

Regulation of tumour necrosis factor α mRNA stability by the mitogen-activated protein kinase p38 signalling cascade

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Abstract The translation of tumour necrosis factor α (TNF α) mRNA is regulated by the stress-activated protein kinase p38, which also controls the stability of several pro-inflammatory mRNAs. The regulation of TNF α gene expression in a mouse macrophage cell line RAW264.7 was re-examined using an inhibitor of stress-activated protein kinases. Stimulation of these cells with bacterial lipopolysaccharide resulted in stabilisation of TNF α mRNA, which was reversed by specific inhibition of p38. An adenosine/uridine-rich element from the TNF α 3' untranslated region conferred p38-sensitive decay in a tetracycline-regulated mRNA stability assay. Therefore the p38 pathway also controls TNF α mRNA turnover. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Tumor necrosis factor α ;
Mitogen-activated protein kinase p38; mRNA stability;
Translation; Macrophage; Lipopolysaccharide

1. Introduction

Mitogen-activated protein kinase (MAPK) p38 is activated by cell stresses such as heat shock or ultraviolet light and (in cells of the myeloid lineage) by bacterial lipopolysaccharide (LPS) [1]. The enzyme activity of p38 is reversibly inhibited by pyridinyl imidazole compounds such as SB203580, which compete with ATP for access to the catalytic site [2]. These compounds inhibit interleukin 1 (IL-1) and tumour necrosis factor α (TNF α) protein synthesis in myeloid cell lines stimulated with LPS, but down-regulate the corresponding mRNAs by 50% at most [3–5]. A mouse knock-out strain lacking the p38 substrate MAPKAPK-2 displays defective translation of TNF α mRNA [6]. These observations indicate a role for the p38 pathway in the regulation of TNF α expression at the translational level.

In contrast, an involvement of this signalling pathway in the regulation of cyclooxygenase-2 (Cox-2) mRNA stability has been demonstrated by actinomycin D chase experiments in human monocytes and HeLa cells [7,8]. This was confirmed using a tetracycline-responsive HeLa cell line [9], in which chimeric β -globin/Cox-2 transcripts were stabilised by the expression of constitutively active MKK6 (an activator of p38) [10,11] or MAPKAPK-2 (a downstream effector of p38) [12,13]. Similar experiments reveal regulation of IL-6 and IL-8 mRNA stability by p38 [14].

The regulation of mRNA stability is often mediated by

adenosine/uridine-rich elements (AREs) within the 3' untranslated regions (UTRs) of the transcripts in question [15,16]. The targeted deletion of a very similar ARE from the mouse genomic TNF α locus causes an apparent increase in TNF α mRNA stability, and a loss of the inhibitory effects of SB203580 upon TNF α biosynthesis [17]. The same ARE is involved in the destabilisation of TNF α mRNA by the RNA binding protein tristetraproline [18,19], which in turn is regulated by the p38 pathway (in preparation). To address the confusion over the regulation of mRNA turnover and translation by the p38 pathway, we re-examined the expression of the TNF α gene in the mouse myeloid cell line RAW264.7.

2. Materials and methods

2.1. Materials

GST-cJun1–135, His-tagged MAPKAPK-2 and a rabbit antiserum to a C-terminal peptide of p38 α are described elsewhere [7]. Recombinant human hsp27 was from StressGen. Sheep anti-rabbit MAPKAPK-2 antibody was from Upstate Biotechnology. SB203580 was from Calbiochem-Novabiochem. [γ - 32 P]ATP (3000 Ci/mmol), [α - 32 P]dCTP (3000 Ci/mmol), [α - 32 P]UTP (800 Ci/mmol), Ready To Go DNA labelling kit, Hybond XL membrane, Microspin S-200 HR columns and a Mono Q column were from Amersham Pharmacia Biotech. *Salmonella typhimurium* LPS was from Sigma-Aldrich, and was used throughout at a concentration of 10 ng/ml. DNase I was from Life Technologies, RNeasy kits and Superfect transfection reagent were from Qiagen, and pBluescript plasmid and Stratalinker UV cross-linker were from Stratagene. Murine GAPDH cDNA, ULTRAhyb hybridisation buffer and Direct Protect ribonuclease protection assay reagents were from Ambion. T7 RNA polymerase and T7 transcription buffer were from Boehringer Mannheim. Murine TNF α enzyme-linked immunosorbent assay (ELISA) reagents were from Pharmingen. The pTetBBB-TNF44 vector was constructed from pTetBBB (gift of A. Shyu) by insertion of a double-stranded oligonucleotide (upper strand sequence GATCCTTGTTGATTATT-TATTATTTATTTATTATTTATTTATTACAGA) at the *Bgl*II site within the β -globin 3' UTR. To generate pCRB-TNF α , a TNF α cDNA fragment was amplified by reverse transcription-polymerase chain reaction using primers GAAGCTGTCTTCAGACAGACATG and GCCGGATCCGCGATCTTTATTTCTCTCAATGACTGTAGG, then cloned into pCRBlunt (Invitrogen).

