Immunity, Vol. 23, 177–189, August, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.immuni.2005.06.009

The Mnks Are Novel Components in the Control of TNFα Biosynthesis and Phosphorylate and Regulate hnRNP A1

Maria Buxadé,[1,2,6] Josep L. Parra,[2,6] Simon Rousseau,[3] Natalia Shpiro,[4] Rodolfo Marquez,[4] Nick Morrice,[3] Jenny Bain,[5] Enric Espel,[1] and Christopher G. Proud[2,6,*]

[1]Departament de Fisiologia
Universitat de Barcelona
Avinguda Diagonal 645
08028 Barcelona
Spain

[2]Division of Molecular Physiology

[3]Medical Research Council Protein Phosphorylation Unit

[4]Division of Biological Chemistry and Molecular Parasitology

[5]Division of Signal Transduction Therapy
School of Life Sciences
University of Dundee
Dundee DD1 5EH
United Kingdom

Summary

Posttranscriptional regulatory mechanisms control TNFα expression through AU-rich elements in the 3′UTR of its mRNA. This is mediated through Erk and p38 MAP kinase signaling, although the mechanisms involved remain poorly understood. Here, we show that the MAP kinase signal-integrating kinases (Mnks), which are activated by both these pathways, regulate TNFα expression in T cells via the 3′UTR. A selective Mnk inhibitor or siRNA-mediated knockdown of Mnk1 inhibits TNFα production in T cells, whereas Mnk1 overexpression enhances expression of a reporter construct containing the TNFα 3′UTR. We identify ARE binding proteins that are Mnk substrates, such as hnRNP A1, which they phosphorylate at two sites in vitro. hnRNP A1 is phosphorylated in response to T cell activation, and this is blocked by Mnk inhibition. Moreover, Mnk-mediated phosphorylation decreases binding of hnRNP A1 to TNFα-ARE in vitro or TNFα-mRNA in vivo. Therefore, Mnks are novel players in cytokine regulation and potential new targets for anti-inflammatory therapy.

Introduction

Tumor necrosis factor (TNF) α plays a crucial role in controlling inflammatory phenomena, and its expression is therefore tightly regulated. TNFα is primarily produced by activated macrophages, but it is also synthesized by T lymphocytes. Its overproduction by these cells is linked to pathological situations such as superantigen-induced septic shock (Miethke et al., 1992), rheumatoid arthritis, and inflammatory bowel disease (Feldmann et al., 1996).

The synthesis of TNFα is under complex control, and regulation occurs at both the transcriptional and posttranscriptional levels. The 3′ untranslated region (UTR) of the TNFα mRNA has been defined as playing the major role in the posttranscriptional control of TNFα expression (Kruys et al., 1992). Its AU-rich elements (AREs) can control the transport of the TNFα mRNA from the nucleus to the cytoplasm (Dumitru et al., 2000), destabilize the message (Kontoyiannis et al., 1999), and inhibit its translation (Han et al., 1990). Consistent with this, transgenic mice expressing TNFα lacking the ARE overexpress TNFα, leading to chronic inflammatory arthritis and Crohn's-like inflammatory bowel disease (Kontoyiannis et al., 1999).

The regulation conferred by the AREs is mediated through proteins that bind to them, the ARE binding proteins (ARE-BPs), which are essential for the posttranscriptional control of TNFα production (Lai et al., 1999; Piecyk et al., 2000). Much work has been done to characterize these ARE-BPs but, although several proteins have been identified, little is known about how the function of the RNA/protein complexes is modulated. It is well established that the regulation of TNFα production in macrophages and T cells involves both the Erk and p38 MAP kinase pathways. Pyridinyl imidazoles such as SB203580, potent inhibitors of p38 MAP kinase α/β (MAPK), were first discovered as anti-inflammatory drugs through their ability to impair TNFα production. The compound PD098059, an inhibitor of MEK1, also blocks TNFα synthesis (Dumont et al., 1998). In peripheral T cells, TNFα production seems to be more dependent on the Erk pathway than on p38 MAPK signaling. Similarly, it has also been shown that TNFα production by human T cells activated by anti-CD3 and anti-CD28 monoclonal antibodies is inhibited by both PD098059 and SB203580. This again suggests that the Erk and p38 pathways both modulate TNFα expression (Ballester et al., 1998; Buxadé et al., 2001; Hoffmeyer et al., 1999). Thus, these enzymes, or downstream kinases activated by them, are important in controlling, for example, the stability and/or translation of the TNFα mRNA in T cells.

To date, MAP kinase-activated protein kinase-2 (MK-2) is the only kinase that has been shown to be involved in regulating specific messages through direct phosphorylation of proteins bound to the 3′UTR. MK-2 is activated by p38 MAPKα/β and is necessary for LPS-induced TNFα biosynthesis in murine macrophages (Kotlyarov et al., 1999). It has been reported that MK-2 phosphorylates three RNA-BPs that associate with TNFα mRNA, tristetraprolin (TTP) (Chrestensen et al., 2004), HuR (Tran et al., 2003) and heterogeneous nuclear (hn) ribonucleoprotein (RNP) A0 (Rousseau et al., 2002). Since TTP and HuR affect the stability of the TNFα mRNA, they may explain how the p38 MAPK pathway regulates TNFα mRNA stability. However, the signaling connections between the p38 MAPK pathway and the control of the translation of the TNFα mRNA

*Correspondence: cgpr@interchange.ubc.ca

[6]Present Address: Department of Biochemistry and Molecular Biology, University of British Columbia, 2350 Health Sciences Mall, Vancouver, British Columbia, V6T 1Z3, Canada.

still remain unclear. Moreover, since MK-2 is not regulated by the Erk pathway, the mechanism(s) by which Erk controls TNFα synthesis is unknown.

The MAP kinase signal-integrating kinases (Mnks) are phosphorylated and activated by both the Erk1/2 and the p38 MAP kinases α/β (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997, 1999). Mnk1 and Mnk2 are encoded by different genes and show different regulatory properties: Mnk2 has high basal activity while Mnk1 is stimulated markedly by signaling through Erk or p38 MAP kinase (Scheper et al., 2001, 2003). The only well-characterized substrate for the Mnks is eukaryotic initiation factor 4E (eIF4E), which binds the 5′ cap structure of eukaryotic cytoplasmic mRNAs (Gingras et al., 1999) to facilitate cap-dependent translation. Phosphorylation of eIF4E at its physiological site, Ser209, reduces its affinity for the cap structure and may stimulate cap-dependent mRNA translation (Scheper et al., 2002). Mnk1 needs to be bound to the scaffold protein eIF4G to phosphorylate eIF4E (Pyronnet et al., 1999; Waskiewicz et al., 1999). eIF4G binds simultaneously to eIF4E and the poly(A) binding protein, thereby promoting mRNA circularization (Mazumder et al., 2003), thus potentially bringing the Mnks close to proteins bound to the 3′UTR.

Here, we provide several lines of evidence that the Mnks play important roles in the posttranscriptional regulation of TNFα synthesis in T cells by mechanisms that involve its 3′UTR. Overexpression of Mnk1 enhances protein expression from a reporter containing the TNFα 3′UTR, whereas inhibition or knockdown of Mnks blocks synthesis of endogenous TNFα. We identify novel substrates for the Mnks that bind specifically to the ARE of TNFα mRNA. One of these, hnRNP A1, is a known ARE binding protein involved in the posttranscriptional regulation of gene expression in both the nucleus and the cytoplasm. T cell activation results in phosphorylation of hnRNP A1 at the Mnk sites, and decreases its ability to bind the TNFα mRNA in vivo, in a Mnk-dependent manner. The Mnks thus appear to regulate the translation of specific messages such as TNFα by phosphorylating proteins that bind its 3′UTR and are therefore promising novel targets for specific anti-inflammatory therapy. Furthermore, these data show that the Mnks can phosphorylate proteins that bind either end of mRNA and regulate specific mRNAs.

Results and Discussion

Regulation of TNFα Synthesis in Jurkat Cells Involves Signaling through the Erk and p38 MAP Kinase Pathways

To address which signaling pathway(s) are involved in regulating TNFα synthesis in T cells, we used PD98059 (a potent inhibitor of MEK1, the upstream activator of Erk [Dudley et al., 1995]) and SB203580 (inhibits p38 MAP kinases α and β [Cuenda et al., 1995]). To verify their efficacy, we examined the phosphorylation of Erk and the activity of MK-2, a downstream effector of p38 MAP kinase α/β (Shi and Gaestel, 2002). PD98059 substantially blocked activation of Erk, as assessed by its phosphorylation at the T-loop sites (Figure 1A). Anti-CD3/anti-CD28 also activate the p38 MAP kinase pathway and this effect was inhibited by SB203580 (Figure 1B). We consistently observed that SB203580 enhanced Erk phosphorylation. This is consistent with other studies showing that activation of p38α enhances its binding to Erk1/2, blocking phosphorylation by MEK1 (Zhang et al., 2001). When used individually, each compound partially inhibited the marked increase in TNFα production caused by anti-CD3/anti-CD28 (Figure 1C). Only in combination did they completely inhibit TNFα production, indicating that both pathways must be blocked to fully inhibit induction. The finding that SB203580 has a smaller effect than PD98059 may either reflect a lesser role for this pathway in activating TNFα production in T cells or its ability to enhance Erk activity.

