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Differential regulation of CHOP translation by phosphorylated eIF4E under stress conditions

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ABSTRACT

Cells respond to environmental stress by inducing translation of a subset of mRNAs important for survival or apoptosis. CHOP, a downstream transcriptional target of stress-induced ATF4, is also regulated translationally in a uORF-dependent manner under stress. Low concentration of anisomycin induces CHOP expression at both transcriptional and translational levels. To study specifically the translational aspect of CHOP expression, and further clarify the regulatory mechanisms underlying stress-induced translation initiation, we developed a CMV promoter-regulated, uORF^{chop}-driven reporter platform. Here we show that anisomycin-induced CHOP expression depends on phosphorylated eIF4E/S209 and eIF2α/S51. Contrary to phospho-eIF2α/S51, phospho-eIF4E/S209 is not involved in thapsigargin-induced CHOP expression. Studies using various kinase inhibitors and mutants uncovered that both the p38MAPK-Mnk and mTOR signaling pathways contribute to stress-responsive reporter and CHOP expression. We also demonstrated that anisomycin-induced translation is tightly regulated by partner binding preference of eIF4E. Furthermore, mutating the uORF sequence abolished the anisomycin-induced association of *chop* mRNA with phospho-eIF4E and polysomes, thus demonstrating the significance of this *cis*-regulatory element in conferring on the transcript a stress-responsive translational inducibility. Strikingly, although insulin treatment activated ERK-Mnk and mTOR pathways, and consequently eIF4E/S209 phosphorylation, it failed to induce phospho-eIF2α/S51 and reporter translation, thus pinpointing a crucial determinant in stress-responsive translation.

INTRODUCTION

Cellular genes are expressed in a coordinated fashion that requires regulation at multiple levels. Among the regulatory mechanisms, translational control is an immediate early response that becomes crucial in the absence of transcription. When mammalian cells encounter stress conditions such as during development, differentiation, nutrient deprivation, chemical exposure and pathogenic infection, a family of protein kinase is activated to phosphorylate eukaryotic initiation factor 2α (eIF2α) (1,2). The phosphorylation of eIF2α on Ser51 results in sequestration of eIF2B and reduction of the eIF2–GTP–tRNAi ternary complex (2,3). Notably, ER stress-induced phosphorylation of eIF2α/S51 is known to up-regulate the translation of a class of stress responsive mRNAs, such as activating transcription factor 4 (ATF4) (4). Through the upstream open reading frame elements (uORFs) in its mRNA, the expression of ATF4 protein is responsive only to ER stress and consequently leads to transcriptional activation of downstream target genes [e.g. CHOP (CCAAT/enhancer-binding protein homologous protein) and GADD34] (5–8). The expression of CHOP are increased at both the transcriptional and translational levels during environmental stress, in line with its important role in various cellular processes such as programmed cell death, growth and differentiation in mammalian cells (9). Like *atf4*, a single uORF element in the 5′UTR of *chop* mRNA is responsible for the increase of CHOP protein level under ER stress conditions (10). Previously, Jousse *et al*. demonstrated a role of uORF from *chop* in limiting ribosomal access to downstream initiation sites (11). It was thought to permit re-initiation by allowing processive scanning of ribosomes after terminating at the uORF stop codon (11). Although the roles of uORF in stress-responsive translation have been analyzed in numerous mRNAs, the underlying molecular mechanisms remain poorly understood. To further investigate the regulation of uORF^{chop}-driven translation, we have created a uORF^{chop}-reporter platform that facilitates

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analysis of stress-induced translational control in the absence of transcription.

Treatment of cells with translation inhibitor anisomycin at high concentration (10 μM) activates p38 MAPK and SAPK/JNK signaling pathways and induces transcription of a number of genes including *chop* while inhibits protein synthesis (12). However, when low concentration (0.5 μM) is used, it activates p38 MAPK signaling pathway and induces transcription of stress response genes such as *chop* without significant inhibition of protein synthesis. The roles for SAPK pathway in the cellular adaptive response to stress have been firmly established (13). On the other hand, anisomycin activation of p38 MAPK and its downstream target Mnk1 (MAPK-activated protein kinase 1) leads to phosphorylation of eIF4G-bound eIF4E at the residue S209 (14–18). The eIF4G serves as a scaffold protein for assembly of eIF4E and eIF4A to form eIF4F, which, with further recruitment of eIF4B, eIF3 and eIF1 and 40S ribosome, underlies formation of pre-initiation complex. Despite the key involvement of eIF4E in cap-dependent translation initiation, the importance of its phosphorylation at S209 remains uncertain as Mnk-deficient mice seems healthy and fertile (19). However, phosphorylated eIF4E/S209 may be required for coping with stress situations. Another critical regulator of stress-responsive translation is the mammalian Target of Rapamycin, (mTOR), which functions in integrating extracellular signals (such as growth factors and hormones), amino-acid availability, and intracellular energy status to control translation rates and additional metabolic processes (20). It affects translation initiation through phosphorylating two major targets: the eIF4E-binding proteins (4E-BPs) and eIF4G, thereby controlling the activity of the eIF4F complex. 4E-BP1, one of the eIF4E-binding proteins, is a repressor of the eIF4F complex. The 4E-BP1/eIF4E complex is regulated at the level of 4E-BP1 phosphorylation: hypophosphorylated 4E-BP1 favors the complex formation, whereas the hyperphosphorylated form favors its dissociation (21).

In this article, we report that the phosphorylation of eIF4E/S209, 4E-BP1 and eIF2α/S51 plays a key role in anisomycin-induced translation of uORF^{chop}-driven reporter or endogenous CHOP. By combining pharmacologic, genetic, biochemical and cellular approaches, we have elucidated a convergence of anisomycin-activated p38MAPK-Mnk1 and mTOR signaling pathways at the level of phosphorylated eIF4E/S209 and 4E-BP1. Furthermore, we demonstrated that anisomycin-induced endogenous CHOP expression was decreased by overexpression of eIF4E/S209A/T210A. In cell line known to have low level of eIF2α (e.g. MCF-7), drug-induced expression of uORF^{chop}-driven reporter or endogenous CHOP is also low as compared to other cell lines. Additionally, insulin failed to induce uORF^{chop}-driven translation albeit it could activate ERK, Mnk1 and mTOR leading to phosphorylation of eIF4E/S209 but not eIF2α/S51. Thus, the fundamental difference between growth factor-induced general translation initiation and anisomycin-induced uORF^{chop}-driven translation hinges on the phosphorylation of both eIF4E/S209 and eIF2α/S51. Taken together, our present results suggest that anisomycin-induced, uORF-driven CHOP translation requires the activation of both mTOR and p38-Mnk1 pathways, in addition to phosphorylated eIF2α/S51.

MATERIALS AND METHODS

Plasmid constructs

All constructs were generated by PCR using LA-Taq polymerase (TaKaRa) from placenta cDNA library. The cDNA of human uORF-CHOP was generated by PCR using the forward 5′-ATGTTAAAGATGAGCGGGTG GCAG-3′ and the reverse or 5′-GATGCTCCCAATCTC GAGTGCTTGGTG-3′ primers. The PCR fragment was cloned into TOPO-TA vector (Invitrogen) and subcloned into pCMV-Tag4 (termed uORF-CHOP-Flag). The uORF fragment was generated by PCR using the forward 5′-ATGTTAAAGATGAGCGGGTGGCAG-3′ and the reverse 5′-GATGCTCCCAATTGTTCATGCTT GGTG-3′ primers and cloned into pcDNA3 (termed pcDNA-uORF). The mutant version of the uORF^{chop} was generated by PCR using forward 5′-AAGTTAAAG AAGAGCGGGTGGCAG-3′ and the reverse 5′-GATGC TCCCAATTGTTCTTGCTTGGTG-3′ primers, and subsequently cloned into pcDNA3 (termed pcDNA-uORF*). The firefly luciferase-reporter (Lu) gene was inserted into HindIII and XbaI restriction sites of the above constructs. To generate the dual monocistronic reporter constructs, CMV promoter-*Renilla* luciferase-reporter (Rlu) gene was inserted into the *Nru*I and *Bg*lII restriction sites of pcDNA-uORF-Lu and pcDNA-uORF*-Lu. The constructs of wild-type eIF4E and its mutants (S209A/T210A and S209D) were generated by PCR using the forward 5′-AATGAATTCAAGATGGC GATCGTC-3′ and reverse 5′-GGCGATATCTTAAAC AACAAACCT-3′ primers for eIF4E, 5′-GGCGATATC TTAAACAACAAACCTATTTTTAGTGGTGTCGCC-3′ for S209D, and 5′-GGCGATATCTTAAACAACAAAC CTATTTTTAGTGGCGGCGCC-3′ for S209A/T210A. The constructsn of wild-type 4E-BP1 and its mutant (F114A) were generated by PCR using the forward 5′-AT AGATATCATGTCCGGGGGCAGC-3′ and reverse 5′-GCGCTCGAGTTAAATGTCCATCTC-3′ primers for 4E-BP1 and 5′-GCGCTCGAGTTAAATGTCCATCTCA GCCTGTGACTC-3′ for F114A. The PCR fragments were cloned into pCMV2B vector (with Flag-tag, Stratagene) by EcoRI-EcoRV (for eIF4E) and EcoRV-XhoI (for 4E-BP1) restriction sites. The constructs of eIF2α/S51 and eIF2α (S51A) form were kindly provided by Dr Woan-Yuh Tarn (Institute of BioMedical Sciences, Academia Sinica, Taiwan), p38 MAPK, and p38 (AGF) were from Dr Jia-Huai Han (Scripps Research Institute).

