Extracellular Signal-regulated Kinase Regulation of Tumor Necrosis Factor-α mRNA Nucleocytoplasmic Transport Requires TAP-NxT1 Binding and the AU-rich Element*

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Tumor necrosis factor-α (TNF-α) production is regulated by transcriptional and posttranscriptional mechanisms. Lipopolysaccharide activates the NFκB pathway increasing TNF-α transcription. Lipopolysaccharide also activates the mitogen-activated protein kinase pathways, resulting in stabilization and enhanced translation of the TNF-α message. In addition, nuclear export of the TNF-α mRNA is a posttranscriptionally regulated process involving the Tpl2-ERK pathway and requiring the presence of the TNF-α AU-rich element (ARE). We demonstrate that nuclear export of the TNF-α message requires not only the TNF-α ARE but also the interaction of the proteins TAP and NxtT1, both of which are involved in nucleocytoplasmic transport of mRNA. Through the use of dominant negative ERK1 and ERK2, we establish that control of TNF-α mRNA nuclear export operates specifically through ERK1. Finally, we examined the role of two established TNF-α ARE-binding proteins, HuR and tristetraprolin, that shuttle between the nucleus and cytoplasm. These data demonstrate that neither tristetraprolin nor HuR is required for TNF-α mRNA export. It is unclear at this time if ARE-binding protein(s) directly interact with the TAP-NxtT1 complex, if each complex is independently targeted by ERK1, or if only one complex is targeted.

Regulation of proinflammatory gene expression is a balance between positive and negative signal transduction pathways. LPS,2 the outer membrane component of Gram-negative bacteria, binds to the TLR4 complex, triggering cascades of intracellular signaling events, resulting in expression of a number of proinflammatory mediators (1, 2). In monocye/macrophages, LPS stimulation is a potent inducer of TNF-α production operating through both transcriptional and posttranscriptional mechanisms (3, 4).

TNF-α gene transcription involves a number of transcription factors, including cAMP-response element-binding protein, LITAF, ATF-2, c-Jun, Egr-1, NFAT, Ets, Elk-1, and Sp1 (5–18). However, it is the NFκB transcription factors that are critically involved in LPS or cytokine stimulation of TNF-α transcription (for a review, see Ref. 19). Posttranscriptional regulation of TNF-α operates through cis-elements in the 3′-untranslated region (3′-UTR), which serve as binding sites for trans-acting proteins. One of the best studied cis-elements, the AU-rich element (ARE), is critical in the regulation of TNF-α message stability and translation (20). In unstimulated cells, the TNF-α message is unstable and translationally repressed, preventing TNF-α protein production (20–25). In response to LPS stimulation, the TNF-α message is stabilized, and translation is derepressed, allowing for the rapid production of TNF-α protein (22–25).

In addition, TNF-α mRNA nuclear export is a regulated event requiring both the ARE and an intact Tpl2/ERK signaling pathway (26). Unlike the Raf-1/ERK pathway, which responds to mitogens, Tpl2 signaling is activated by TLR or proinflammatory cytokine stimulation (26–28). In an interesting convergence of regulation, Tpl2 is bound by NFκB in unstimulated cells, which prevents Tpl2 phosphorylation of MEK1 (29). Tpl2−/− mice exhibit a reduction in TNF-α nucleocytoplasmic mRNA transport (26). The nuclear/cytoplasmic ratio of TNF-α mRNA shifts from 20% nuclear and 80% cytoplasmic in Tpl2+/− animals to about 60% nuclear and 40% cytoplasmic in Tpl2−/− animals.

We examined the effect of combined MAP kinase pathway inhibition on TNF-α posttranscriptional expression in unstimulated and LPS-stimulated RAW264.7 cells, a mouse macrophage cell line widely used to study TNF-α production. Although these studies confirmed previous findings regarding the importance of the p38 pathway and TNF-α posttranscriptional regulation, our results using a TNF-α 3′-UTR reporter failed to recapitulate the previously reported effect of ERK inhibition on TNF-α nuclear export. The TNF-α gene contains three introns and intron splicing results in deposition of exon junction complex (EJC) proteins on the message (30–32). Following splicing, proteins of the EJC recruit proteins that regulate mRNA nuclear export (33). We hypothesized that EJC recruitment was required for the ERK regulation of TNF-α mRNA nuclear export.

To test this hypothesis, we generated constructs containing different TNF-α introns as well as artificial introns. These data

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‡ The abbreviations used are: LPS, lipopolysaccharide; UTR, untranslated region; ARE, AU-rich element; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; TTP, tristetraprolin; RNAi, RNA interference; MOPS, 4-morpholinopropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; JNK, c-Jun N-terminal kinase; DN, dominant negative; RLU, relative light units; NT, no treatment.
demonstrate that ERK inhibition did not alter the nuclear cytoplasmic distribution of intronless or ARE−/− TNF-α 3′-UTR message constructs. In contrast, TNF-α constructs containing both an ARE and an intron were retained in the nucleus to the same degree as endogenous TNF-α message in macrophages treated with PD98059 (26). Further work demonstrated that the effect of ERK inhibition is mediated specifically by ERK1, as dominant negative ERK2 did not reduce TNF-α 3′-UTR-mediated luciferase expression from intron-containing constructs.