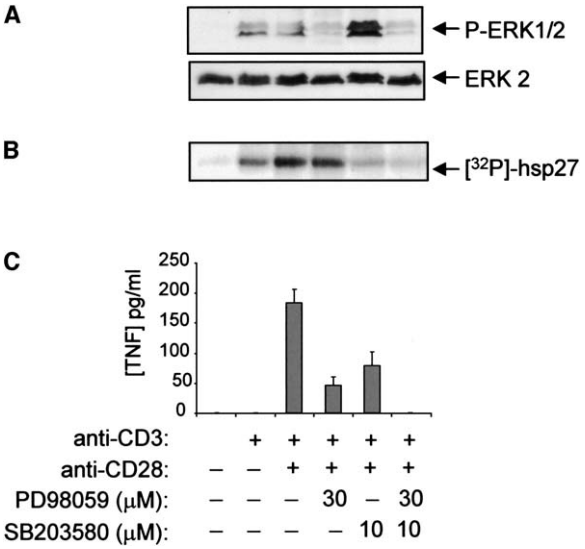

Figure 1. Roles of Erk and p38 MAP Kinase Pathways in TNFα Production in Jurkat Cells

Jurkat cells growing in DMEM plus 10% FCS were treated (where shown) for 1 hr with 30 μM PD98059, 10 μM SB203580 or DMSO (vehicle), and then stimulated with anti-CD3 or anti-CD3/anti-CD28 before harvesting.

(A) 30 min later, the activation states of Erk1/2 were determined by immunoblotting with phospho-Erk antibodies (upper panel). As loading control, the same membrane was reprobed with anti-Erk2 (lower panel).

(B) Activation of p38 MAPK signaling was assayed by an in vitro kinase assay using hsp27 (a substrate for MK-2, which is activated by p38 MAPKα/β), 30 min after stimulation. An autoradiograph of the gel is shown.

(C) TNFα secretion in cell supernatants was measured by ELISA 90 min after stimulation. The data represent the average ± SD of three independent experiments.

These data imply that expression of endogenous TNFα is regulated through signaling events that involve both Erk and p38 MAP kinase. Extensive work has been carried out to define how p38 MAP kinase controls TNFα synthesis in macrophages (Salituro et al., 1999). MK-2 has been implicated in regulating the stability and/or translation of the TNFα mRNA (Neininger et al., 2002). While this enzyme may, at least in part, explain the input from p38 MAP kinase, MK-2 is not activated by Erk and cannot explain its input. As Mnk1 is activated by both these pathways, we asked whether control of TNFα production involved this enzyme.

In Vivo Production of TNFα Is Inhibited by the Mnk Inhibitor

To study the role of the Mnks in the production of endogenous TNFα, we used the Mnk inhibitor 4-amino-3-(*p*-fluorophenylamino)pyrazolo[3,4-*d*]pyrimidine (Knauf et al., 2001; CGP57380). T cell activation markedly increased eIF4E phosphorylation, and this was prevented by CGP57380 in a dose-dependent manner, being almost completely blocked at 40 μM (Figure 2A). Production of TNFα, measured by ELISA in cell supernatants, was also markedly inhibited by this compound in a dose-dependent manner (Figure 2B), consistent with direct involvement of the Mnks in regulating TNFα synthesis in vivo. Importantly, CGP57380 had no effect on the phosphorylation of Erk or hsp27, a specific substrate for MK-2, which is activated by p38 MAP kinases α/β (Figure 2C), indicating that CGP57380 does not interfere with activation or activity of the Erk pathway, p38 MAP kinases α/β or MK-2.

To explore further the specificity of CGP57380, we assessed whether it inhibited a range of other relevant protein kinases that are related to the Mnks (e.g., RSK1 or MSK1) involved in pathways that regulate the Mnks (ERK2, p38 MAPKα, β, γ, and δ, and MKK1) or activated by MAP kinases (PRAK, MK2). In vitro, CGP57380 did not inhibit p38 MAP kinase β at all, and the IC_{50} for MK2, p38 γ and δ, MSK1, and PRAK was about 70–100 times higher than the IC_{50} against Mnk1 or Mnk2. CGP57380 showed slight inhibitory action against MKK1, ERK2, RSK1, and p38 MAPKα but only at concentrations 25–40 times higher than those that inhibit Mnk1/2 (Table S1 in the Supplemental Data available with this article online). Therefore, the effects of CGP57380 on TNFα synthesis are not due to inhibition of either of the upstream signaling pathways or of other related protein kinases.

It was possible that inhibition of the Mnks, and thus dephosphorylation of eIF4E, might simply impair total protein synthesis and thereby block TNFα synthesis. To assess this, we measured [^{35}S]methionine incorporation in cells stimulated with anti-CD3/anti-CD28 plus increasing amounts of CGP57380. Although at 40 μM, CGP57380 completely blocked eIF4E phosphorylation and inhibited TNFα production up to 75%, total protein synthesis was only slightly inhibited (by <20%; Figure 2D). RNAse Protection Assay (RPA) analysis was performed to assess whether CGP57380 affected the levels of the TNFα mRNA. The data (Figures 2E and 2F) suggest there may be a modest decrease (25%, when normalized to the GAPDH control) but that this is much smaller than the marked effect on overall TNFα production (75% inhibition). Thus, although Mnk inhibition may conceivably affect both the stability and translation of the TNFα mRNA, it seems that the Mnks regulate TNFα production mainly by modulating its translational efficiency. Therefore, it was possible that Mnk1 and MK-2 cooperate to regulate TNFα production posttranscriptionally. Consistent with this, the partial effect of SB203580 (which would block MK-2 activation) was substantially increased by cotreatment with CGP57380 (data not shown).

Although CGP57380 clearly does not impair the signaling pathways that lead to activation of Erk or p38 MAP kinase/MK-2, we wanted to rule out the possibility that it might exert its effects by interfering with other signaling events. We therefore tested a closely related compound (SHN-093) in which a single hydrogen is replaced by a methyl group (Figure 3A). As shown in Figures 3B and 3C, SHN-093 does not inhibit Mnk activity in vitro or within cells. It also fails to block TNFα production by Jurkat cells (Figure 3D). Because it seems most unlikely that the single substitution would result in loss of activity both against the Mnks and against any hypothetical second relevant target, these data reinforce the evidence that the inhibition of TNFα production by CGP57380 reflects blockade of Mnk function. SHN-093 may be a useful negative control for CGP57380.

Because CGP57380 inhibits Mnk1 and Mnk2, we cannot distinguish from the data in Figure 2 which is involved in regulating TNFα production. However, while Mnk2 has high basal activity that is quite resistant to upstream signaling blockade (Scheper et al., 2001), Mnk1 has low basal activity that is stimulated via either the Erk or p38 MAP kinase signaling pathways that are activated upon T cell stimulation (Wang et al., 1998). Mnk1 is thus a better candidate than Mnk2 for mediating the effects of activation of these pathways on TNFα expression. To explore further the possible role of Mnk1 in TNFα biosynthesis, we exploited small inhibitory RNA-mediated interference (siRNA) to decrease Mnk1 levels in Jurkat T cells and assessed its effect on TNFα output. O'Loghlen et al. (2004) have previously shown that in HEK293T cells, siRNA is effective in decreasing Mnk1 levels and impairing eIF4E phosphorylation. Here, we used the same siRNAs. Cells were either "mock" transfected, transfected with a negative control pair of oligonucleotides (siRNA-NS), or with a pair designed to target Mnk1 (siRNA-M3). At intervals of 24, 48, 72, or 96 hr after transfection, cells were either lysed and analysed for Mnk1 or actin (loading control) by Western blot (Figure 3E, lower part) or challenged with anti-CD3/anti-CD28 for 2.5 hr, after which TNFα production was assessed by ELISA (Figure 3E, upper part). Treatment of cells with siRNA-M3 reduced Mnk1 levels significantly at all times tested, although the effect was partial. Using the same oligonucleotides, O'Loghlen et al. (2004) achieved greater reduction of Mnk1 expression: possible reasons for the partial effect seen here include lower transfection levels with the Jurkat cells and, perhaps, lower efficiency of siRNA in these cells (this varies greatly between cell types). No change in Mnk1 levels was seen in mock-transfected cells or in ones that received siRNA-NS (Figure 3E). Strikingly, TNFα production was markedly and reproducibly impaired in the Mnk1-siRNA treated cells, relative to the two sets of controls, confirming further a role for Mnk1 in TNFα production. The partial inhibition of TNFα synthesis likely reflects the partial nature of Mnk1 knockdown, but could also be due to roles for Mnk2.