Cell culture, transfection and drug treatment

293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) and PC3 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (HyClone) and 100 U/ml penicillin-streptomycin (Invitrogen) at 37°C in humidified incubator containing

5% CO_2. Cells were seeded in a 10-cm dish the day before transfection. 293T cells at a concentration of 5×10^6 cells per dish were transfected with 0.25 μg (for uORF's constructs) or 5 μg (for eIF2α, eIF4E, eIF4G and 4E-BP1 constructs) DNA using calcium phosphate precipitation method. PC3, MCF-7 and HeLa cells at a concentration of 1×10^7 cells per dish were transfected with 0.25 μg (for uORF's constructs) or 5 μg (for eIF2α and eIF4E constructs) DNA using Lipofectamine™ 2000 method (Invitrogen). After 16 h, the cultures were changed to fresh medium for 1.5 h and then added DMSO, 1 μM thapsigargin (Sigma), 0.5 μM anisomycin (Sigma), 6 nM to 60 μM cycloheximide (Sigma), 1 μM SB203580 (Sigma), 1 μM SP600125 (Biomol), 1 μM U0126 (Biomol), 10 μM CGP57380 (Sigma), 1 μM Rapamycin (Bioaustralis) or 2.5 μM insulin. Cells were harvested after 0–4 h post-treatment.

Western blot analysis and dual luciferase assay

The cells were harvested and centrifuged for 5 min at 6000 *g*, and washed twice with PBS. Cell pellets were re-suspended by gently pipetting in whole cell extraction (WCE) buffer [20 mM Hepes (pH 7.6), 10% glycerol, 0.4 M NaCl, 0.5% Triton X-100, 0.5 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin A] at 0.5×10^6 to 1.0×10^6 cells/100 μl. Cell lysate was prepared by vortexing at 4°C for 30 min. Protein concentration was determined by the Bradford reagent (Bio-Rad). Western blot analysis was performed after electrophoretic separation of polypeptides by 7.5–15% SDS–PAGE and transfer to PVDF membranes. Blots were probed with the indicated primary and appropriate secondary antibodies and detected by ECL chemiluminescence (Amersham Biosciences). Monoclonal antibody to anti-tubulin (clone 10D8) and rabbit anti-mTOR, anti-eIF2α, anti-eIF4E, anti-CHOP and anti-eIF4G antibodies were generated by our own laboratory. Rabbit antibodies to phospho-mTOR/S2481 (#2974), phospho-eIF4E/S209 (#9741), phospho-eIF4G/S1108 (#2441), phospho-S6K1/T389 (#9206), phospho-ERK/T202/Y204 (#4376), phospho-p38/T180/Y182 MAPK (#9211), phospho-4E-BP1/S65 (#9451), phospho-Mnk1/T197/202 (#2111), Mnk1 (#2195), S6K1 (#9202) and 4E-BP1 (#9452) were obtained from Cell Signaling. Anti-p38 MAPK (sc-535) and anti-ERK1 (sc-93) antibodies were obtained from Santa Cruz Biotech. Dual luciferase assays were carried out according to manufacturer's instructions (Promega). 1 μg of extract was assayed for firefly and *Renilla* luciferase activities. Ratio is the unit of firefly luciferse after normalized with *Renilla* luciferase, and each value was derived from three independent experiments.

RNA extraction and RT–PCR

Total RNAs were extracted from cell cultures using an *EZ* EXTRACTION Solution Kit (Genomics Biosci & Tech) supplemented with RNasin (Promega). Contaminating DNA was digested with RNase-free DNase according to the manufacturer's instructions (Promega). For RT–PCR analysis, 2.5 μg of total RNA was reverse-transcribed into cDNA using random primers (Invitrogen) and Superscript II Reverse Transcriptase (Invitrogen) Kit. The following primers were used for PCR (5 min denaturation at 94°C and then 30 cycles of amplification at 94°C for 30 s, 50°C for 30 s and 72°C for 45 s):

CHOP, 5′-AGAGATGGCAGCTGAGTCATTGCC-3′, and 5′-GCAGATTCACCATTCGGTCA-3′;
GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′, and 5′-GTCGCTGTTGAAGTCAGAGGAGAC-3′; CHOP-Flag, 5′-ATGTTAAAGATGAGCGGGTGGCAG-3′, and T7 primer;
Lusiferase, 5′-CCATGGAAGACGCCAAAAAC-3′, and 5′-CTTGTCCCTATCGAAGGACTCT-3′.

Polysome profile analysis by sucrose gradient centrifugation

Polysome profile by sucrose gradient was done as described in a previous study (22). After transfection for 16 h, cells were cultured with fresh media for 1.5 h and treated with DMSO or anisomycin (0.5 μM) for 1 h. Next, cells were incubated with cycloheximide (100 μg/ml) for 3 min at 37°C and washed three times with 1x PBS. Lysis buffer (15 mM Tris–HCl, pH 7.4, 15 mM $MgCl_2$, 200 mM NaCl, 1% Triton X-100 (v/v), 100 μg/ml cycloheximide, 1 mg/ml heparin) was used to harvest cells directly. The cell lysates were clarified by centrifuging at 12 000 *g* for 10 min at 4°C, and the resulting supernatants were applied to the 10–50% (w/v) sucrose gradient and centrifuged at 15 100 g for 4 h in a SW41 rotor at 4°C. Using the fraction collector and a syringe pump system, fractions were collected from the top to bottom. To purify RNA, we took 400 μl of each fraction and add 600 μl of guanidine-HCL and 600 μl of isopropanol. After vigorous vortexing, the samples were incubated at −20°C O/N and then centrifuged at 9 100 g for 25 min. The pellet was subsequently washed by 70% EtOH and resuspend in 100 μl of water. Equal volumes of fractionated RNA were subjected to RT–PCR and amplified by T7 and Lusiferase primers (5′-CTTGTCCC TATCGAAGGACTCT-3′).

Immunoprecipitation and RNA immunoprecipitation (RNA-IP)

Cells were harvested by spinning for 5 min, washed with PBS and resuspended (0.5×10^6 to 1.0×10^6 cells/400 μl) by gently pipetting in cold whole cell extraction (WCE) buffer and vigorously vortexed for 30 min at 4°C. After centrifugation for 15 min in 12 740 g at 4°C, the cell extracts were incubated with protein G-Sepharose beads (Pharmacia) for 1 h rotating at 4°C. The pre-cleared extracts were incubated with desired antibodies for 2–3 h rotating at 4°C. The beads were washed with WCE buffer for three times followed by SDS–PAGE and western blot analysis. For RNA-IP, M2 beads (contained protein and RNA, Sigma) were extracted directly by using an *EZ* EXTRACTION Solution Kit and subjected to RT–PCR. We used the T7 and Lusiferase primers (5′-CT TGTCCCTATCGAAGGACTCT-3′) in the cDNA amplification step.

RNA interference

The pSUPER shRNA-producing plasmid (Oligoengine) was directed against the following target sequence: 4E-BP1-5′-GTTTGAGATGGACATTTAA-3′ (23). HEK 293T cells were co-transfected with 0.25 μg of dual construct and 5 μg of pSUPER-4E-BP1 or pSUPER (as control). After 16 h, the cultures were changed to fresh medium for 24 h and then changed to fresh medium again for 1.5 h. After being treated with DMSO, 1 μM thapsigargin, and 0.5 μM anisomycin for 2 h, cells were harvested for luciferase assay and western blot analysis.

Statistical and quantitative analysis

The Student's *t*-test was used to estimate statistical significance of differences between two groups. Differences between groups were considered statistically significant at $P < 0.05$. Quantitative ratios were presented as protein or RNA levels that were normalized with internal controls (such as tubulin or GAPDH) through using the NIH ImageJ software.

RESULTS

Stress-responsive expression of uORF^{chop}-driven reporter and endogenous CHOP

When used at high concentration (i.e. 10 μM), anisomycin could induce *chop* mRNA transcription while inhibit its translation. However, low concentration (i.e. 0.5 μM) of anisomycin could induce both transcription of *chop* mRNA and translation of CHOP protein (Figure 1A, upper and lower panels). To investigate the underlying mechanisms that govern the uORF-driven CHOP translation, we constructed a plasmid that directs the transcription of uORF-CHOP under a CMV promoter control and fused Flag-tag at its C-terminus (uORF-CHOP-Flag, Figure 1B, upper panel). The results of western blot analysis indicated that CHOP-Flag expression was also repressed by high concentration of anisomycin but induced by low-concentration treatment (Figure 1B, lower panel). RT–PCR confirmed that the mRNA of CHOP-Flag was constitutively expressed without response to anisomycin treatment (Figure 1B, upper panel), therefore excluding the possibility that stress-responsive reporter induction is mediated at the transcriptional level. For an efficient assay platform, we constructed a plasmid that directs the transcription of uORF-firefly luciferase (CMV-uORF^{chop}-Lu, Figure 1C, upper panel) under a CMV promoter control. Under such setting, the reporter firefly luciferase (FL) transcript was translated in a uORF^{chop}-driven manner while the control *Renilla* luciferase (RL) transcript (CMV-Rlu) was translated in a non-uORF-driven manner. The FL expression depends almost exclusively on stress conditions (i.e. anisomycin treatment, Figure 1C). In contrast, we found that reporter expression from the dual reporter system was not induced by treatment with cycloheximide (Figure 1C, lower panel), although *de novo* CHOP synthesis under stress condition was shown repressed by this eukaryotic protein synthesis inhibitor (24). Both thapsigargin and anisomycin could induce uORF^{chop}-driven FL expression effectively (Figure 1D, upper panel). RT–PCR also confirmed that the expression of FL mRNA was not responsive to either thapsigargin or anisomycin treatment (Figure 1D, lower panel). Our results further demonstrated that the kinetics of FL expression from this dual reporter system is faster than that of the endogenous CHOP induction under anisomycin treatment (Figure 1C, lower panel). This difference in induction kinetics is likely due to the availability of reporter versus endogenous gene transcripts, which are generated by different promoters, at the time of the drug treatment. Together these results show that the dual reporter plasmid is a specific and effective platform for addressing the mechanisms of anisomycin-induced uORF^{chop}-driven translation.