2.2. Cell culture

RAW 264.7 and HeLa Tet-off cells were maintained in Dulbecco's modified Eagle's medium/10% (v/v) foetal calf serum in a humidified atmosphere of 5% (v/v) CO₂ at 37°C.

2.3. Kinase assays

Cells (2×10^6) were stimulated with 10 ng/ml LPS for the time periods indicated, and lysates were prepared as described [7]. MAPK p38 was immunoprecipitated from 500 μ g of lysate, and kinase assays were performed as described [7], using 1 μ g recombinant MAPKAPK-2 as substrate. SB203580 is a reversible inhibitor of p38, which is lost during washing of immunoprecipitates. To measure the inhibition of p38 by SB203580, RAW264.7 cells were stimulated with

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10 ng/ml LPS for 20 min, the p38 substrate MAPKAPK-2 was immunoprecipitated and used in kinase assays with 1 μ g recombinant hsp27 as described [7].

Cells (10^8) were stimulated for 20 min with 10 ng/ml LPS, then a lysate was prepared and subjected to anion exchange chromatography as described [7], except that a total of 2 mg of protein was chromatographed. Fractions were assayed for cJun N-terminal kinase (JNK) activity using GST-cJun1–135 substrate in the presence of vehicle (dimethyl sulfoxide; DMSO) or SB203580 as indicated. Kinase activities were quantified by phosphorimager (Fuji FLA2000; Fuji, Tokyo, Japan).

2.4. Measurement of TNF α protein and mRNA

TNF α protein was quantitated by ELISA as described [20]. Total RNA was isolated using the RNeasy kit. Riboprobe was generated from pCRB-TNFr by in vitro transcription as described [9]. A TNF α cDNA probe was generated by random prime labelling of a 960 bp *SpeI*–*SspI* fragment from pUC18mTNFloc. The GAPDH probe is described elsewhere [7]. Northern blotting was performed as described [7]. Where riboprobe was used, blots were washed at 65°C in 2 \times , 1 \times and 0.1 \times SSC containing 0.1% SDS. Quantitation of mRNA was by phosphorimager.

2.5. Transfection of HeLa Tet-off cells, and ribonuclease protection assays

Superfect was used to transfect HeLa Tet-off cells with 100 ng pGL3c, 100 ng pTetBBB-TNF44, with or without 100 ng of pCMV-MKK6E and carrier DNA to a total of 1 μ g. After 24 h, 1 μ M SB203580 or vehicle (DMSO) was added. After a further 30 min, tetracycline was added to a final concentration of 100 ng/ml, and cells were harvested in Direct Protect lysis buffer at the time intervals indicated. Ribonuclease protection assays were performed as described [9], using riboprobes for β -globin and for endogenous GAPDH, and quantified by phosphorimager.

3. Results

3.1. Time course of activation of MAPK p38 by LPS

MAPK p38 activity was almost undetectable in unstimulated RAW264.7 cells, but was rapidly and strongly activated by LPS treatment, with a peak of activity at 20 min post-stimulus (Fig. 1). The activity subsequently declined, but remained significantly elevated above basal for at least 4 h after the stimulus. Effects of p38 inhibition were later examined in cells