Expression of Mnk1 Enhances Translation of a Reporter mRNA Containing the TNFα 3′UTR

It is hard to explain the effect of Mnk1 on translation of a specific mRNA through its ability to phosphorylate the known substrate, eIF4E, as it binds all cytoplasmic mRNAs. We therefore wondered whether the Mnks might exert an effect via the regulatory region of TNFα

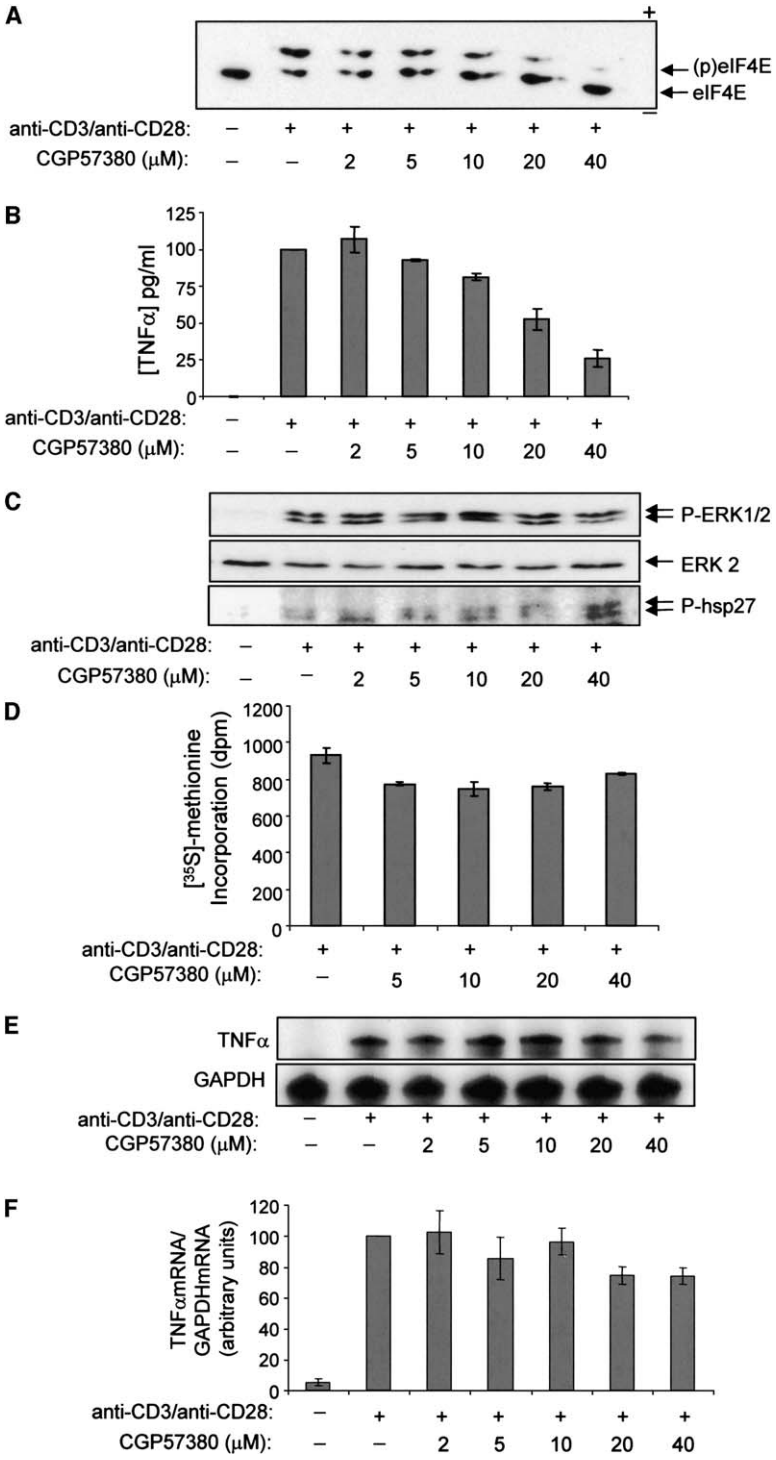

Figure 2. Mnk Inhibition Blocks the In Vivo Production of TNFα

Jurkat cells growing in DMEM 10% FCS were pretreated for 1 hr with increasing concentrations of CGP57380 or DMSO (vehicle) and then stimulated with anti-CD3 plus anti-CD28 before harvesting.

(A) eIF4E phosphorylation was analyzed by isoelectric focusing (IEF) and immunoblotting with anti-eIF4E 30 min after stimulation. Positions of phospho- and dephospho-eIF4E are shown. One representative experiment from a total of three is shown.

(B) TNFα secretion was determined by ELISA in cell supernatants 90 min after stimulation. The data represent the average ± SD of three independent experiments.

(C) Phosphorylation states of Erk1/2 and hsp27 were examined by immunoblotting with phosphospecific antibodies 30 min after stimulation. Anti-Erk2 was used for loading controls.

(D) 30 min after stimulation, ^{35}S-methionine was added and the cells were incubated for a further 90 min. Cells were then extracted and samples processed to measure incorporation of label into trichloroacetic acid-precipitable material. Incorporation was normalized to the protein content of each sample. The mean ± SD of two independent experiments assayed in triplicate is shown.

(E) Endogenous mRNA levels for TNFα and GAPDH were measured by RPA. Only the parts of the urea-denaturing gel containing the protected TNFα and GAPDH probes are shown. A representative experiment from three performed is shown.

(F) Radioactivity in protected bands in (E) was quantified by phosphorimager analysis. The ratio between the signals for the bands corresponding to TNFα and GAPDH mRNAs was calculated. The data represent the average ± SD of three independent experiments.

mRNA, which lies within the 3′UTR. To eliminate the effects of T cell activation on transcription and splicing, it was preferable to use a transiently transfected reporter vector containing the regulatory elements of the TNFα mRNA (or appropriate negative controls) rather than looking at the endogenous mRNA.

To validate the use of GFP reporter constructs to study the role of the TNFα 3′UTR in its posttranscriptional regulation, we checked expression levels of both reporter constructs and their ability to respond to T cell activation. GFP expression was 15-fold higher in cells transfected with a vector encoding GFP fused to the globin 3′UTR (glob-GFP-glob) than in cells that received the vector for GFP fused to the TNFα 3′UTR (glob-GFP-tnf) (Figures S1A–S1C); at the transcript level, the former construct gave 2.5-fold higher mRNA

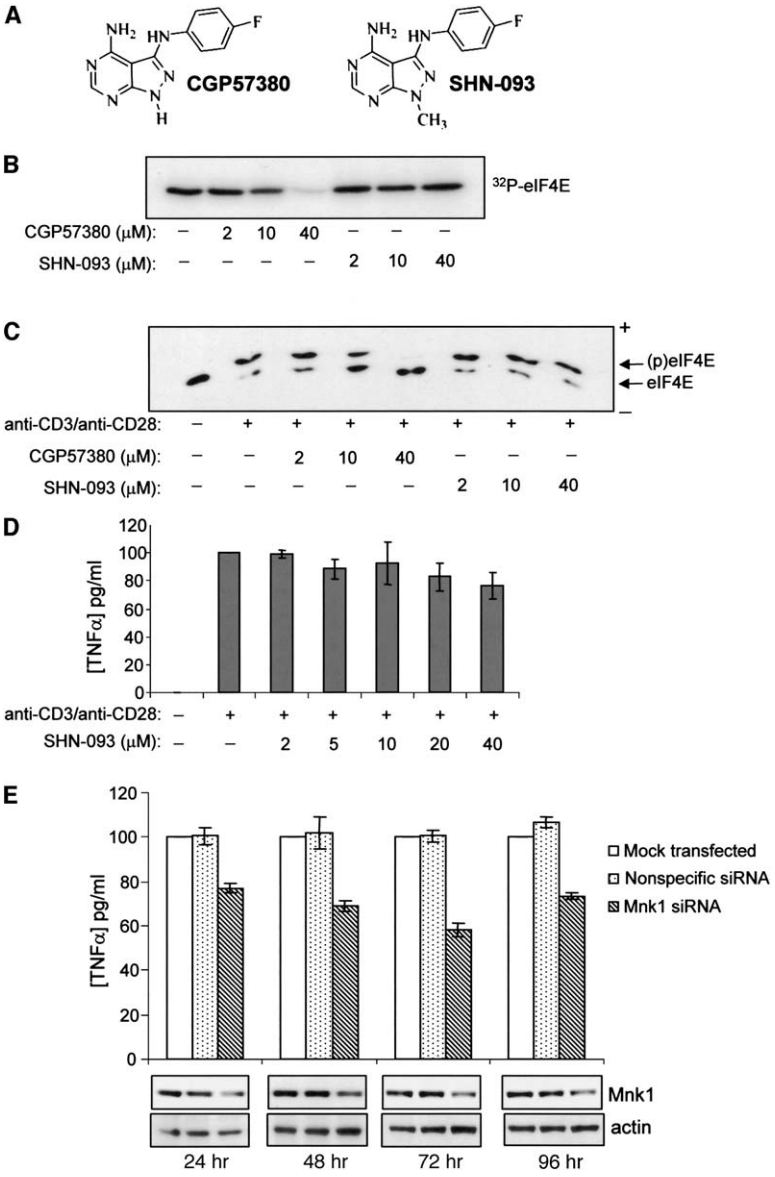

Figure 3. SHN-093 Does Not Inhibit the Mnks or TNFα Production

(A) Structures of CGP57380 and SHN093.

(B) The activity of recombinant Mnk1 was tested in an in vitro kinase assay using recombinant eIF4E in the presence of CGP57380 or SHN-093. The part of the autoradiogram showing labeled eIF4E is shown.

(C) Jurkat cells growing in DMEM plus 10% FCS were pretreated for 1 hr with increasing concentrations of CGP57380 or SHN-093 and then stimulated with anti-CD3 plus anti-CD28 for 30min. Cell lysates were prepared, and the state of eIF4E phosphorylation was determined.

(D) TNFα secretion was analysed by ELISA in cell supernatants 90 min after stimulation. The data represent the average ± SD of three independent experiments.