Role of eIF2α/S51 phosphorylation in thapsigargin- or anisomycin-induced uORF^{chop}-driven reporter translation

The ER stress-activated phosphorylation of translation initiation factor eIF2α/S51 is known to be important for thapsigargin-induced ATF4 or CHOP translation (5,10). To examine whether phosphorylated eIF2α/S51 is crucial for both thapsigargin- and anisomycin-induced uORF^{chop}-FL expression, we tested the effect of eIF2α/S51A mutation on the expression of FL. We found that thapsigargin was more effective than anisomycin in inducing phosphorylation of eIF2α/S51 (Figure 1D, lower panel). Furthermore, when the non-phosphorylatable eIF2α/S51A mutant was over-expressed, the thapsigargin-induced FL expression was almost completely abolished while anisomycin-induced expression was only partially reduced (Figure 1E, upper panel). Anisomycin-activated p38 MAPK-Mnk pathway leads to phosphorylation of eIF4E/S209 (25). We next sought to analyze whether Mnk1 and eIF4E are involved in the anisomycin-induced uORF^{chop}-driven FL expression. When cell lysates were probed with phosphospecific antibodies to p38, Mnk1 and eIF4E, the phosphorylation levels of all three proteins were found increased after anisomycin treatment (Figure 1E, lower panel). In sharp contrast, thapsigargin treatment had no effect on the phosphorylation levels of these proteins. These results thus suggest that thapsigargin-induced uORF^{chop}-FL reporter expression is dependent exclusively on phosphorylated eIF2α/S51 while such modification showed minor effects for the anisomycin-induced reporter translation.

The p38/MAPK-Mnk-eIF4E pathway plays an essential role in the anisomycin-induced translation of uORF^{chop}-driven reporter

To analyze whether the p38/MAPK-Mnk pathway is involved in the anisomycin-induced uORF^{chop}-driven FL expression, cells were treated with p38 inhibitor. As shown in Figure 2A (upper panel), inhibition of p38 activity by SB203580 led to a significant reduction of anisomycin-induced uORF^{chop}-driven FL expression, but had no effect on thapsigargin treatment. Western blot results showed that, while SB203580 treatment had no effect on

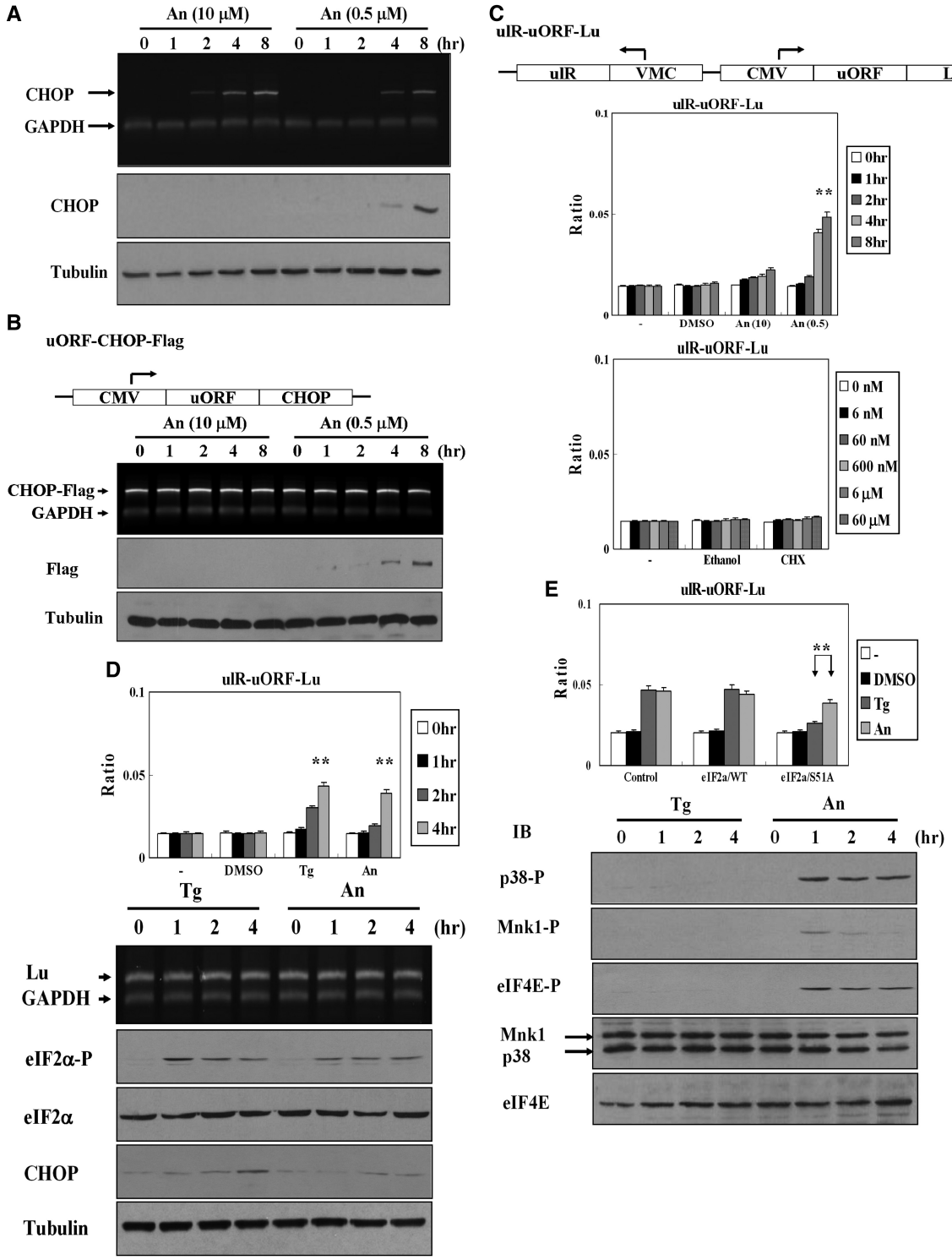

Figure 1. Induction of CHOP expression by anisomycin. (**A**) 293T cells were treated with high (10 μM) or low (0.5 μM) dose of anisomycin for the indicated time lengths (0–8 h) and analyzed by western blot (lower panel) with the indicated antibodies (anti-CHOP and anti-tubulin) or by RT–PCR (upper panel). (**B**) Cells were transfected with the CMV promoter-driven uORF-CHOP-Flag construct (upper panel) as described in the 'Materials and Methods' section. Cells were subsequently treated with DMSO, 10 μM or 0.5 μM anisomycin (An) for 0–8 h, and harvested for RT–PCR and western blot analysis. (**C**) Schematic representation of the dual reporter construct, ulR-uORF-Lu (upper panel). Compare to firefly luciferase (FL), we named the *Renilla* luciferase (Rlu) as ulR because the direction of it is antisence/reverse in relation to the FL. 293T cells were transfect with this construct and subjected to drug treatment as in (B) or treatment with indicated concentrations of cycloheximide for 4 h (lower panel), and subsequently harvested and assayed with Dual-Luciferase Reporter Assay system (Promega). Ratio represents the activity of firefly luciferase divided by that of *Renilla* luciferase. Experiments were done in triplicate (mean ± SD, $P < 0.05$). (**D**) The dual-reporter construct (ulR-uORF-Lu) were transfected into cells, which were subsequently treated with DMSO, 1 μM thapsigargin (Tg), or 0.5 μM anisomycin (An). After 0–4 h, cells were harvested for Dual-Luciferase Reporter Assay (upper panel, mean ± SD, $P < 0.05$), RT–PCR and western blot analysis (lower panel). Blots were probed with the indicated primary (anti-CHOP, anti-tubulin, anti-phosphorylated eIF2α/S51 and anti-eIF2α) and appropriate secondary antibodies. (**E**) The dual reporter construct (ulR-uORF-Lu) was co-transfected with vector (as negative control), eIF2α (wild-type, wt), or eIF2α (S51A) into 293T cells, and subsequently subjected to drug treatment as in (D). After 4 h, cells were harvested for Dual-Luciferase Reporter Assay (upper panel, mean ± SD, $P < 0.05$). Cell lysates (from Figure 1D) were probed with antibodies against phosphorylated p38 MAPK (p38-P), p38 MAPK, phosphorylated Mnk1 (Mnk1-P), Mnk1, phosphorylated eIF4E/S209 (eIF4E-P) and eIF4E, as indicated on the left of lower panel.

