorylation of eIF-4E may cause a conformational change of the eIF-4E structure which allows eIF-4G to remain associated with eIF-4E despite binding of eIF-4E by 4E-BP1. This hypothesis is supported by our results showing the correlation between reduced phosphorylation of eIF-4E and decreased association between eIF-4E and eIF-4G after the addition of CGP57380 in cells treated with etoposide. Interestingly, suppression of eIF-4E phosphorylation by CGP57380 during the recovery of human 293 kid-

ney cells from hypertonic stress [18] also caused reduced interaction between eIF-4E and eIF-4G.

Our findings further indicate that the JNK signaling pathway may lead to eIF-4E phosphorylation in response to etoposide. This is consistent with a previous report that JNK is an upstream kinase which induces eIF-4E phosphorylation in response to anisomycin [6]. However, it challenges the current consensus that ERK1/2 and p38 MAPK are two direct upstream kinases that phosphorylate Mnk1 [19,20]. We examined the phosphorylation of Mnk1 in etoposide-treated MCF-7 cells with an antibody specifically targeting the two sites (Thr-197 and Thr-202) of Mnk1 phosphorylated by p38 and ERK and did not detect the signal of Mnk1 phosphorylation at these two sites (data not shown). These results again suggest a JNK or a JNK-like kinase may be a new, potential upstream kinase of Mnks in response to etoposide treatment.

Despite recent advances in studies regarding the protein translation initiation process, the function of eIF-4E phosphorylation in translation initiation remains controversial [14]. Our studies provide new insight into this important question. Our findings, along with others, suggest that phosphorylation of eIF-4E is a main factor in regulating protein synthesis during cytotoxic or genotoxic stress. Since currently IRES-mediated protein translation is the only known mechanism that leads to protein synthesis under cellular stress [11], our discovery provides a novel approach for the regulation of protein synthesis when cap-dependent synthesis is compromised by 4E-BP1.

Acknowledgments

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