(E) Jurkat cells were transfected with the indicated siRNAs as described in Supplementary Material. At 24, 48, 72, and 96 hr after transfection, cells were stimulated with anti-CD3/anti-CD28 for 2.5 hr and the levels of TNFα secretion determined by ELISA (± SD, n = 3). The data represent the average ± SD of three independent experiments. The same cells were analyzed for Mnk1 levels by immunoblotting (upper panels). As loading control, membranes were probed with anti-actin (lower panels).

expression (Figures S1D and S1E). These data thus indicate that the 3′UTR of the TNFα message negatively modulates mRNA stability and translation in T cells. We next tested the effect of T cell activation on expression of the constructs. While glob-GFP-tnf responded to TCR and CD28 engagement, the control construct did not. GFP expression from the glob-GFP-glob mRNA was almost unchanged after stimulation, while the level of GFP derived from the glob-GFP-tnf mRNA rose quickly and markedly after treatment with anti-CD3/anti-CD28, showing a 12-fold increase at the level of protein (Figure S2A) and a 4-fold increase in transcript levels (Figures S2B and S2C). Because both reporters are driven by the same promoter and lack introns, the differences cannot be due to changes in transcription or splicing and likely reflect modulation of transcript stability and/or translation after T cell activation. Because endogenous TNFα induction is regulated through signaling events that require both the Erk and the p38 MAP kinase pathways, we tested whether these signaling events regulated the reporter containing the 3′UTR of the TNFα mRNA. We observed that, in combination, PD98059 and SB203580 almost completely blocked the enhanced expression of GFP from the glob-GFP-tnf reporter at both protein (Figure S3A) and transcript levels (Figures S3B and S3C). It therefore appears that, in T cells, these pathways regulate both the translation of the glob-GFP-tnf reporter mRNA and its level of expression (presumably via changes in mRNA stability). These effects could be exerted through a common target of the Erk and p38 MAP kinase pathways such as Mnk1.

To study further the role of Mnk1 in regulating TNFα synthesis, we cotransfected Jurkat cells with our reporter constructs and a vector encoding wild-type (WT) Mnk1 or the corresponding empty vector. We also used the T2A2 mutant of Mnk1, in which Thr197 and 202 in

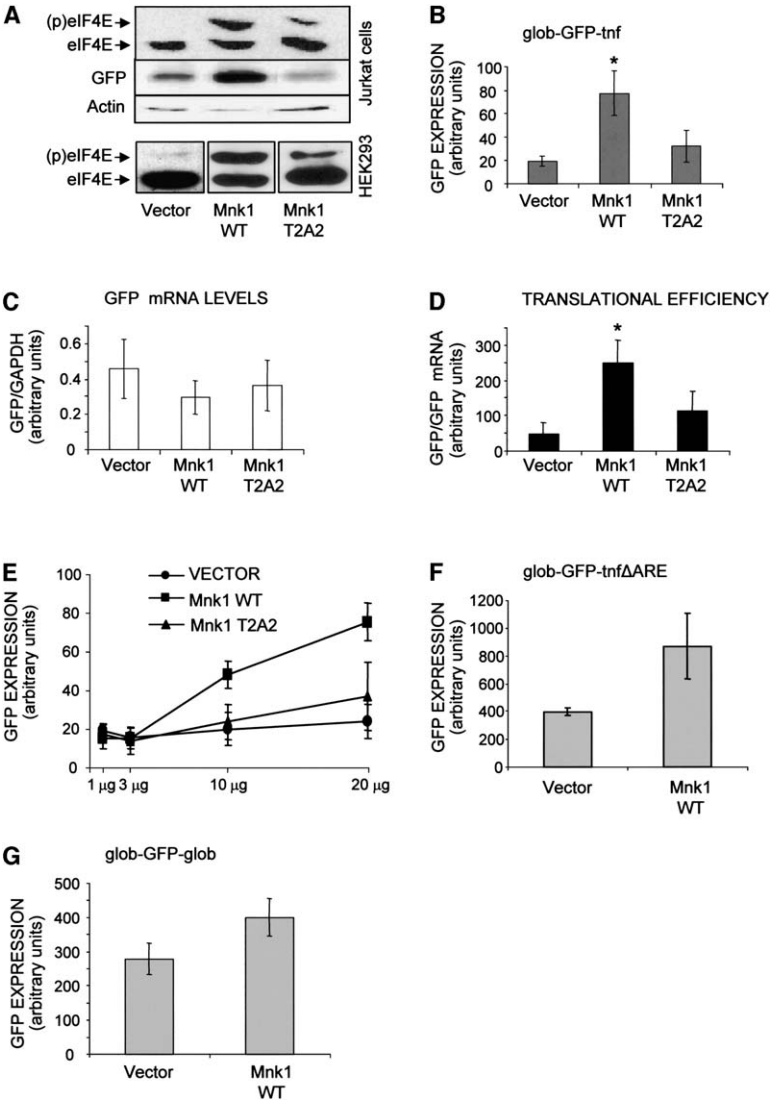

Figure 4. Mnk1 Increases the Expression of GFP from the glob-GFP-tnf mRNA

Jurkat cells were transfected with 10 μg of glob-GFP-tnf and 20 μg of Mnk1 constructs coding wild-type Mnk1 (Mnk1 WT), a low activity form (Mnk1 T2A2) or with empty vector (Vector). 24 hr after transfection, cells were harvested to analyze eIF4E phosphorylation and GFP expression.

(A) Upper section: levels of phosphorylation of endogenous eIF4E in transfected cells were determined as described in Figure 2. Levels of GFP in transfected cells was determined by immunoblotting of total cell lysates with anti-GFP. To assess the loading, the Western blot was reprobed with anti-actin. Lower section: HEK293 cells were transfected with the same plasmids and phosphorylation of endogenous eIF4E was determined as for the Jurkat cells.

(B) GFP fluorescence of transfected cells. GFP expression is calculated as the median fluorescence intensity of the cell population. The data represent the average ± SD of five independent experiments, each performed in duplicate.

(C) Total RNA was extracted from aliquots of the same cells to analyze specifically the levels of GFP and GAPDH mRNAs by RNase protection. The amount of radioactivity in protected bands was determined by phosphorimager analysis and is shown as GFP/GAPDH ratio. The average ± SD of three independent transfections is presented.

(D) Translational efficiency (GFP protein)/(GFP mRNA) was calculated for samples represented in (B) and (C). The figure shows the mean ± SD of the ratio [(GFP protein)/(GFP mRNA)] from three independent experiments shown in (B) and (C).

(E) Jurkat cells were transfected with 10 μg of glob-GFP-tnf and increasing amounts of the Mnk1 constructs or empty vector. 24 hr after transfection, cells were harvested to analyse GFP expression by FACS. The mean ± SD of three independent transfections, each with duplicate determinations, is shown.

(F and G) Jurkat cells were transfected with 10μg of glob-GFP-tnfΔARE (F) or glob-GFP-glob (G) and 20μg of wild-type Mnk1 (Mnk1 WT) or empty vector (Vector). 24 hr after transfection, GFP expression in live cells was determined by FACS analysis. The mean ± SD of three independent transfections is shown. The asterisk represents data significantly differing from "vector" values ($p < 0.01$, Student's t test).

the T-loop have been mutated to alanines. This mutant does not undergo autophosphorylation and has much lower activity against eIF4E than WT Mnk1 (Waskiewicz et al., 1999). As expected, transfection of cells with WT Mnk1 resulted in a marked increase in eIF4E phosphorylation (Figure 4A, top section), demonstrating that the overexpressed Mnk1 is active in these cells. The T2A2 mutant caused only a small increase in eIF4E phosphorylation either in Jurkat or in HEK293 cells (Figure 4A, lower panel). Thus, although it has previously been described as inactive (Waskiewicz et al., 1997), this T loop mutant actually shows low residual activity.

Expression of WT Mnk1 markedly increased the expression of GFP encoded by glob-GFP-tnf, whereas the T2A2 mutant had no obvious effect (Figure 4A). We also quantified GFP expression by FACS analysis of the cells (Figure 4B). Again, it is clear that WT Mnk1 strongly increases GFP expression, whereas Mnk1[T2A2] exerted only a slight stimulatory effect. Thus, Mnk1 can increase the expression of a protein encoded by an mRNA containing the TNFα 3′UTR.

In principle, Mnk1 could enhance GFP expression from the glob-GFP-tnf reporter either by enhancing the stability of the mRNA or its translation. In fact, expression of WT Mnk1, if anything, slightly repressed the level of the glob-GFP-tnf reporter mRNA, although this effect was not statistically significant ($p = 0.22$, Figure 4C). A modest trend towards decreased transcript levels was also seen in cells expressing Mnk1[T2A2]. This indicated that the stimulatory effect of Mnk1 on GFP expression is not due to increased mRNA levels. To assess its effects on the translation of the mRNA, we calculated the translational efficiency: GFP protein levels/transcript levels (Han et al., 1990). Expression of WT Mnk1 greatly enhanced this ratio, while expression of Mnk1[T2A2] had a smaller stimulatory effect (Figure 4D).

To study further the relationship between Mnk1 and

GFP expression, we examined the effect of increasing amounts of Mnk1 or Mnk1[T2A2]. As the amount of Mnk1 DNA used in the transfection was increased, so the level of GFP expression also rose (Figure 4E). There may be a threshold effect here, as low levels of DNA (1 or 3 μg) had no detectable effect on GFP expression while larger amounts elicited a marked increase. Taken together, these data provide evidence for direct involvement of Mnks in the translation of a specific mRNA, i.e., one containing the TNFα 3′UTR.