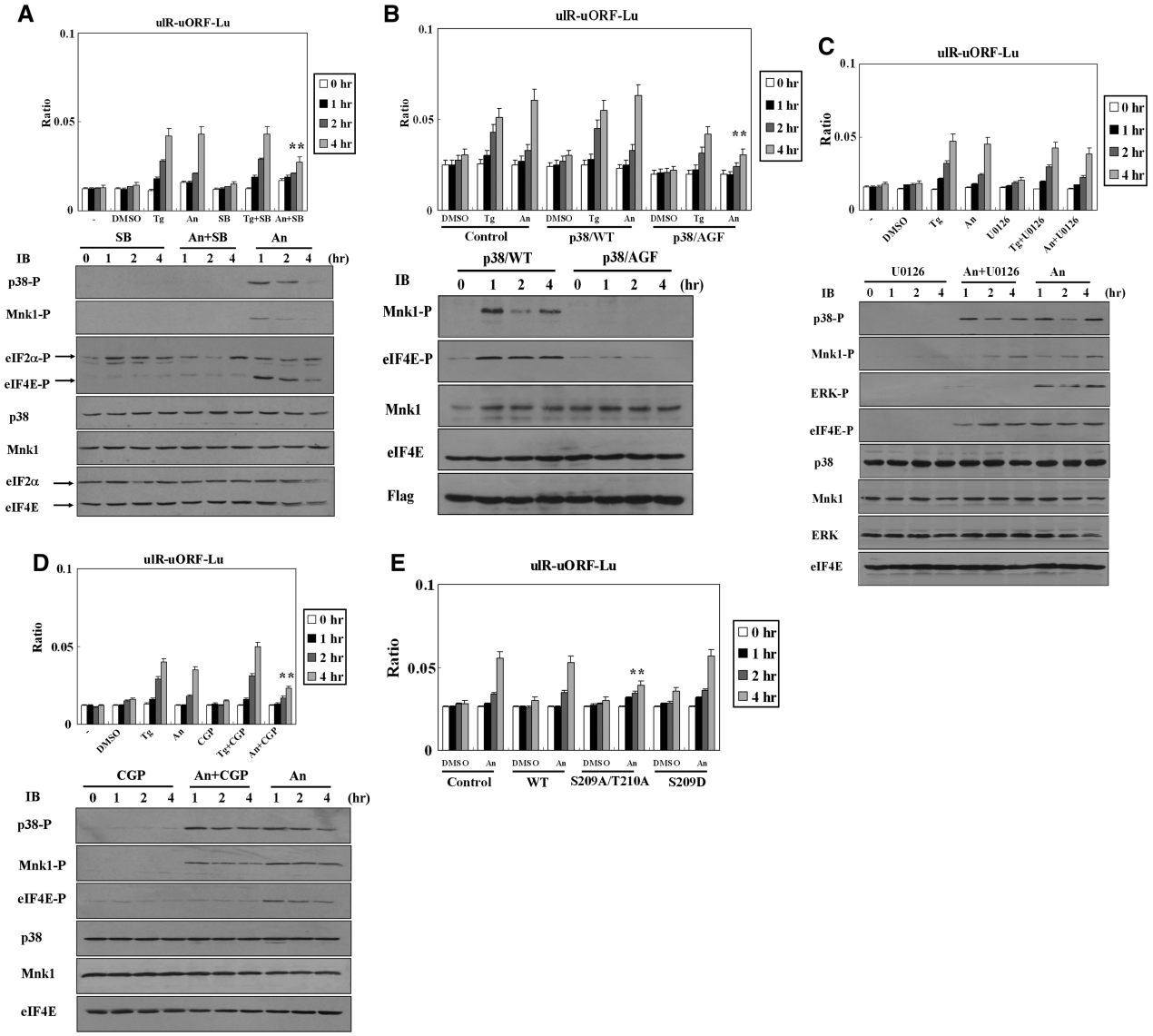

Figure 2. The p38 MAPK signaling pathway is required for anisomycin-, but not thapsigargin-induced uORF^{chop}-driven translation. (**A**) The dual reporter construct (ulR-uORF-Lu) was transfected into 293T cells as described in the 'Materials and Methods' section. Cells were subsequently treated with DMSO, 1 μM thapsigargin (Tg), 0.5 μM anisomycin (An), 1 μM SB203580, thapsigargin plus SB203580 (Tg + SB), or anisomycin plus SB203580 (An + SB). After 0–4 h, cell extracts were prepared for Dual-Luciferase Reporter Assay (upper panel, mean ± SD, $P < 0.05$) and western blot analysis (lower panel). Blots were probed with the indicated antibodies. (**B**) The dual reporter construct was co-transfected with vector (as negative control), p38 MAPK (WT), or p38 MAPK dominant negative mutant (AGF) into 293T cells. After 0–4 h of treatment with DMSO, thapsigargin (Tg), or anisomycin (An), cells were harvested for Dual-Luciferase Reporter Assay (top panel) and western blot analysis (bottom panel). Ratio in each experiment represents the activity of firefly luciferase divided by that *Renilla* luciferase (mean ± SD, $P < 0.05$). (**C**) The dual reporter construct was transfected into 293T cells, which were subsequently treated with DMSO, thapsigargin (Tg), anisomycin (An), 1 μM U0126, thapsigargin plus U0126 (Tg + U0126), or anisomycin plus U0126 (An + U0126). After 0–4 h, cell extracts were prepared for Dual-Luciferase Reporter Assay (top panel) (mean ± SD, $P < 0.05$) and western blot analysis (bottom panel). (**D**) The dual reporter construct was transfected into cells, which were then treated with DMSO, thapsigargin (Tg), anisomycin (An), 10 μM CGP57380, thapsigargin plus CGP57380 (Tg + CGP), or anisomycin plus CGP57380 (An + CGP). The cultures were subjected to luciferase assay and western blot analysis as detailed in (A). (**E**) Dual reporter constructs (ulR-uORF-Lu) was co-transfected with vector (as negative control), eIF4E-WT, or eIF4E mutants (S209A/T210A and S209D) into 293T cells. Cells were treated with anisomycin (An) for the indicated time lengths and subsequently harvested for reporter assay analysis. The asterisk indicates significant increase of this ratio as compared to the control.

the protein levels of p38, Mnk1, eIF2α and eIF4E (Figure 2A, lower panel), it decreased the phosphorylated eIF2α/S51 level slightly after 1–2 h treatment (Figure 2A, lower panel). To further characterize the involvement of p38, we performed genetic experiments by co-transfecting wild-type or dominantly negative mutant p38 (i.e. p38/AGF) with the uORF^{chop}-luciferase plasmid. Western blot results showed that p38/AGF blocked the phosphorylation of Mnk1 and eIF4E under anisomycin treatment (Figure 2B, lower panel), implicating these factors in the p38 signaling pathway under stress. We further found that overexpression of p38/AGF efficiently diminished

anisomycin-, but not thapsigargin-induced luciferase expression (Figure 2B, upper panel), strengthening the role of p38 and its associated signaling cascade in this translational control.

To address whether other MAPKs may also be involved in the anisomycin-induced reporter protein translation, we tested the effect of ERK and JNK inhibitors. ERK is an extracellular signal regulated kinase that has been reported to regulate translation through phosphorylating Mnk1 and Mnk2 (26,27). The expression of uORF^{chop}-driven FL decreased only slightly when the activity of ERK is inhibited by U0126 (Figure 2C, upper panel). We further found that while ERK phosphorylation was inhibited by U0126 under anisomycin treatment, phosphorylation of p38, Mnk1 and eIF4E remained unchanged (Figure 2C, lower panel). While stress-activated protein kinase 1 (SAPK1/JNK) was also known to be activated by anisomycin (28), its activity was dispensable in the anisomycin-induced reporter expression based on studies using JNK inhibitor SP600125 (data not shown). Therefore, these data indicate that p38 MAPK is the major kinase for activating the anisomycin-mediated induction of uORF^{chop}-mediated FL expression.

To further demonstrate that the downstream target of p38, Mnk1, is also important for the anisomycin-mediated induction of uORF-luciferase, we treated the cells with Mnk inhibitor, CGP57380. Anisomycin-, but not thapsigargin-induced FL expression was inhibited by CGP57380 (Figure 2D, upper panel). CGP57380 had no effect on the protein levels of p38, Mnk1 or eIF4E, or the activity of p38 (i.e. phosphorylated p38). However, in the presence of this inhibitor, the phosphorylation of Mnk1 was decreased while that of eIF4E/S209 was completely inhibited (Figure 2D, lower panel). These results suggest that the Mnk1-mediated phosphorylation of eIF4E/S209 plays a crucial role for anisomycin-induced uORF^{chop}-driven luciferase expression. To further strengthen this notion, we performed co-transfection of wild-type, phospho-defective (S209A/T210A) or phospho-mimetic (S209D) variant of eIF4E with the uORF^{chop}-FL plasmid into 293T cells and tested their effect on drug-induced reporter expression. As shown in Figure 2E, the induction of uORF^{chop}-driven FL by anisomycin was compromised by eIF4E/S209A/T210A, but not by eIF4E/S209D, signifying the importance of eIF4E phosphorylation. On the contrary, both eIF4E mutants have no effects on thapsigargin-induced uORF^{chop}-driven FL expression (data not shown). Collectively, these findings and the results of Figure 2 indicate that the phosphorylated eIF4E/S209 is crucial for the induction of uORF^{chop}-driven FL by anisomycin, but does not play considerable role in the thapsigargin-driven induction.