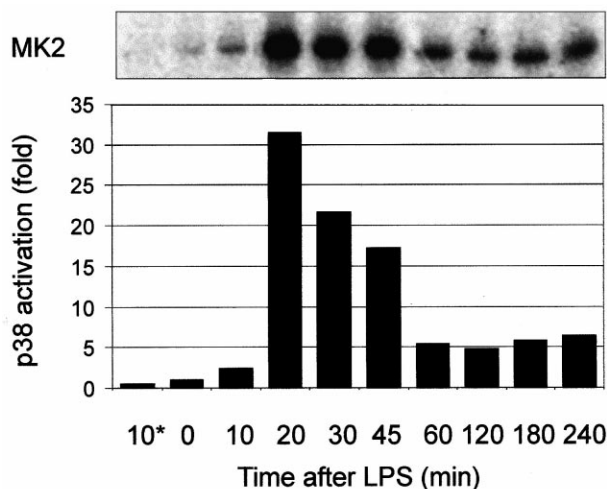


Fig. 1. Time course of activation of p38 by LPS. RAW264.7 cells were stimulated with 10 ng/ml LPS for the times indicated, then harvested. Immunoprecipitations were performed using an antiserum to p38, except in the first track (marked with an asterisk), where the corresponding pre-immune serum was used. Kinase assays were then performed using the substrate MAPKAPK-2 (MK2).

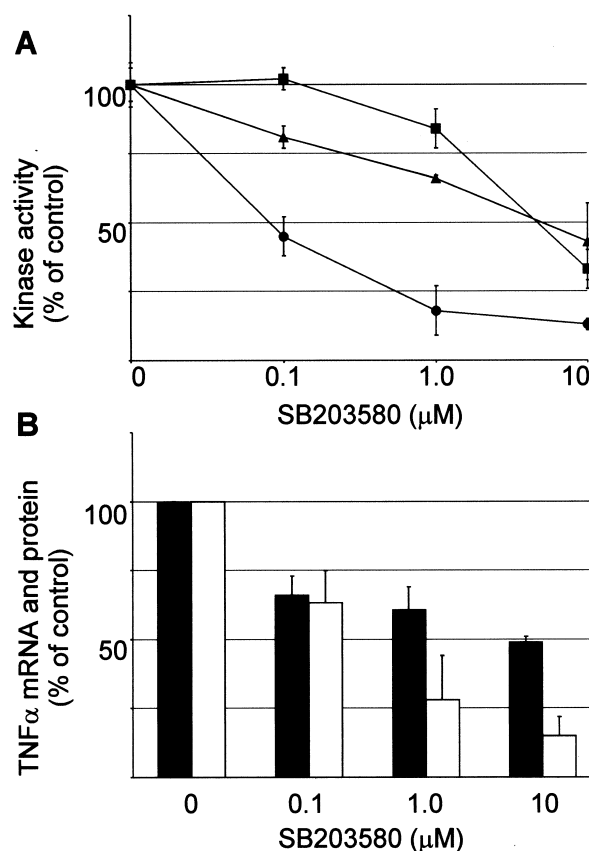


Fig. 2. Inhibition of stress-activated protein kinases and TNF α gene expression by SB203580. A: Inhibition of p38 in vivo (●) and of two chromatographic peaks of JNK activity in vitro (■ and ▲) was measured in triplicate. Means \pm S.D. are indicated. B: RAW264.7 cells were stimulated for 4 h with 10 ng/ml LPS, then TNF α mRNA (closed bars) and protein (open bars) were quantified. Means \pm S.D. of three independent experiments are indicated.

treated with LPS for 4 h. In this context the sustained activation of p38 by LPS is significant. A similar pattern of activation of JNK was observed following LPS stimulation (data not shown).

3.2. Dose responses of inhibition of p38 and JNK by SB203580

Inhibition of stress-activated protein kinases was measured in extracts of LPS-stimulated RAW264.7 cells (Fig. 2A). As expected, p38 was inhibited with an IC_{50} of approximately 0.1 μ M. Two chromatographically distinct JNK fractions were purified from these cells and contained approximately equal activity (data not shown). Although much less sensitive to SB203580 than p38, both were significantly inhibited at higher doses of the inhibitor in vitro. Total JNK activity was inhibited by 10, 25 and 60% at 0.1, 1.0 and 10 μ M SB203580. Inhibition of JNK isoforms by the 'p38-specific inhibitor' SB203580 has previously been reported [7,21,22], but appears somewhat cell type specific, and has not been examined in RAW264.7 cells.