To test whether the Mnks were targeting the AU-rich element within the TNFα 3′UTR, we used a reporter construct containing the 3′UTR of TNFα from which the ARE was deleted (glob-GFP-tnfΔARE). As expected, since the ARE is important for destabilizing and translationally repressing the mRNA, the basal level of GFP expression from the ΔARE construct was much higher than for the one containing WT TNFα 3′UTR (compare Figures 4F and 4B). Although transfection with Mnk1 did increase the expression of GFP from this construct, the effect was only half that seen with the full-length 3′UTR, showing that the ARE is required for the full effect of the Mnks. The fact that Mnk1 still enhanced GFP expression from the ΔARE construct likely indicates that other features of the 3′UTR are also involved in its regulation, e.g. hnRNP A1 could also be binding outside the ARE. To test this, we analysed the complexes formed between hnRNP A1 and TNF mRNA by UV crosslinking (data not shown). The experiment showed that hnRNP A1 protects two fragments of the TNFα mRNA from digestion with RNase T1, which might represent the ARE and an additional region. This suggests that focusing on the ARE might overlook other regions in the 3′UTR or elsewhere in the mRNA that are also important in the regulation of translation of the TNFα mRNA. After all, while the minimal AU-rich sequence necessary to confer instability has been identified, the features determining the translational rate of an mRNA have not yet been fully characterized.

To test further the specificity of this effect, we used the glob-GFP-glob reporter since this 3′UTR lacks any relevant regulatory elements. Although transfection with Mnk1 did slightly increase expression of GFP from this construct, the effect barely reached significance (Figure 4G) and was much smaller than the four-fold increase seen for GFP expression from the glob-GFP-tnf reporter ($p < 0.01$). This small increase might just reflect a role for the Mnks in general protein synthesis. Because inhibition of the Mnks slightly decreases total protein synthesis (Figure 2D), one would expect their overexpression to increase general mRNA translation, including the glob-GFP-glob construct.

Given this, and the data in Figure 2D, it is hard to explain the effect of Mnk1 on the translation of mRNAs containing the TNFα 3′UTR through changes in the phosphorylation of eIF4E, which binds all cytoplasmic mRNAs. The effects of Mnk1 expression or inhibition are more likely to reflect its action on specific target proteins, e.g., ones that bind specifically the 3′UTR of the TNFα mRNA. We therefore tested whether the Mnks could phosphorylate ARE binding proteins (ARE-BPs), substrates that associate specifically with such mRNAs and may regulate their translation (Figure 5A).

Mnks Phosphorylate Proteins that Bind to the 3′UTR of the TNFα mRNA

To do this, we used a biotinylated oligoribonucleotide consisting of the ARE of TNFα to pull down ARE-BPs from Jurkat cell lysates. We then tested whether they could be phosphorylated by the Mnks. The same several polypeptides became phosphorylated in this assay using either Mnk1 (data not shown) or Mnk2 (Figure 5B). No labeling was observed when the bound proteins were incubated with p38 MAP kinase plus SB203580 at the concentration used to block any "spurious phosphorylation" due to this enzyme, which is used to activate the Mnks. Thus, these proteins are phosphorylated by the Mnks, not by any residual contaminating p38 MAP kinase. Moreover, in separate experiments (not shown), we confirmed that hnRNP A1 is not a substrate for p38 MAP kinase.

The labeled polypeptides were then identified by (tryptic) peptide mass fingerprinting. All three are hnRNP proteins, i.e., hnRNPs A0, A1 and JKT BP. The first two are already known to bind to AREs (Rousseau et al., 2002; Hamilton et al., 1997). To further confirm the binding specificity of these hnRNPs, we tested by Western blotting for the presence of hnRNP A1 and hnRNPA0 in a pull down with an oligo RNA encoding either for the TNFα ARE or an irrelevant RNA (Figure 5C). Both hnRNPs were found to bind only to the ARE-containing oligo RNA. Because hnRNP A0 is known to be phosphorylated by MK-2 (Rousseau et al., 2002), we decided to focus on hnRNP A1, which plays important roles in RNA metabolism (Hamilton et al., 1997). To confirm that hnRNP A1 is indeed an Mnk1 substrate we expressed it in *E. coli* and incubated it with Mnk1 (Figure 5D). Mnk1 clearly phosphorylates recombinant hnRNP A1 in vitro, although slightly less efficiently than eIF4E, a positive control.

Identification of the Sites within hnRNP A1 Phosphorylated by Mnk1

To further characterize hnRNP A1 as a substrate for the Mnks, it was important to identify the sites phosphorylated by the Mnks, as a necessary step to determining whether hnRNP A1 is phosphorylated in vivo at these residues by the Mnks.

Recombinant hnRNP A1 made in *E. coli* was phosphorylated by Mnk1 in vitro, and then digested with trypsin. The tryptic peptides were separated by chromatography by reverse-phase HPLC on a C_{18} column. Two major ^{32}P-labeled species were observed, that eluted close together on the HPLC (Figure 5E) and also migrated similarly on the two-dimensional map (Figure 5F). The masses of the peptides (m/z = 1774.67 and m/z = 1277.48) corresponded to that of monophosphorylated forms of the tryptic peptides comprising residues 184-194 (QEMASASSSQR) and 301-318 (NQGGYGGSSSSSYGSGR). This was confirmed by Edman sequencing, which identified Ser192 as the site of phosphorylation in one peptide ("b" in Figure 5F and Figure 6), and Ser310, Ser311 and Ser312 in the second peptide (peptide "a"). The ms/ms spectrum for the second peptide did not contain any species where Ser313 was phosphorylated, and thus the ^{32}P at this position in the cycle burst is due to trailing from Ser312. The mass spectrometry showed that the peptide containing Ser310/1/2 was a

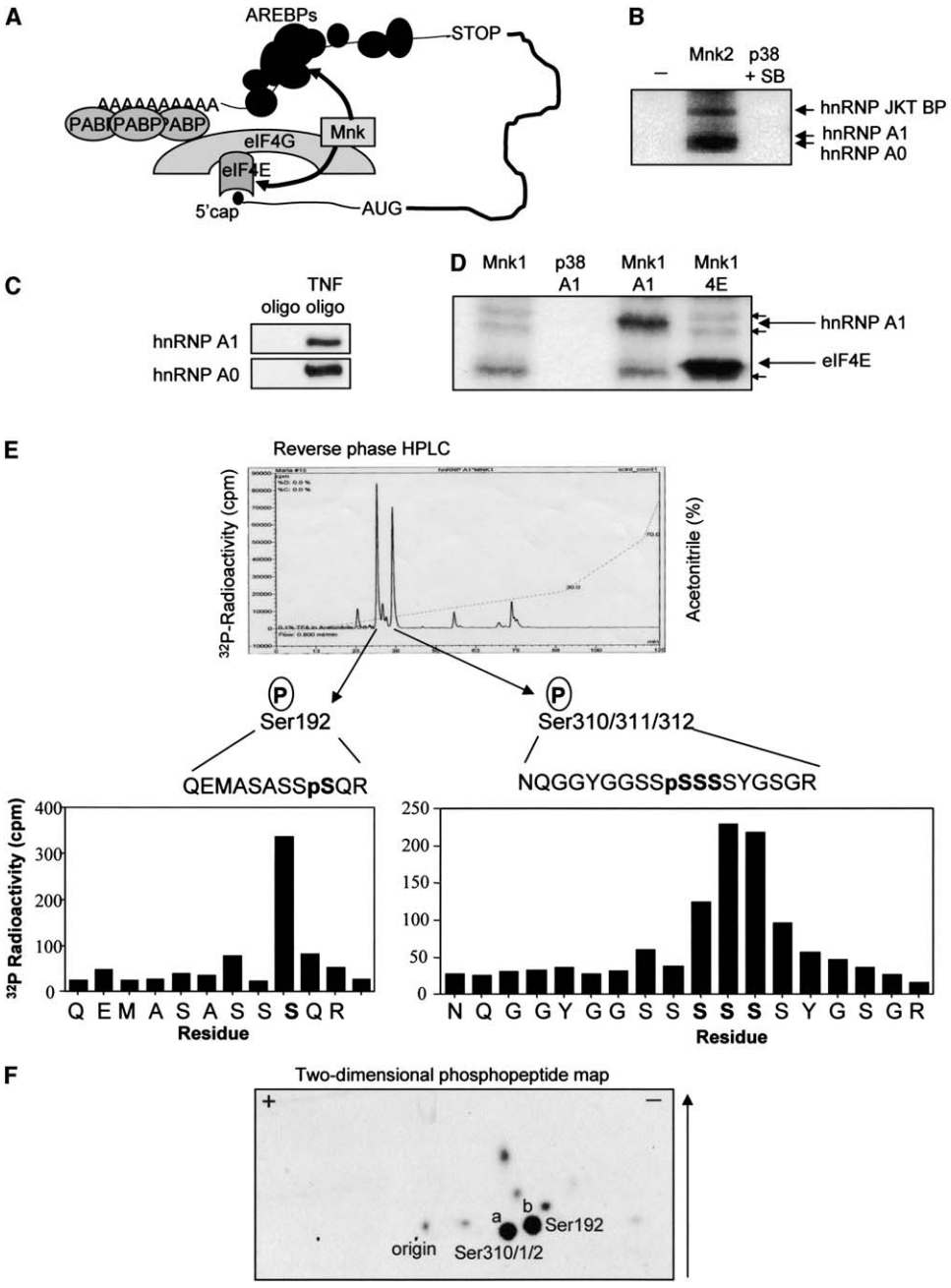

Figure 5. Mnks Phosphorylate Proteins that Bind to the 3′UTR of the TNFα mRNA In Vitro

(A) Schematic diagram of recruitment of Mnks to the eIF4F complex bound to the 5′ cap of an mRNA. Mnks bind the C terminus of eIF4G, a scaffold protein that also binds eIF4E and PABP thereby potentially circularizing the mRNA. Arrows indicate phosphorylation of eIF4E by the Mnks and putative in vivo phosphorylation of proteins bound to the ARE of the TNFα mRNA.