Preferential translation of CHOP under stress condition requires an intact uORF element and the involvement of phosphorylated eIF4E

To assess whether the stress-responsive translation of the uORF-containing mRNA is dependent on the start codons within this *cis*-regulatory sequence, we generated a variant of the ulR-uORF-Lu reporter constructs in which the AUGs of uORF^{chop} were mutated to AAGs (ulR-uORF*-Lu). Upon treatment with thapsigargin or anisomycin, we found that, while reporter induction was evident from the wild-type dual reporter construct, the uORF-minus (ulR-Lu) or the uORF mutant (ulR-uORF*-Lu) constructs did not exhibit such induction (Figure 3A). Furthermore, the basal reporter expression from the latter two constructs was significantly elevated as compared to the wild-type construct, consistent with the notion that uORF plays a repressive role in general mRNA translation. Next, to directly examine the role of uORF^{chop} in mediating mRNA translation during the stress condition, lysates from the transfected cells previously treated with DMSO or anisomycin were subjected to polysome profiling analysis (Figure 3B). Upon fractionation, RNA was extracted and subjected to RT–PCR analysis for the luciferase reporter mRNA. As shown in Figure 3C, mutant uORF-containing mRNA (from ulR-uORF*-Lu) displayed a greater degree of polysome association (fractions 7–10) under non-stress condition, whereas association of wild-type transcripts was minimal. This observation is in line with the reporter assay in Figure 3A, which illustrated that the loss of an intact uORF element derepresses mRNA translation, particularly in a non-stress context. Interestingly, under anisomycin treatment, there was a significant increase in the presence of the wild-type mRNA in the polysome fractions, signifying a rise in translation. On the other hand, the association of the uORF-mutated mRNA with polysomes underwent a slight reduction. Therefore, our results pinpoint the uORF element of the *chop* mRNA as an important determinant for regulating downstream transcript translation.

To further characterize the mechanism underlying the uORF^{chop}-driven translation during anisomycin treatment, we next set out to examine the involvement of eIF4E phosphorylation. To this end, we transfected the wild-type eIF4E and its mutants (S209A/T210A and S209D) into 293T cells and performed RNA immunoprecipitation (RNA-IP) on the lysates to dissect their association with the uORF-containing reporter mRNA. As shown in Figure 3D, anisomycin treatment promoted the co-precipitation of the wild-type uORF-containing mRNA with eIF4E/WT (lower panel). However, under the same condition, association of RNA with eIF4E/S209A/T210A was weakened. Furthermore, while there was a basal interaction between the mutant uORF-containing mRNA and eIF4 proteins, such association did not exhibit any stress-responsive changes. Together with the above polysome profiling data, our results provide strong evidence that the phosphorylated eIF4E/S209 is crucial for the induction of uORF^{chop}-driven FL by anisomycin.

To further confirm the function of eIF4E in CHOP regulation, we attempted to perform siRNA-mediated knockdown of eIF4E expression in 293T cells. However, such approach did not permit further functional analysis due to side effects from down-regulating this essential gene (data not shown). Published results have documented that the

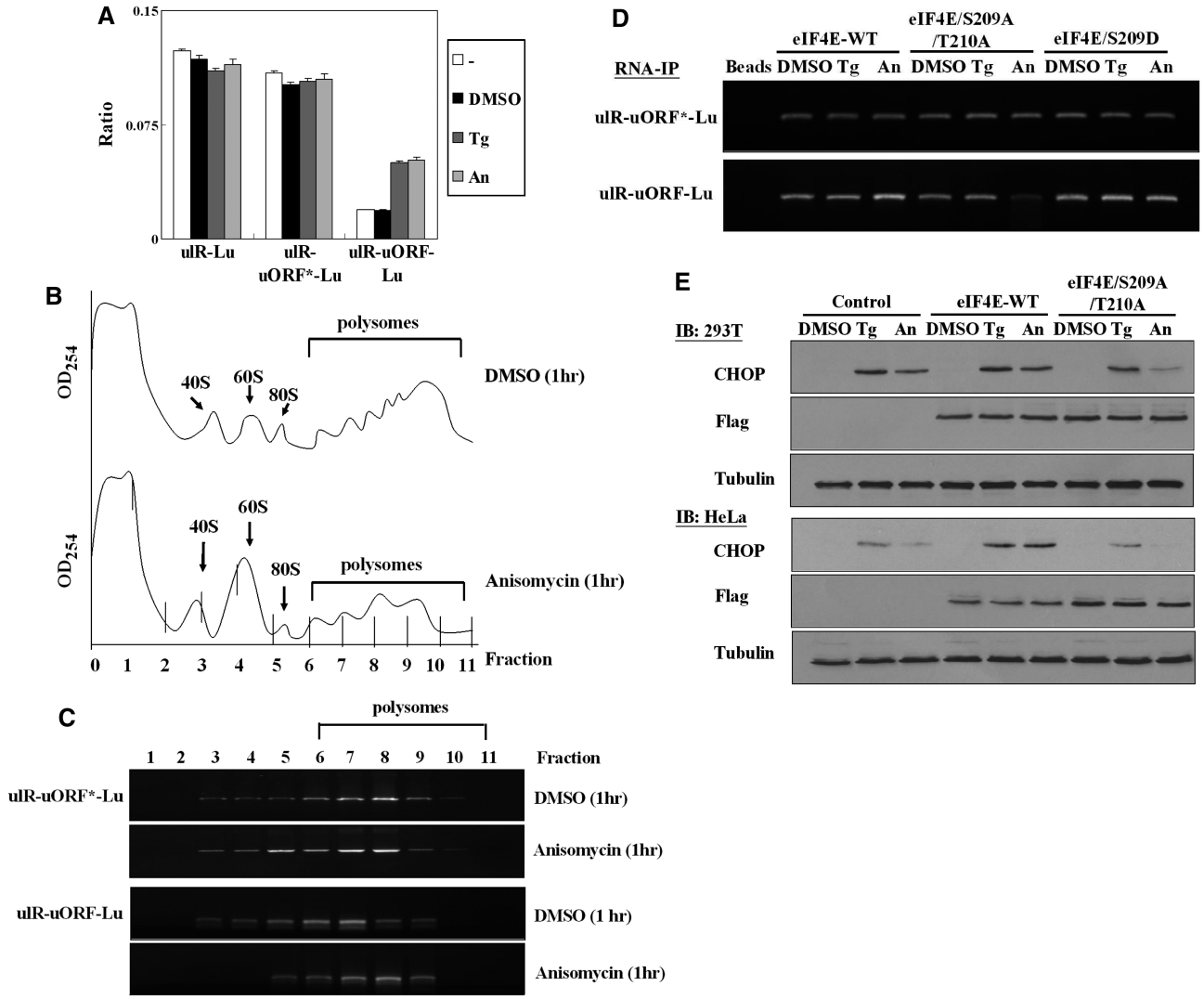

Figure 3. Anisomycin induces translation of the reporter mRNA in a phosphorylated eIF4E/S209- and uORF-dependent manner. (**A**) 293T cells were transfected with dual reporter construct (ulR-uORF-Lu) or its mutant version in which the $uORF^{chop}$ was altered (ulR-uORF*-Lu) and subjected to drug treatment as in Figure 1B, and subsequently harvested for Dual-Luciferase Reporter Assay system (mean ± SD, $P < 0.05$). (**B** and **C**) In addition, after DMSO or anisomycin treatment for 1 h, the cell lysates of the transfectants were also applied to polysome profile analysis (B), with resulting fractions subsequently subjected to RT–PCR assay (C). The top to bottom of the sucrose gradient was shown from left to right, respectively. The sedimentation of the 40S, 60S, 80S and polysomes are also indicated. (**D**) The constructs of eIF4E-WT, or eIF4E mutants (S209A/T210A and S209D) were transfected into 293T cells. Cells were treated with drugs for 1 h and subsequently harvested for the RNA-IP analysis (see 'Materials and Methods' section). (**E**) The eIF4E-WT or eIF4E mutant (S209A/T210A) was transfected into 293T and HeLa cells. After 16 h, the culture was changed to fresh medium and cultured for additional 3 h, then treated with DMSO, thapsigargin (Tg) or anisomycin (An) for 8 h. Cell extracts were analyzed by western blot and probed with anti-CHOP, anti-Flag and anti-tubulin antibodies, as indicated.

rate of protein synthesis in the cells is regulated by eIF4E and eIF2α, and that the activity and expression of both factors vary in different cancer cell lines (29,30). First, we analyzed the protein level of eIF2α and eIF4E in 293T, MCF-7, HeLa and PC3 cells by western blot analysis (Supplementary Data, Figure S1A, upper panel). Quantitative determination revealed that expression of eIF4E was at a higher level in 293T than other three cells while lower level of eIF2α was detected in MCF-7 as compared with others (Supplementary Data, Figure S1A, lower panel). To further investigate the effects of eIF2α and eIF4E expression levels on CHOP expression in different cancer cells, 293T, PC3, HeLa and MCF-7 cells were treated with thapsigargin and anisomycin for 8 h. The RT–PCR results showed that both thapsigargin and anisomycin could significantly induce *chop* mRNA level in PC3 cells (Supplementary Data, Figure S1B). However, drug-induced *chop* mRNA expression varied slightly in other cells, with the lowest level of induction evident in HeLa cells during anisomycin treatment. Furthermore, we also analyzed the protein level of CHOP under stress condition. Both thapsigargin and anisomycin induced high level of CHOP expression in both PC3 and 293T cells (Supplementary Data, Figure S1C). However, the induced CHOP protein level did not correlate with the drug-induced mRNA levels. These results thus suggest that eIF4E level may be crucial in regulating CHOP expression at the translation

initiation step. Next, we over-expressed wild-type eIF4E in HeLa cells, which was shown to express a relatively lower level of endogenous eIF4E, and subsequently observed an elevated anisomycin-induced CHOP expression as compared with the plasmid control (Figure 3E, lower panel). Such further induction was not seen in the 293T cells, which already expressed high level of eIF4E. Additionally, over-expression of the eIF4E phospho-defective mutant (eIF4E/S209A/T210A) decreased anisomycin-induced CHOP expression both in HeLa and 293T cells (Figure 3E). Together, these results demonstrated that both the protein and phosphorylation levels of eIF4E are required for anisomycin-induced CHOP expression.