3.3. Differential regulation of TNF α protein and mRNA expression in RAW264.7 cells

The effects of SB203580 upon TNF α protein and mRNA accumulation were examined in RAW264.7 cells preincubated for 1 h with the inhibitor, then stimulated for 4 h with LPS.

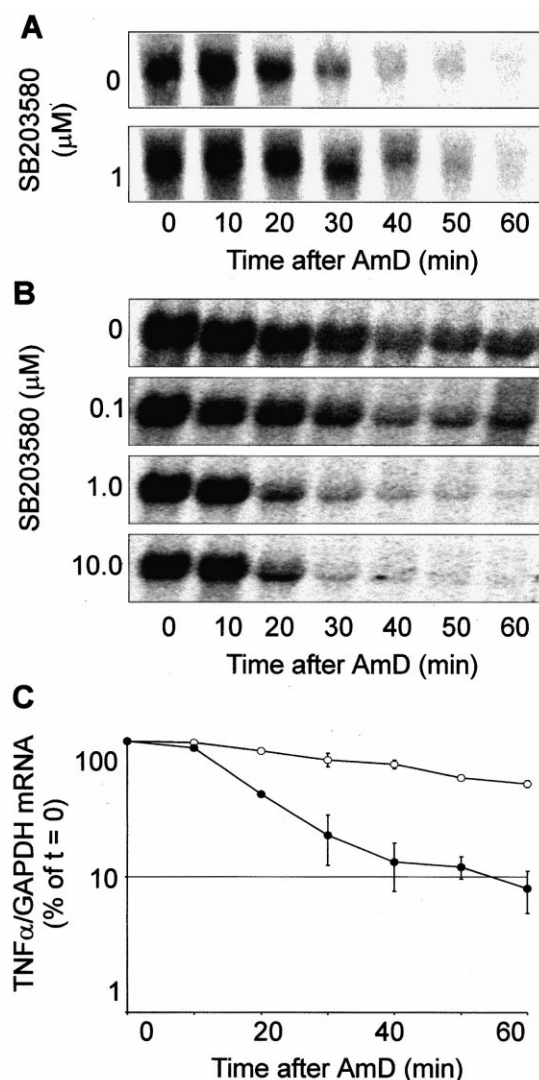


Fig. 3. p38 regulates the stability of TNF α mRNA. A: RAW264.7 cells were treated with actinomycin D (AmD, 10 $\mu\text{g}/\text{ml}$) in the presence of SB203580 or vehicle, harvested at the time intervals shown, and Northern blotted for TNF α mRNA using a high specific activity riboprobe. B: RAW264.7 cells were stimulated with 10 ng/ml LPS for 4 h, then actinomycin D (AmD, 10 $\mu\text{g}/\text{ml}$) was added in the absence or presence of SB203580 as indicated, cells were harvested at the time intervals shown and Northern blots were done using a TNF α cDNA probe. C: Mean TNF α mRNA levels, corrected for GAPDH mRNA, from three independent experiments performed as in B, using a 1 μM dose of SB203580. \circ , -SB203580; \bullet , +SB203580. Error bars indicate S.D.

TNF α mRNA expression was inhibited by around 50% at most, even at a 10 μM concentration of SB203580 (Fig. 2B). Protein expression was more strongly inhibited, with an IC_{50} close to 0.1 μM . The differential regulation of protein and mRNA expression at higher doses of SB203580 is consistent with translational regulation of TNF α gene expression. However it is difficult to be certain whether this is mediated entirely by p38, or whether a significant role is played by JNK [23].

3.4. Regulation of TNF α mRNA stability by SB203580

At 0.1 or 1.0 μM concentration SB203580 partially, but reproducibly, blocked the accumulation of TNF α mRNA in

response to an LPS challenge. In preliminary experiments (data not shown) we found no evidence for the regulation of TNF α gene transcription by SB203580, therefore the regulation of TNF α mRNA stability by the p38 pathway was examined. In unstimulated cells TNF α mRNA could be detected by Northern blotting with a high specific activity riboprobe. Decay of this transcript was rapid and insensitive to SB203580 (Fig. 3A). In cells stimulated for 4 h with LPS, the stability of TNF α mRNA was greater, but was decreased significantly by 0.1 μM , and very strongly by 1.0 or 10 μM SB203580 (Fig. 3A,B). The estimated transcript half-lives were 26, 60 and 11 min in unstimulated cells, in stimulated cells and in stimulated cells treated with 1 μM SB203580. This suggests an LPS-responsive stabilisation of TNF α mRNA, which is dependent upon p38. Such stabilisation can be detected within 30 min of LPS treatment (data not shown).