(B) ARE-BPs immunoprecipitated with an oligoribonucleotide corresponding to the ARE of the TNFα mRNA were incubated in the presence/absence of activated Mnk2 and [γ-^{32}P]ATP. Proteins were separated by SDS-PAGE and analyzed by autoradiography. Labeled bands were identified by tryptic mass fingerprint. As negative control, the p38 MAPK used to activate Mnk2 was tested in the presence of SB203580 (this compound is always present in the Mnk assays to inhibit any residual p38 MAPK).

(C) Western blot for hnRNP A0 or hnRNP A1 from pull-downs performed with either an irrelevant oligo or one containing the TNFα ARE.

(D) An in vitro kinase assay was performed with Mnk1 in the presence of hnRNP A1 or eIF4E. As a control, the p38MAPK was also used in the presence of SB203580. Arrowheads indicate bands derived from the recombinant Mnk1 used.

(E) Purified hnRNP A1 was phosphorylated in vitro by activated Mnk1 and subjected to SDS-PAGE. The band corresponding to hnRNP A1 was excised, digested with trypsin and the resulting peptides were separated by reverse-phase chromatography on a C18 column equilibrated in 0.1% trifluoroacetic acid and developed with acetonitrile. ^{32}P-radioactivity is shown by the solid line and the acetonitrile gradient by the broken line. The masses of the two major tryptic phosphopeptides were obtained by MALDI-TOF. The sequence of the peptides and the sites of phosphorylation were identified by solid-phase sequencing.

(F) Two-dimensional tryptic phosphopeptide map of hnRNP A1 phosphorylated in vitro by Mnk1. The positions of the origin and the peptides "a" and "b" containing the identified phosphorylation sites are indicated. The direction of chromatography (vertical arrow) and polarity of electrophoresis (+/–) are shown.

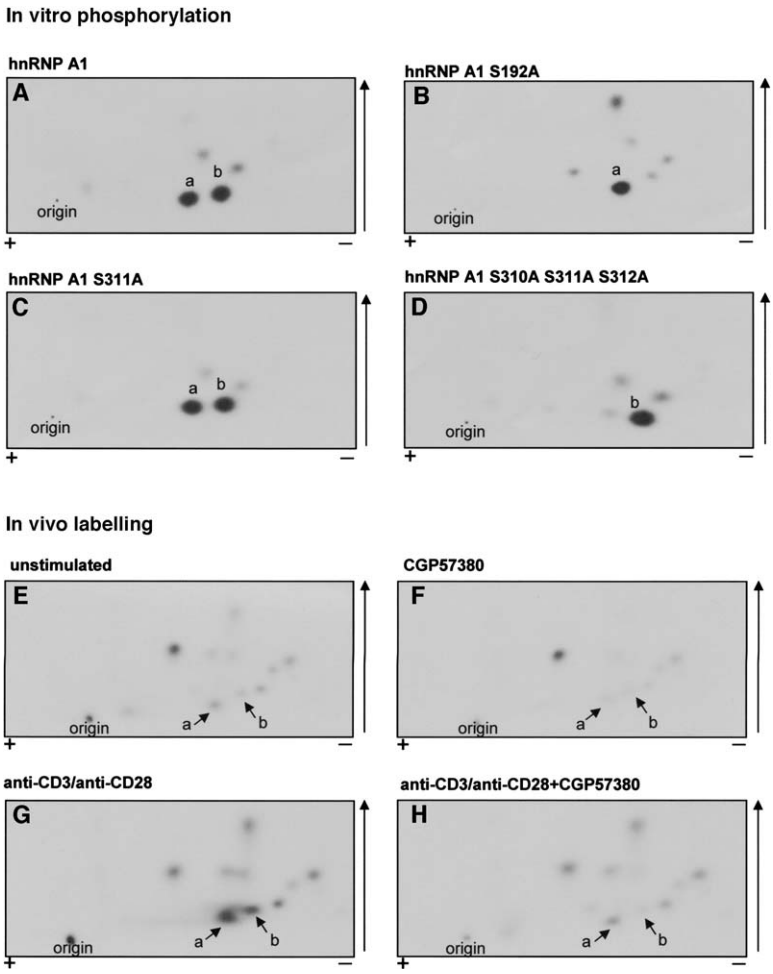

Figure 6. Analysis of Phosphorylation of Recombinant and Endogenous hnRNP A1

(A–D) Two dimensional analysis of tryptic peptides from bacterially expressed hnRNP A1 wild-type (A), hnRNP A1 S192A (B), hnRNP A1 S311A (C) and hnRNP A1 S310A S311A S312A (D), phosphorylated in vitro by Mnk1. The peptides "a" and "b" containing the Mnk phosphorylation sites identified in vitro are indicated.

(E–H) Endogenous hnRNP A1 was immunoprecipitated from metabolically labeled Jurkat cells, digested with trypsin, and the peptides were resolved on two-dimensional maps. Maps correspond to hnRNP A1 from unstimulated Jurkat cells (E), cells incubated with CGP57380 for 45 min (CGP57380, F), stimulated with anti-CD3/anti-CD28 for 30 min (anti-CD3/anti-CD28, G), or pretreated with CGP57380 prior to stimulation (anti-CD3/anti-CD28+CGP57380, H). Peptides "a" and "b" containing the Mnk phosphorylation sites identified in vitro are indicated by labeled arrows.

mixture of monophosphorylated peptides and did not contain any bis- or tris-phosphorylated forms, suggesting that, although Mnk can phosphorylate more than one residue in this peptide, it phosphorylates only one at a time. hnRNP A1 mutants in which all four serines were changed to alanines could not be phosphorylated in vitro by Mnk1 and allowed us to determine which spot in the 2D map corresponded to which of the phosphopeptides "a" and "b" (Figures 6A–6D).

The sequences flanking the phosphorylation sites identified in hnRNP A1 do not resemble those around the Mnk-site in eIF4E (Ser209). Because the Mnks can only phosphorylate eIF4E when it is properly folded and not when it is denatured, we believe that the Mnks recognize a higher order structure not primary sequence.

Phosphorylation of hnRNP A1 In Vivo at Mnk1 Sites

To determine whether hnRNP A1 is phosphorylated in vivo at the sites identified in vitro as targets for Mnk1, Jurkat cells were metabolically labeled with ^{32}P-orthophosphate, and hnRNP A1 was immunoprecipitated from the cell lysates. The antibody used to immunoprecipitate hnRNP A1 binds to the protein whether or not it is associated with hnRNP complexes. In vivo phosphorylation of hnRNP A1 was examined in stimulated cells in the presence or absence of the Mnk inhibitor. After tryptic digestion of the immunoprecipitated hnRNP A1, peptides were separated on two-dimensional maps. In unstimulated cells, hnRNP A1 is already phosphorylated at several residues as indicated by the different radiolabeled peptides obtained (Figure 6E). The basal phosphorylation of hnRNP A1 might actually be due to the stress derived from the incubation of these cells with phosphate-free medium, which they did not tolerate well. In unstimulated cells, two faint spots matched the peptides phosphorylated in vitro (Figure 6E), and these were completely eliminated by the addition of the Mnk inhibitor (Figure 6F). Moreover, T cell stimulation increased phosphorylation of these peptides (Figure 6G), and this was also prevented by pretreatment of cells with CGP57380 (Figure 6H).

This experiment shows that hnRNP A1 is phosphorylated in vivo upon T cell stimulation at residues 192 and 310/1/2 and that this phosphorylation is sensitive to the Mnk inhibitor. Thus, hnRNP A1 appears to be an in vivo Mnk substrate. Altogether, the data argue in favor of an in vivo role for the Mnks in regulating cytokine production through phosphorylation of ARE binding proteins such as hnRNP A1. The next step was to examine