Involvement of mTOR signaling in anisomycin-induced uORF^{chop}-driven translation

The mechanism underlying the involvement of phosphorylated eIF4E/S209 in anisomycin-induced uORF^{chop}-mediated FL expression remains unclear. The competitive interaction of eIF4E with either 4E-BPs or eIF4G underlies the cap-dependent translation and is known to be regulated by mTOR (20). We therefore tested whether rapamycin could inhibit the anisomycin-induced reporter expression. As shown in Figure 4A (upper panel), rapamycin treatment diminished anisomycin-mediated induction, but did not show any evident effect on the thapsigargin-induced expression. Intriguingly, we found that the phosphorylation of S2481 on mTOR was stimulated by anisomycin and was only partially inhibited with the additional treatment of rapamycin. Additionally, the protein level of mTOR was slightly elevated by anisomycin or anisomycin and rapamycin treatment (Figure 4A, lower panel). We also assessed the phosphorylation levels of several mTOR downstream targets (S6K, 4E-BP1 and eIF4G), as an indication of the activity of mTOR (Figure 4A). Such persisted activation of mTOR may explain why the induction of uORF^{chop}-driven reporter expression by anisomycin cannot be inhibited completely by rapamycin (Figure 4A, upper panel).

Paradoxically, western blot results showed that the phosphorylation levels of both Mnk1 and eIF4E/S209 were elevated by rapamycin treatment while the level of phosphorylated p38 was not affected (Figure 4A, lower panel). These results thus imply that there might be another kinase affecting the phosphorylation of Mnk1 in a rapamycin-dependent manner. Results from a previous report suggested that ERK could be a candidate Mnk-1-targeting kinase (27). Indeed, ERK was activated by rapamycin or anisomycin treatment (Figure 4A, lower panel). However, such activation was suppressed when cells were treated with both of these drugs (Figure 4A, lower panel). In line with our observations on the rapamycin treatment, knockdown of mTOR was shown to abrogate rapamycin-induced eIF4E/S209 phosphorylation via Mnk-dependent mechanism (31). Our results therefore suggest that rapamycin-induced phosphorylation of eIF4E/S209 may be through ERK-driven phosphorylation of Mnk1. Furthermore, levels of both the protein and S2481 phosphorylation of mTOR were stimulated by anisomycin (Figure 4A, lower panel), thus suggesting that both mTOR and Mnk1 activities are important for the anisomycin-induced uORF^{chop}-driven FL expression.

Unlike anisomycin treatment, uORF^{chop}-driven FL expression did not increase under rapamycin treatment even though Mnk1 and eIF4E/S209 were phosphorylated (Figure 4A). We thus postulate that the phosphorylation of eIF4E/S209 per se may not be sufficient for a full induction of uORF^{chop}-driven FL translation. It is a formal possibility that, despite the rapamycin-induced phosphorylation, eIF4E may exist in a complex with 4E-BP1 and is thus still translation-incompetent. To resolve this issue, we performed immunoprecipitation experiments using anti-eIF4E antibody. We found that the level of 4E-BP1 associated with eIF4E or phosphorylated eIF4E/S209 was dramatically enhanced by rapamycin treatment (Figure 4B). Under the same condition, the phosphorylaion of eIF4G/S1108 (Figure 4A) and the association of eIF4G with eIF4E (Figure 4B) underwent significant reduction. These results are thus consistent with the notion that mTOR plays a pivotal role in regulating the assembly of the eIF4F complex that contains both eIF4E/pS209 and eIF4G/pS1108 and, consequently, the uORF^{chop}-driven FL translation under anisomycin treatment.

We next sought to determine whether the observed effects of the various kinase inhibitors on the anisomycin-induced reporter expression (Figures 2 and 4) also reflect the response of endogenous CHOP expression regulation under stress. To this end, cells were treated with inhibitor of the p38, Mnk, ERK or mTOR signaling pathway in the presence or absence of anisomycin. As shown in Figure 4C, we found that addition of SB, CGP or rapamycin effectively abolished the anisomycin-induced CHOP protein expression. In contrast, the ERK inhibitor imparted little effect on the CHOP induction. These results thus demonstrate the relevance of our dual reporter platform in assessing the stress-responsive regulation of endogenous CHOP protein.

Anisomycin-induced uORF^{chop}-driven translation is tightly regulated by partner binding preference of eIF4E

To further assess the mechanism underlying the involvement of eIF4E in drug-induced translation, we next performed immunoprecipitation assays to characterize eIF4E-associated complex formation. We first found that the interaction between eIF4E and 4E-BP1 underwent a discernable decrease at 2–4 h post-anisomycin treatment, but remained unchanged during the tharpsgargin treatment (Figure 5A, upper panel). Next, we examined whether the eIF4E-4E-BP1 association depends on the phosphorylation of eIF4E, which was previously shown to increase in response to drug treatment. Western blots of immunoprecipitates showed that, as compared to the wild-type and S209A/T210A mutant of eIF4E, less 4E-BP1 was associated with the eIF4E/S209D variant (Figure 5A, lower panel) in the

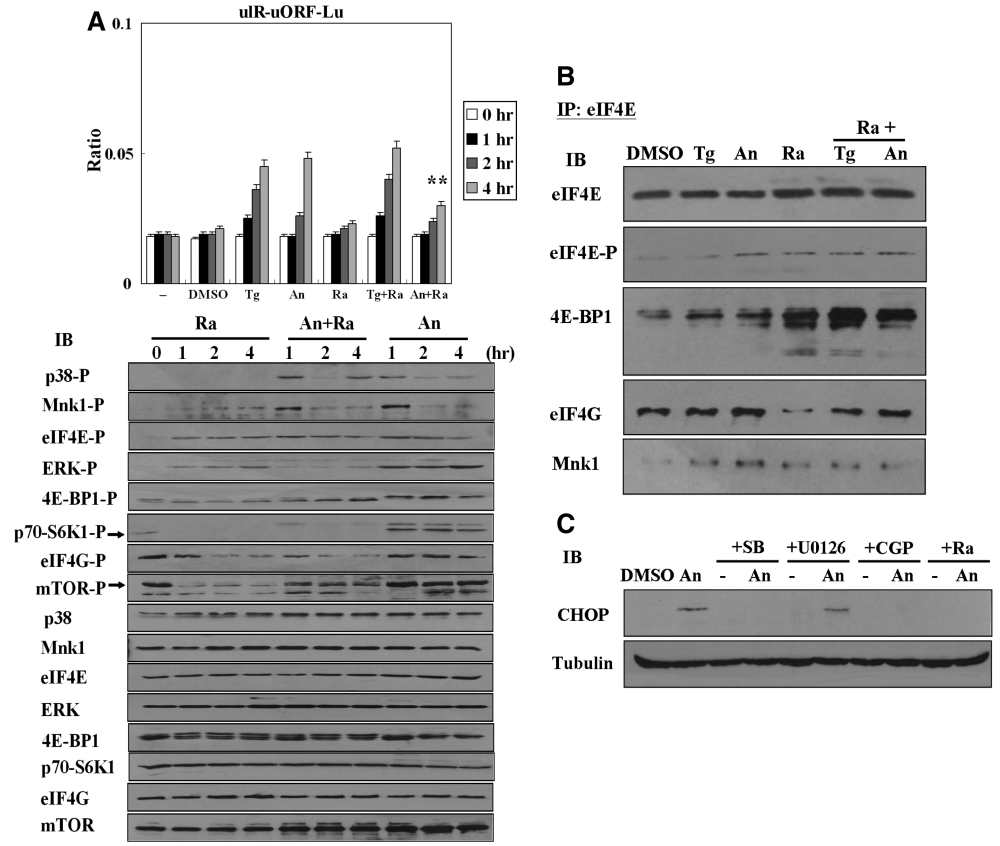

Figure 4. Rapamycin treatment represses anisomycin-, but not thapsigargin-induced uORF^{chop}-driven translation. (**A**) 293T cells were transfected with dual reporter construct (ulR-uORF-Lu) and treated with anisomycin (An, 0.5 μM), thapsigargin (Tg, 1 μM), rapamycin (Ra, 1 μM) or a combination of An + Ra or Tg + Ra. Cell extracts were harvested for Dual-Luciferase Reporter Assay (upper panel, mean ± SD, $P < 0.05$) and western blot analysis (lower panel). The blots were probed with the indicated antibodies. Anisomycin treatment induced both the protein level and the activated form of mTOR (i.e. phosphorylated mTOR/S2481) and its targets, such as eIF4G, S6K1, and 4E-BP1. (**B**) Extracts from cells subjected to drug treatment (1 h) as in (A) were immunoprecipitated with anti-eIF4E antibody followed by western blot analysis. The blots were probed with anti-eIF4E, anti-eIF4E/S209 (eIF4E-P), anti-4E-BP1, anti-eIF4G and anti-Mnk1 antibodies. (**C**) Cells were treated with anisomycin, or with kinase inhibitor targeting either p38, ERK, Mnk or mTOR, in the presence or absence (−) of anysomycin. Cell extracts were harvested for western blot analysis using antibody against CHOP. The asterisk indicates significant increase of this ratio as compared to the control.