To address the possibility that regulation of mRNA stability by p38 is an actinomycin D artefact, effects of SB203580 upon TNF α mRNA levels were examined in the absence of actinomycin D, following stimulation of cells for 4 h with LPS (Fig. 4). In the absence of SB203580, TNF α mRNA levels remained roughly constant over 1 h, suggesting that transcription and degradation rates were approximately balanced during this time. Following the addition of the inhibitor, steady state TNF α mRNA levels rapidly decreased to about 50% of their starting level but no further, suggesting that degradation of TNF α mRNA is p38-sensitive in the absence of actinomycin D.

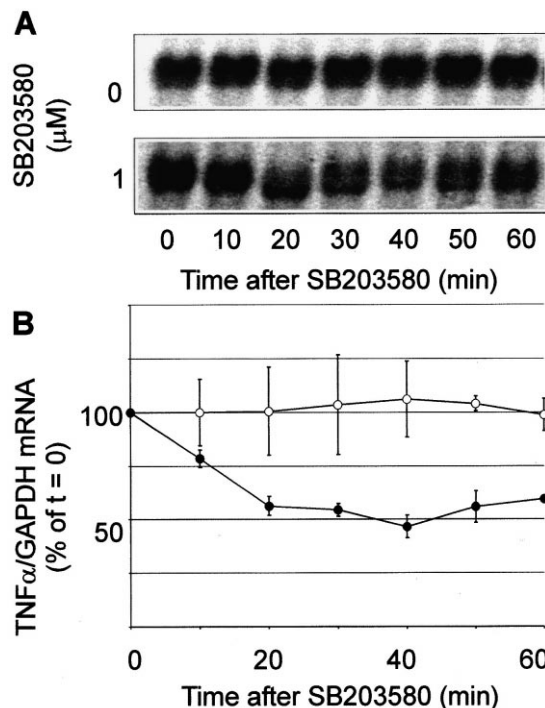


Fig. 4. SB203580 down-regulates TNF α mRNA in the absence of actinomycin D. A: RAW264.7 cells were stimulated for 4 h with 10 ng/ml LPS, then treated with 1 μM SB203580 or vehicle (DMSO). At the time intervals shown, cells were harvested and Northern blots performed using a TNF α cDNA probe. B: Mean TNF α mRNA levels, corrected for GAPDH mRNA, from three independent experiments performed as in A. \circ , -SB203580; \bullet , +SB203580. Error bars indicate S.D.

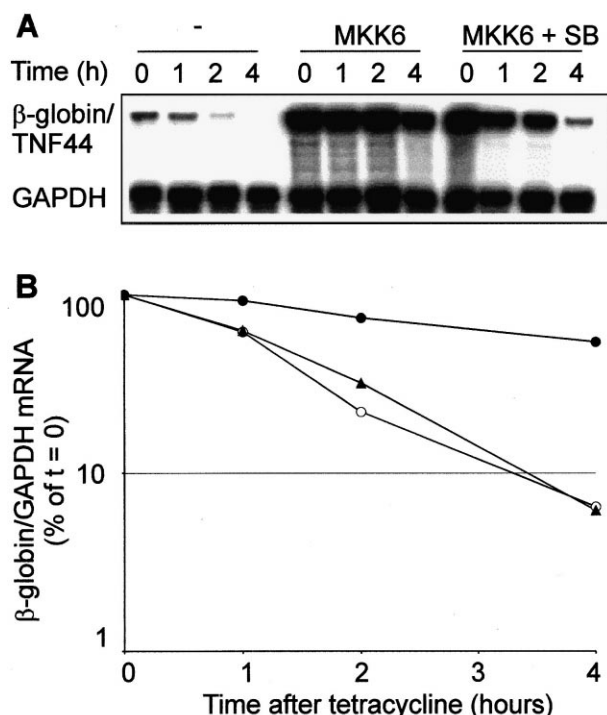


Fig. 5. The TNF α ARE confers p38-responsive mRNA decay in HeLa cells. A: HeLa Tet-off cells were transfected with the construct pTetBBB-TNF44, with or without a vector expressing constitutively active MKK6. After 24 h, SB203580 (1 μ M) or vehicle was added. After a further 30 min, tetracycline was added to a final concentration of 100 ng/ml, cells were harvested at the time intervals indicated and ribonuclease protection assays carried out. This experiment was performed three times with qualitatively identical results. B: Graphical representation of the experiment shown in A. ○, -MKK6; ●, +MKK6; ▲, +MKK6+SB203580.