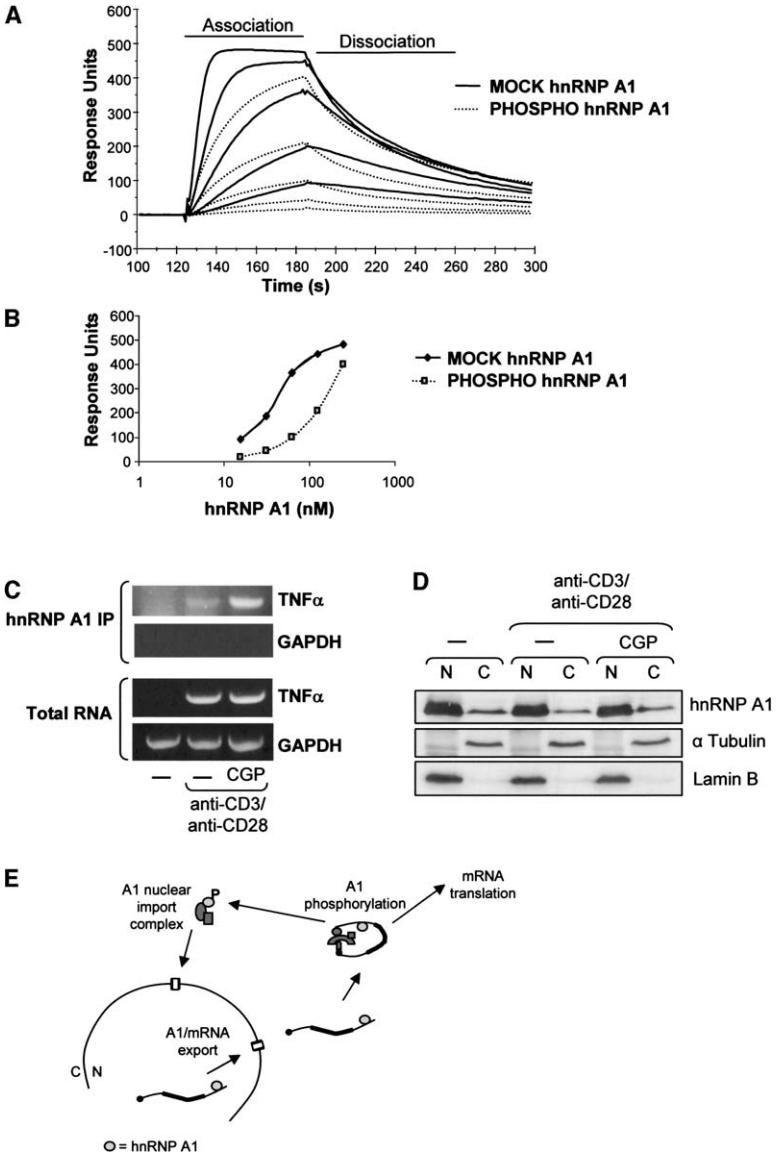

Figure 7. hnRNP A1 Binding to TNFα mRNA

(A and B) Binding of bacterially expressed hnRNP A1 to the TNFα oligoribonucleotide was analysed by surface plasmon resonance BiaCore analysis as described in Experimental Procedures. (A) Binding profile. The oligoribonucleotide containing the TNFα ARE (sample channel) or the irrelevant oligo (control channel) were attached to the sensor chip. Association and dissociation profiles were recorded for five different concentrations (15.6, 31.25, 62.5, 125, and 250 nM) of either phosphorylated or mock phosphorylated hnRNP A1 (incubated with inactive Mnk1) (B) The response level at steady-state binding was plotted vs. the log of the hnRNP A1 concentration. Binding to the biotinylated irrelevant oligoribonucleotide was used as a measurement of nonspecific binding and subtracted from the binding observed with the TNFα oligoribonucleotide.

(C) Jurkat cells were either untreated or stimulated with anti-CD3/anti-CD28 in the absence or presence of CGP57380. After 1 hr of stimulation cells were harvested and RNA extracted from either total cell lysates or hnRNP A1 IPs (see Supplemental Experimental Procedures for details). From the RNA obtained, RT-PCR reactions were performed for TNFα and GAPDH. Levels of the PCR products from the TNFα and GAPDH mRNAs present in the hnRNP A1 IPs (upper panels) or in total RNA extracts (lower panels) are shown.

(D) Extracts from Jurkat cells treated as in C, were fractionated to give cytoplasmic (C) and nuclear (N) fractions as described in Supplemental Data. Aliquots representing 7% of each fraction were analyzed by Western blotting with antibodies directed against hnRNP A1, lamin B and α-tubulin.

(E) Possible model for the regulation of hnRNP A1 by Mnk-mediated phosphorylation. For details, see main text.

whether Mnk-mediated phosphorylation affected hnRNP A1 function.

Phosphorylation of hnRNP A1 by the Mnks Reduces Its Binding to the 3′UTR

Our hypothesis is that the Mnks regulate specific messages via phosphorylation of ARE-BPs. This phosphorylation may disrupt the protein complexes bound to the 3′UTR responsible for the translational repression of the message. To test whether Mnk-mediated phosphorylation of hnRNP A1 modulated its binding to the RNA, we performed surface plasmon resonance (SPR) binding studies, as described in the Supplemental Experimental Procedures. As shown in Figures 7A and 7B, bacterially expressed hnRNP A1 phosphorylated in vitro with active Mnk1 bound with lower affinity to the immobilized TNFα oligoribonucleotide. This suggests that phosphorylation negatively regulates binding of hnRNP A1 to the ARE of the TNFα mRNA.

We attempted to confirm the binding of hnRNP A1 to the TNFα mRNA by gel mobility shift analysis. RNAase digestion to remove unbound RNA was required to study the binding of purified hnRNP A1 and UV crosslinking was needed prior to digestion. Unfortunately, the crosslinking between TNFα and hnRNP A1 was inefficient, rendering this approach unsuitable for in vitro assessment of the affinity of hnRNP A1 for the TNFα mRNA (data not shown).

We considered it important to examine the interaction between hnRNP A1 and the TNFα mRNA in vivo. To do this, we performed ribonucleoprotein (RNP) immunoprecipitation (IP) assays (Niranjanakumari et al., 2002). Jurkat cells stimulated in the presence or absence of CGP57380, were fixed in vivo with formaldehyde and the crosslinked RNP complexes were then immunoprecipitated with an anti-hnRNP A1 antibody. To allow further characterization of the immunoprecipitated components, the crosslinks were reversed. The

RNA was then extracted from the samples and analyzed by RT-PCR. No products were amplified from the sample obtained with anti-hnRNP A1 using primers for GAPDH (negative control, Figure 7C upper panel; data in lower panel confirm the efficacy of the primers), indicating the specificity of the IP. In contrast, the TNFα mRNA clearly coimmunoprecipitated with hnRNP A1 in stimulated T cells, indicating that this protein:RNA interaction occurs in cells. Strikingly, the level of binding was much greater when cells were pretreated with CGP57380 prior to TCR stimulation. The fact that we saw obvious alterations in the amount of TNFα mRNA that was amplified shows that the PCR reaction is occurring within a range where differences are clearly detected. Thus, Mnk inhibition enhances the association of the TNFα mRNA with hnRNP A1, in agreement with the SPR data where phosphorylation of hnRNP A1 decreased its affinity for the TNFα ARE. Total TNFα mRNA and GAPDH were also analysed by RT-PCR on total RNA extracted from whole-cell lysates (Figure 7C, lower panels). While TNFα mRNA is undetectable in unstimulated cells, it is greatly induced upon activation of T cells. The levels reached upon stimulation are not affected by the Mnk inhibitor, further confirming that the Mnks may regulate the translational efficiency of the TNFα mRNA rather than its transcription, processing or stability. GAPDH levels were constant in all conditions tested.

We therefore conclude that phosphorylation of hnRNP A1 by the Mnks decreases its binding to the TNFα 3′UTR both in vitro and in vivo. Consistent with a translation repressor role for hnRNP A1, it has recently been found to act cooperatively with CUGBP2 during translational blockade of the COX-2 mRNA (Mukhopadhyay et al., 2003). The COX-2 mRNA contains an ARE very similar to that of TNFα, likely explaining why hnRNP A1 binds both mRNAs.

Moreover, our results agree with the proposed model for the nucleo-cytoplasmic shuttling of hnRNP A1. hnRNP A1 is believed to exit the nucleus bound to mRNA and to re-enter in a complex with other proteins (Weighardt et al., 1995). However, although the Mnks might be involved in the recycling of hnRNP A1, they do not affect the overall cellular distribution of the protein. Neither treatment of cells with the Mnk inhibitor nor their stimulation with anti-CD3/anti-CD28 affected the nucleocytoplasmic distribution of hnRNP, which was primarily nuclear, although a substantial proportion was also recovered in the cytoplasm (Figure 7D).

We have shown that in the cytoplasm, phosphorylation by the Mnks decreases the affinity of hnRNP A1 for the mRNA, leading to its release. We suggest that this contributes to the derepression of the translation of the TNFα mRNA (Figure 7E), although the exact mechanisms by which hnRNP A1, and other ARE binding proteins, controls translation remain to be elucidated.

Conclusions

The present data provide several lines of evidence that the Mnks play a key role in the regulation of the synthesis of the inflammatory cytokine TNFα and suggest that this involves phosphorylation by the Mnks of proteins (e.g. hnRNP A1) that bind the regulatory AREs of the 3′UTR of its mRNA. However, formal proof that the effects of the Mnks on TNFα expression are mediated through phosphorylation of hnRNP A1 is still lacking. The Mnks thus phosphorylate proteins that bind either to the 5′- or 3′UTRs of eukaryotic mRNAs and this is likely facilitated by the Mnks association with the eIF4F complex which interacts with both ends of the mRNA. Since Mnk1 is activated by Erk and p38 MAP kinases, it may account for the link between Erk signaling and the posttranscriptional regulation of cytokine expression.

In summary, this study identifies the Mnks as potentially important novel players in the control of TNFα production. Our initial data (unpublished) suggest they may be also involved in the regulation of other cytokines. The Mnks are consequently promising novel targets for specific anti-inflammatory therapy. It will be important to establish whether cytokine production is compromised, for example, in the mice lacking Mnk1 and Mnk2 that were recently described (Ueda et al., 2004). Although the lack of phenotype reported by these workers may at first appear surprising given our data showing a role for the Mnks in TNFα synthesis, it should be noted that no phenotype was initially seen for the MK-2 knock-out mice under sterile controlled laboratory conditions (Kotlyarov et al., 1999) even though it is now well established that this enzyme does play an important role in regulating cytokine biosynthesis.