absence of drugs, thus indicating a negative role of phosphorylation in such complex formation. Paradoxically, uORF^{chop}-driven luciferase activity did not increase significantly when we over-expressed the eIF4E/S209D mutant in the cells (Figure 2E), raising the possibility that phosphorylated 4E-BP1 may be also involved in regulating reporter expression. To address this issue, we created a variant of 4E-BP1 with a mutation in the TOS motif (F114A), which renders 4E-BP1 unable to interact with and be targeted by mTOR/Raptor (32). By over-expressing 4E-BP1 or its mutant (4E-BP1/F114A), we observed a reduction or even absence of drug-responsive reporter induction (Figure 5B). Furthermore, knock-down of the endogenous 4E-BP1 in the cells by shRNA (Figure 5C, lower panel) led to a more rapid induction of the uORF^{chop}-driven luciferase activity under anisomycin treatment as compared to the control (Figure 5C, upper panel). Additionally, we demonstrated that down-regulation of 4E-BP1 reversed the negative effect of rapamycin on the anisomycin-induced reporter expression, thus implying that the inhibition by rapamycin may be mediated through 4E-BP1 (Figure 5D). Collectively, these data suggest that anisomycin induces a dissociation of the eIF4E-4EBP complex that consequently contributes to the anisomycin-induced reporter expression.

Furthermore, our results also showed that the phosphorylation of eIF4E affects its association with eIF4G (Figure 5E). We next characterized the formation of eIF4F complex under anisomycin treatment. When treated with anisomycin, eIF4E mutant (S209A/T210A) partially lost its interaction with eIF4G, demonstrating that eIF4E phosphorylation may be critical for the formation of eIF4F under drug treatment. In a reverse co-immunoprecipitation experiment, our results further showed that levels of eIF4E, phospho-eIF4E, and Mnk1 immunoprecipitated with Flag-eIF4G after anisomycin treatment were higher than those in the DMSO control or thapsigargin treatment (Figure 5E). Together with the above results, these findings indicate that a phosphorylation-dependent shift in the interacting partners of eIF4E may be a critical determinant in the stimulation of uORF^{chop}-driven translation initiation.

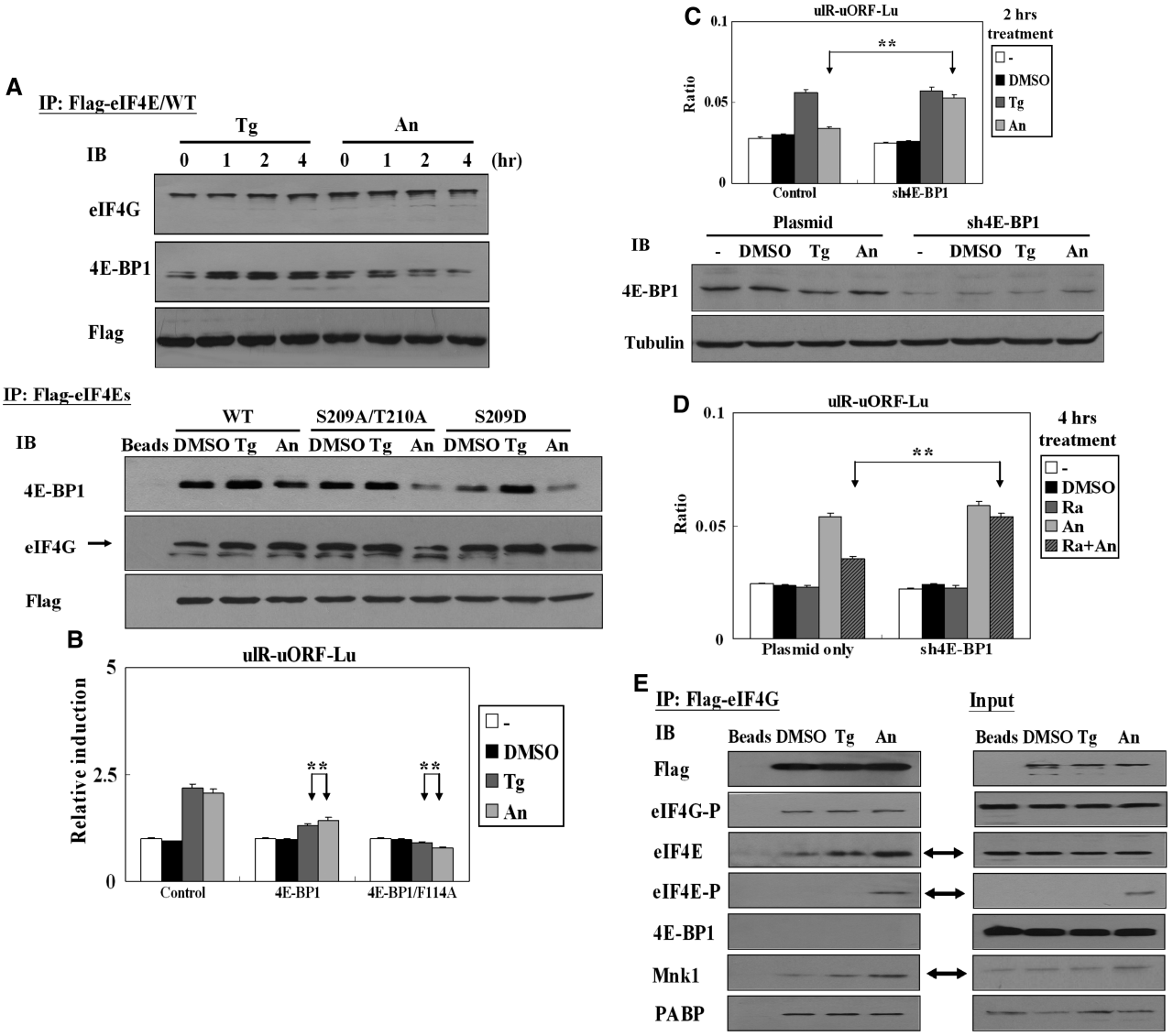

Figure 5. Regulation of uORF^{chop}-driven translation depends on dissociation of eIF4E/4E-BP1 and association of eIF4E/eIF4G. (**A**) 293T cells were transfected with wild-type Flag-eIF4E (WT). After the drug treatment (for 0–4 h, as indicated), cell extracts were immunoprecipitated with anti-Flag antibody followed by western blots with anti-4E-BP1 and anti-Flag antibodies (upper panel). The eIF4E-WT or eIF4E mutants (S209A/T210A and S209D) were transfected into 293T cells. After drug treatment, cell extracts were immunoprecipitated with anti-Flag antibody followed by western blots with anti-4E-BP1, anti-eIF4G and anti-Flag antibodies (lower panel). (**B**) 293T cells were co-transfected with dual reporter construct (ulR-uORF-Lu) and wild-type 4E-BP1 (WT) or mutant 4E-BP1/F114A. Cells were then treated with DMSO, thapsigargin (Tg), or anisomycin (An) for 4 h, and haravested for Dual-Luciferase Reporter Assay (Promega). The relative induction represents the ratio of drug treatment divided by the ratio of mock treatment. (**C**) The dual reporter construct (ulR-uORF-Lu) was co-transfected with vector or sh4E-BP1 into 293T cells. After 16 h, the culture was changed to fresh medium and cultured for 24 h. The culture was again changed to fresh medium culture for additional 1.5 h before being treated with DMSO, thapsigargin (Tg) or anisomycin (An) for 2 h. Cells were harvested for Dual-Luciferase Reporter Assay (upper panel) and western blot analysis (lower panel). (**D**) Transfection was done as in (C) for 16 h, after which transfectants were treated with anisomycin, rapamycin or a combination of An + Ra. Cell extracts were harvested for Dual-Luciferase Reporter Assay (mean ± SD, $P < 0.05$). (**E**) 293T cells were transfected with Flag-eIF4G and subjected to drug treatment as above with DMSO, thapsigargin (Tg), or anisomycin (An) for 2 h. Cell extracts were immunoprecipitated with anti-Flag antibody and probed with the indicated antibodies. The asterisk indicates significant increase of this ratio as compared to the control.

Stress-induced uORF^{chop}-driven translation is fundamentally different from growth factor (i.e. insulin)-induced translation

Non-stress condition, such as insulin treatment, is known to induce the activation of ERK and Mnk1, also leading to phosphorylation of eIF4E/S209 (27). To test whether such similar activation of signaling pathways could also induce uORF^{chop}-driven translation, we performed reporter expression and western blot assays of extracts derived from insulin-treated transfectants. Insulin, when compared to the anisomycin, induced minimal reporter expression (Supplementary Data, Figure S2A, upper panel). However, consistent with previous results, this treatment efficiently induced phosphorylation of ERK

and Mnk1, while phosphorylation of eIF4E/S209 was observed only at 4 h post-treatment. In contrast, insulin failed to induce phosphorylations of both p38 and eIF2α/S51 (Supplementary Data, Figure S2A, lower panel).