3.4. An ARE derived from the TNF α 3' UTR mediates regulation of mRNA stability by the MAPK p38 cascade

To further analyse the regulation of TNF α mRNA stability, a tetracycline-regulated reporter gene system [24,25] was employed (Fig. 5). In HeLa Tet-off cells β -globin mRNA was expressed under the control of a tetracycline-responsive promoter, which can be switched off by addition of 100 ng/ml tetracycline to the culture medium. Decay of the β -globin mRNA was scarcely detectable under these conditions (data not shown). The insertion of a short (44 nt) TNF α adenosine/uridine-rich stretch resulted in the destabilisation of the β -globin reporter mRNA. The chimeric β -globin-TNF44 mRNA was stabilised by coexpression of the p38 activator MKK6, and this stabilisation was reversed upon addition of SB203580. The increased β -globin-TNF44 mRNA seen at the zero time point in the presence of MKK6 is the consequence of stabilisation during the 24 h prior to the tetracycline chase. MAPK p38-responsive mRNA instability was not conferred by a fragment containing five overlapping AUUUA motifs (data not shown), and is therefore not a general feature of AREs. Although TNF α mRNA is not normally expressed by HeLa cells, these observations clearly demonstrate that the transcript contains p38-responsive stability determinants.

4. Discussion

Both JNK and p38 signal transduction pathways were acti-

vated in LPS-stimulated RAW264.7 cells, but were differentially inhibited by SB203580. Effects observed at high concentrations of SB203580 cannot safely be ascribed to p38 inhibition, because of significant inhibition of JNK activity under these conditions. Thus, the differential regulation of TNF α gene expression at protein and mRNA levels is consistent with translational control, however this may be mediated in part by JNKs [23]. In the absence of reliable assays of translational control *in vivo*, the contributions of the two stress-activated protein kinase pathways are difficult to assess.

In contrast, TNF α mRNA was clearly destabilised by 1 μ M SB203580, strongly implicating p38 in the regulation of TNF α mRNA stability. Several observations suggest that this is not an artefact arising from the use of actinomycin D. In actinomycin D chase experiments the stability of TNF α mRNA was increased by LPS treatment of cells. Stability was sensitive to p38 inhibition only in LPS-stimulated cells, therefore SB203580 does not simply interfere with an artifactual stabilisation caused by actinomycin D. Whilst there was no evidence for an involvement of p38 in the LPS-stimulated transcription of the TNF α gene, SB203580 significantly down-regulated steady state TNF α mRNA levels in the absence of actinomycin D, implying an effect upon mRNA stability. SB203580 influenced TNF α mRNA stability in the presence of α -amanitin, a transcriptional inhibitor which is functionally distinct from actinomycin D (data not shown). Finally, a short adenosine/uridine-rich fragment of the TNF α 3' UTR conferred p38-responsive mRNA instability in HeLa cells. It is possible that changes in TNF α mRNA stability in RAW264.7 cells are masked by ongoing transcription, which is not sensitive to SB203580, resulting in the relatively minor differences at the steady state mRNA level which are described here and elsewhere [5].

Both translation and mRNA stability are coupled to the regulation of poly(A) tail length [26]. Deadenylation is implicated in the regulation of TNF α mRNA stability by both TTP and the protein kinase C pathway [19,27,28]. In the absence (Fig. 3B) or presence (Fig. 4) of actinomycin D, the addition of SB203580 appeared to be followed by a rapid shortening of the TNF α transcript. It remains to be shown that this corresponds to the shortening or removal of the poly(A) tail, however the regulation of poly(A) tail length by the p38 pathway may provide an explanation for the apparent regulation of translation or of mRNA stability by the p38 pathway in different studies.

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