Supplemental Data

Supplemental Data include Experimental Procedures, four additional figures, a table, and Supplemental References and can be found with this article online at http://www.immunity.com/cgi/content/full/23/2/177/DC1/.

Acknowledgments

We thank J. Comas (Universitat de Barcelona) for expert assistance in flow cytometry analysis; M. Kleijn, G. Scheper (both University of Dundee), and S. MacKenzie (Universitat de Barcelona) for technical advice; I. Puga (Universitat de Barcelona) and M. Wilson (University of Dundee) for technical help; M. Peggie (MRC Protein Phosphorylation Unit, Dundee) for the hnRNP A1 clone; and D. Lamont and K. Beattie (University of Dundee) for MALDI-TOF MS analyses. This research was supported by the Fondo de Investigación Sanitaria (E.E.), Ministerio de Educación y Cultura (M.B.), the European Union (M.B., E.E., and C.G.P.) and the Medical Research Council (C.G.P.).

Received: December 17, 2004
Revised: June 8, 2005
Accepted: June 15, 2005
Published: August 23, 2005

References

Ballester, A., Velasco, A., Tobeña, R., and Alemany, S. (1998). Cot kinase activates tumor necrosis factor-α gene expression in a cyclosporin A-resistant manner. J. Biol. Chem. *273*, 14099–14106.

Buxadé, M., Ramírez-Alvarado, M., Fernández-Troy, N., MacKenzie, S., Casaroli-Marano, R.P., Vilella, R., and Espel, E. (2001). Integrating signals from T-cell receptor and serum by T cells enhance translation of TNF-alpha. Immunology *102*, 416–425.

Chrestensen, C.A., Schroeder, M.J., Shabanowitz, J., Hunt, D.F., Pelo, J.W., Worthington, M.T., and Sturgill, T.W. (2004). MAPKr4tAP

kinase 2 phosphorylates tristetraprolin on in vivo sites including Ser178, a site required for 14-3-3 binding. J. Biol. Chem. *279*, 10176–10184.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., and Lee, J.C. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. *364*, 229–233.

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., and Saltiel, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. USA *92*, 7686–7689.

Dumitru, C.D., Ceci, J.D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J.H., Patriotis, C., Jenkins, N.A., Copeland, N.G., Kollias, G., and Tsichlis, P.N. (2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell *103*, 1071–1083.

Dumont, F.J., Staruch, M.J., Fischer, P., DaSilva, C., and Camacho, R. (1998). Inhibition of T cell activation by pharmacological disruption of the MEK1/ERK MAP kinase or calcineurin signaling pathways results in differential modulation of cytokine production. J. Immunol. *160*, 2579–2589.

Feldmann, M., Brennan, F.M., and Maini, R.N. (1996). Role of cytokines in rheumatoid arthritis. Annu. Rev. Immunol. *14*, 397–440.

Fukunaga, R., and Hunter, T. (1997). MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J. *16*, 1921–1933.

Gingras, A.-C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors:effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. *68*, 913–963.

Hamilton, B.J., Burns, C.M., Nichols, R.C., and Rigby, W.F. (1997). Modulation of AUUUA response element binding by heterogeneous nuclear ribonucleoprotein A1 in human T lymphocytes. The roles of cytoplasmic location, transcription, and phosphorylation. J. Biol. Chem. *272*, 28732–28741.

Han, J., Brown, T., and Beutler, B. (1990). Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. J. Exp. Med. *171*, 465–475.

Hoffmeyer, A., Grosse-Wilde, A., Flory, E., Neufeld, B., Kunz, M., Rapp, U.R., and Ludwig, S. (1999). Different mitogen-activated protein kinase signaling pathways cooperate to regulate tumor necrosis factor alpha gene expression in T lymphocytes. J. Biol. Chem. *274*, 4319–4327.

Knauf, U., Tschopp, C., and Gram, H. (2001). Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2. Mol. Cell. Biol. *21*, 5500–5511.

Kontoyiannis, D., Pasparakis, M., Pizarro, T.T., Cominelli, F., and Kollias, G. (1999). Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity *10*, 387–398.

Kotlyarov, A., Neininger, A., Schubert, C., Eckert, R., Birchmeier, C., Volk, H.D., and Gaestel, M. (1999). MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. Nat. Cell Biol. *1*, 94–97.

Kruys, V., Kemmer, K., Shakov, A., Jongeneel, V., and Beutler, B. (1992). Constitutive activity of the TNF promoter is canceled by the 3′-UTR in nonmacrophage cell lines: a trans-dominant factor overcomes this suppressive effect. Proc. Natl. Acad. Sci. USA *89*, 673–677.

Lai, W.S., Carballo, E., Strum, J.R., Kennington, E.A., Phillips, R.S., and Blackshear, P.J. (1999). Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol. Cell. Biol. *19*, 4311–4323.

Mazumder, B., Seshadri, V., and Fox, P.L. (2003). Translational control by the 3′-UTR: the ends specify the means. Trends Biochem. Sci. *28*, 91–98.

Miethke, T., Wahl, C., Heeg, K., Echtenacher, B., Krammer, P.H., and Wagner, H. (1992). T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. J. Exp. Med. *175*, 91–98.

Mukhopadhyay, D., Houchen, C.W., Kennedy, S., Dieckgraefe, B.K., and Anant, S. (2003). Coupled mRNA stabilization and translational silencing of cyclooxygenase-2 by a novel RNA binding protein, CUGBP2. Mol. Cell *11*, 113–126.

Neininger, A., Kontoyiannis, D., Kotlyarov, A., Winzen, R., Eckert, R., Volk, H.D., Holtmann, H., Kollias, G., and Gaestel, M. (2002). MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels. J. Biol. Chem. *277*, 3065–3068.

Niranjanakumari, S., Lasda, E., Brazas, R., and Garcia-Blanco, M.A. (2002). Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo. Methods *26*, 182–190.

O'Loghlen, A., Gonzalez, V.M., Salinas, M., and Martin, M.E. (2004). Suppression of human Mnk1 by small interfering RNA increases the eukaryotic initiation factor 4F activity in HEK293T cells. FEBS Lett. *578*, 31–35.

Piecyk, M., Wax, S., Beck, A.R., Kedersha, N., Gupta, M., Maritim, B., Chen, S., Gueydan, C., Kruys, V., Streuli, M., and Anderson, P. (2000). TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha. EMBO J. *19*, 4154–4163.

Pyronnet, S., Imataka, H., Gingras, A.-C., Fukunaga, R., Hunter, T., and Sonenberg, N. (1999). Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E. EMBO J. *18*, 270–279.

Rousseau, S., Morrice, N., Peggie, M., Campbell, D.G., Gaestel, M., and Cohen, P. (2002). Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs. EMBO J. *21*, 6505–6514.

Salituro, F.G., Germann, U.A., Wilson, K.P., Bemis, G.W., Fox, T., and Su, M.S. (1999). Inhibitors of p38 MAP kinase: therapeutic intervention in cytokine-mediated diseases. Curr. Med. Chem. *6*, 807–823.

Scheper, G.C., Morrice, N.A., Kleijn, M., and Proud, C.G. (2001). The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. Mol. Cell. Biol. *21*, 743–754.

Scheper, G.C., Parra, J.L., Wilson, M., Van Kollenburg, B., Vertegaal, A.C., Han, Z.G., and Proud, C.G. (2003). The N and C termini of the splice variants of the human mitogen-activated protein kinase-interacting kinase Mnk2 determine activity and localization. Mol. Cell. Biol. *23*, 5692–5705.

Scheper, G.C., van Kollenburg, B., Hu, J., Luo, Y., Goss, D.J., and Proud, C.G. (2002). Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. J. Biol. Chem. *277*, 3303–3309.

Shi, Y., and Gaestel, M. (2002). In the cellular garden of forking paths: how p38 MAPKs signal for downstream assistance. Biol. Chem. *383*, 1519–1536.

Tran, H., Maurer, F., and Nagamine, Y. (2003). Stabilization of urokinase and urokinase receptor mRNAs by HuR is linked to its cytoplasmic accumulation induced by activated mitogen-activated protein kinase-activated protein kinase 2. Mol. Cell. Biol. *23*, 7177–7188.

Ueda, T., Watanabe-Fukunaga, R., Fukuyama, H., Nagata, S., and Fukunaga, R. (2004). Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. Mol. Cell. Biol. *24*, 6539–6549.

Wang, X., Flynn, A., Waskiewicz, A.J., Webb, B.L., Vries, R.G., Baines, I.A., Cooper, J.A., and Proud, C.G. (1998). The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. J. Biol. Chem. *273*, 9373–9377.

Waskiewicz, A.J., Flynn, A., Proud, C.G., and Cooper, J.A. (1997). MAPKs activate the ser/thre kinases Mnk1 and Mnk2. EMBO J. *16*, 1909–1920.

Waskiewicz, A.J., Johnson, J.C., Penn, B., Mahalingam, M., Kimball, S.R., and Cooper, J.A. (1999). Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. Mol. Cell. Biol. *19*, 1871–1880.

Weighardt, F., Biamonti, G., and Riva, S. (1995). Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains in hnRNP A1. J. Cell Sci. *108*, 545–555.

Zhang, H., Shi, X., Hampong, M., Blanis, L., and Pelech, S. (2001). Stress-induced inhibition of ERK1 and ERK2 by direct interaction with p38 MAP kinase. J. Biol. Chem. *276*, 6905–6908.