It has been shown that MCF-7 cell line contains lower level of eIF2α than other cells (Supplementary Data, Figure S1A, upper panel). When cells over-expressing eIF2α or its phosphorylation-site mutant (S51A) were treated with anisomycin and thapsigargin, the uORF^{chop}-driven FL expression can be restored by over-expressing eIF2α/WT in MCF-7 cells, especially thapsigargin (Supplementary Data, Figure S2B). These results thus suggest that phosphorylated eIF2α/S51 functions in conjunction with phosphorylated eIF4E/S209 for activating uORF^{chop}-driven stress gene expression.

DISCUSSION

In this study, we reported the use of a reporter platform for the analysis of the regulatory mechanisms underlying stress-induced expression of the CHOP protein, particularly at the translation initiation level. Expression of our reporter plasmid, which is under a constitutive CMV promoter and encodes a short half-life of protein (33), bypasses the transcriptional and protein stability control and exhibited a kinetic response that was expectedly faster than that of endogenous CHOP protein under anisomycin treatment. Unexpectedly, a slightly delayed response (i.e. 2–4 h) to anisomycin treatment was observed. Based on our findings, this delayed activation can be explained by a two-layered regulation: dissociation of eIF4E–4E–BP1 complex and formation of eIF4E–eIF4G complex. Results from kinetic analysis showed that anisomycin-induced uORF^{chop}-driven translation was tightly controlled by the dissociation of phosphorylated eIF4E/S209 from 4E-BP1 (Figure 5), which is likely the rate-limiting step in uORF^{chop}-driven translation.

A key finding of our study is that the phosphorylation of both eIF4E/S209 and eIF2α/S51 is crucial for anisomycin-induced uORF-driven CHOP translation. Our results delineated the upstream signaling pathways associated with such translational regulation, and further revealed the distinct signaling consequences between anisomycin and thapsigargin treatments (Figure 6). In sharp contrast to anisomycin, thapsigargin-induced uORF-driven CHOP translation is independent of phosphorylated eIF4E/S209. Intriguingly, although activation of both Mnks and mTOR by anisomycin is required for translation of CHOP, our results showed that mere activation of Mnks and mTOR by insulin-mediated signaling pathway could not fully induce uORF^{chop}-driven translation. The key difference between insulin-induced general cap-dependent translation and anisomycin-induced uORF^{chop}-driven translation lies in the phosphorylation of eIF2α/S51 by anisomycin treatment. Although low concentration of anisomycin is generally regarded as non-ER stress inducing reagent, the kinase cascade involved in anisomycin-induced phosphorylation of eIF2α/S51 may be mediated by

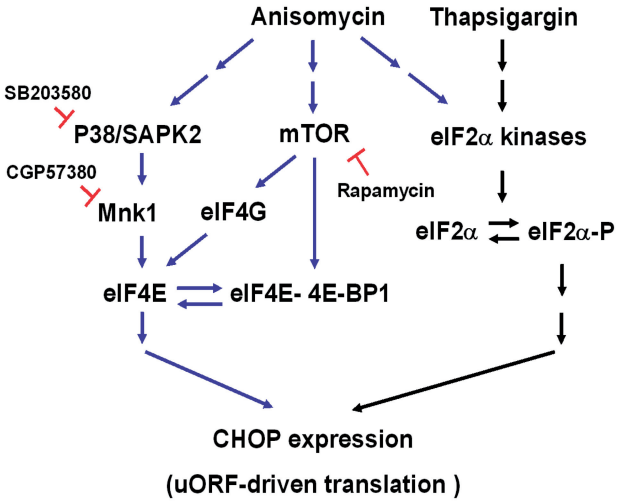

Figure 6. Schematic model depicting the involvement of phosphorylated eIF4E and eIF2α in stress-responsive, uORF-mediated translation regulation. Upon anisomycin treatment, p38, Mnk1 and mTOR signaling pathways converge at eIF4E for the uORF^{chop}-driven translation, with phospho-eIF2α also playing certain role in this regulation. In contrast, under thapsigargin treatment, uORF^{chop}-driven translation depends exclusively on the induced phosphorylation of eIF2α/S51. See text for detailed discussion on the roles of these signaling pathways and their interplay.

anisomycin-activated PKR (as suggested in Ref. 34) as opposed to the thapsigargin-activated PERK. It is well-established that phosphorylated eIF2α/S51 is crucial for translation of some stress-induced proteins, either in a uORF- or uAUG-mediated (cap-dependent) or IRESs-mediated (cap-independent) manner (35). Interestingly, over-expression of eIF2α/S51A could abolish thapsigargin—but decrease only slightly anisomycin-induced uORF^{chop}-driven expression of our reporter construct. Furthermore, while the induction of ATF4 translation correlated with the level of phosphorylated eIF2α/S51 induced by ER stress, our results showed that uORF^{atf4}-driven translation could not be induced by anisomycin (data not shown). These observations suggest that stress-responsive translation of various uORF-driven mRNA may occur in a context-dependent manner and respond differently to stress-activated translation initiation factors. The specific roles of as well as the interplay between the phosphorylated eIF2α/S51 and Mnk-phosphorylated eIF4E/S209 under these conditions are intriguing issues that remain to be addressed.

The uORF^{chop} was thought to promote re-initiation by allowing the terminating ribosome to resume scanning after termination at the uORF stop codon, and it appeared that initiation factor eIF4G must be retained during translation of the uORF for scanning to resume (36). Our present work demonstrated that eIF4E, when phosphorylated at S209 under anisomycin treatment, was preferentially recruited to the eIF4G complex (Figure 5E), further linking phospho-eIF4E to the uORF-driven translation. The most likely scenario for anisomycin-induced formation of this phosphorylated eIF4E/S209-containing eIF4F complex lies in the

mTOR-dependent dissociation of phosphorylated eIF4E/S209-4E-BP1 and subsequent association with eIF4G and eIF3. This postulation is consistent with a previous report on mTOR-dependent stimulation of cap-dependent translation initiation through association of eIF4G and eIF3 under insulin treatment (37). As effective cap-dependent translation initiation requires the interaction between eIF3 and eIF4F (15), our present results thus suggest that interaction between phosphorylated eIF4E/S209-containing eIF4F and eIF3 may be essential also for anisomycin-induced uORF^{chop}-driven translation.

mTOR is known to affect translation initiation through phosphorylating two major targets: the eIF4E binding proteins (4E-BPs) and eIF4G. However, our results showed that treatment with rapamycin failed to completely inhibit the anisomycin-induced activation of mTOR as well as uORF^{chop}-driven reporter expression (Figure 4A). Such outcome may be attributed to the alteration of two signaling pathways. First, the protein level of mTOR was elevated by anisomycin treatment, even in the presence of rapamycin (Figure 4A), signifying up-regulation of mTOR translation or down-regulation of mTOR degradation or both by anisomycin. Both possibilities have important clinical implications as the potential treatment of cancers by rapamycin may be compromised under stress conditions. How anisomycin stimulates mTOR is thus an important issue to be addressed in future studies. Second, perhaps more paradoxically, rapamycin treatment increased both Mnk1 and eIF4E phosphorylation (Figure 4A). These results are consistent with the published data on the increased phosphorylation of eIF4E by rapamycin or by silencing of either mTOR or Raptor, which is likely mediated by a PI3K-dependent and Mnk-dependent mechanism (38). Despite the increased phosphorylation of eIF4E/S209, rapamycin treatment alone did not result in induction of uORF^{chop}-driven reporter expression (Figure 4A). This may be due to the lack of 4E-BP1 phosphorylation as a result of mTOR inhibition, consequently rendering the phospho-eIF4E inactive. Taken together, these findings strengthened the important role of mTOR-associated signaling in the uORF^{chop}-driven translational regulation.

Our present work demonstrated that the Mnk-eIF4E pathway played an essential role in anisomycin-induced translation of CHOP. The eIF4E is a major substrate of Mnk, as indicated by the studies on the Mnk1-Mnk2 double knockout mice, in which no phosphorylated eIF4E was detected in any tissue studied, even after LPS or insulin injection (39). Furthermore, Mnk-mediated phosphorylation of eIF4E/S209 is required for its *in vivo* oncogenic activity (40). While the Mnk-eIF4E signaling pathway is dispensable for normal development in mammals, a number of studies clearly indicate that Mnks and phosphorylated eIF4E/S209 are needed for cellular stress response. Inhibition of eIF4E/S209 phosphorylation by Mnk inhibitor in keratinocytes dramatically decreases the anisomycin-induced protein release of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), IL-1β and IL-6 as well as the IL-1β-induced protein release of TNF-α (41). Such inhibition similarly leads to repressed TNF, TTP and IL6 production in RAW264.7 macrophage cells in response to LPS (42). Shiga toxin 1-induced cytokine expression is also mediated by activation of Mnk1 and phosphorylation of eIF4E/S209 (43). These examples suggest that Mnk activated under cellular stress leads to the phosphorylation of eIF4E/S209 and activation of cytokines and other pro-inflammatory factors production, and further implicate this kinase in adaptive immune response and innate immunity. With regard to the molecular mechanism underlying Mnk's cellular role, ours and the published results have together suggested that phosphorylated eIF4E/S209 may be required for translation of a class of mRNAs under certain stress or growing conditions. Anti-cancer drugs based on inhibitors of PI3K, Akt and mTOR all target at inhibiting the phosphorylation of 4E-BPs (37,38,40). By combining the inhibition of phosphorylation of 4E-BPs and the enhanced expression of CHOP by anisomycin, cancer cells may be readily killed as a result of enhanced apoptosis. Conceivably, one of these inhibitors, when used in combination with anisomycin, may be much more effective anti-cancer drug than a single inhibitor alone.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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