We tested two known TNF-α ARE binding proteins, tristetraprolin (TTP) and HuR, to determine if either was necessary for ERK-regulated TNF-α mRNA nuclear export. Using a TTP-negative cell line and HuR RNAi, these data demonstrate that neither TTP nor HuR is required for ERK-regulated TNF-α nuclear export. In order to examine which EJC proteins are involved, we performed tethering assays, in which EJC proteins are bound to target RNAs via a heterologous RNA-binding domain. TAP, NxT1, Aly, and UAP56 were fused to the bacteriophage P22 N-peptide and used with constructs containing the B-box nucleotide sequence. The N-peptide specifically binds to the B-box sequence in the mRNA, tethering the EJC protein to the message in the absence of an intron. These studies demonstrate that the interaction of the TAP-NxT1 complex, but not Aly or UAP56, was required for ERK1-regulated nuclear export. Together, these data establish a link between proteins bound to the ARE and proteins of the ECJ in the regulation of mRNA metabolism.

MATERIALS AND METHODS

Study Design

Materials—LPS (Escherichia coli 026:B6), and Me2SO were purchased from Sigma. The p38 inhibitor SB202190 (2.5 μM), ERK inhibitor PD98059 (20 μM), and JNK inhibitor SP600125 (20 μM) were purchased from Calbiochem. Vectors—TNF-α luciferase constructs were generated from genomic DNA and mRNA using either the pGLO3 control vector or pRL-CMV Renilla vector (Promega, Madison, WI). The same primer pairs were used to generate constructs from both genomic and message sources. Constructs made from mRNA were as follows: pGLO3-TNF 3′-UTR-complete human TNF-α 3′-UTR, nucleotides 855–1643; pGLO3 TNF No Intron 3, nucleotides 396–1643; TNF Renilla, nucleotides 855–1643 in the pRL-CMV vector. Genomic constructs were as follows: pGLO3 TNF Intron 3, nucleotides 1833–3381; TNF Intron 3 ΔARE, nucleotides 1833–2909 and 3131–3381 generated by cutting pGLO3 TNF Intron 3 with PstI and BaeI. pGLO3 TNF Intron 2 was made by amplifying the second intron using the primers CTAGTGCCCTCTGGCGAGGACT (sense) and ACTAGTAGGCTTGTCACTCGGGTGTCG (antisense) and cloning this fragment into an engineered SpeI site of the TNF 3′-UTR constructs. The SpeI site is 5 nucleotides downstream from the luciferase stop codon. Finally, we cloned four repeats of the luciferase stop codon. We tested two known TNF-α ARE binding proteins, tristetraprolin (TTP) and HuR, to determine if either was necessary for ERK-regulated TNF-α mRNA nuclear export. Using a TTP-negative cell line and HuR RNAi, these data demonstrate that neither TTP nor HuR is required for ERK-regulated TNF-α nuclear export. In order to examine which EJC proteins are involved, we performed tethering assays, in which EJC proteins are bound to target RNAs via a heterologous RNA-binding domain. TAP, NxT1, Aly, and UAP56 were fused to the bacteriophage P22 N-peptide and used with constructs containing the B-box nucleotide sequence. The N-peptide specifically binds to the B-box sequence in the mRNA, tethering the EJC protein to the message in the absence of an intron. These studies demonstrate that the interaction of the TAP-NxT1 complex, but not Aly or UAP56, was required for ERK1-regulated nuclear export. Together, these data establish a link between proteins bound to the ARE and proteins of the ECJ in the regulation of mRNA metabolism.

RESULTS

MAP kinase pathway activation is critical in the posttranscriptional control of TNF-α expression (1, 40). Numerous studies have examined the effect of individual pathway inhibition (23, 26, 41–50); however, none have examined the effect of combinations of MAP kinase pathway inhibition on TNF-α posttranscriptional expression in unstimulated and LPS-stimulated macrophages. For these studies, we utilized the mouse macrophage cell line RAW264.7, which is widely employed to study the regulation of TNF-α production, as well as an established transfection system using luciferase reporters (Table 1) (37–39). The use of TNF-α 3′-UTR luciferase reporters allows
for the specific analysis of posttranscriptional events by eliminating the effects of MAP kinase inhibition on the TNF-α promoter from the analysis. Use of the pGL3 control vector allows for control of the transcriptional and other vector effect of LPS stimulation and MAP kinase inhibition. RAW cells were transfected with either pGL3 control or TNF-α 3′-UTR luciferase reporters. Two hours after the addition of serum to the transfections, the p38 inhibitor SB202190 (2.5 μM), ERK inhibitor PD98059 (20 μM), and/or the JNK inhibitor SP600125 (20 μM) were added. The next day, cells were treated with or without LPS (10 ng/ml, 2 h), following which luciferase values were quantified as outlined under "Materials and Methods."

Fig. 1 demonstrates the effect of inhibiting the MAP Kinase pathways, individually and in combination, on TNF-α posttranscriptional expression in unstimulated and LPS-stimulated RAW264.7 cells. As we have previously demonstrated, LPS stimulation results in a significant increase in TNF-α 3′UTR luciferase expression (p < 0.001) (37–39). Consistent with previous work, p38 inhibition significantly inhibited TNF-α 3′-UTR expression in all combinations in both unstimulated and LPS-stimulated RAW cells (p < 0.001) (for a review, see Refs. 51 and 52). JNK inhibition had a differential effect on TNF-α posttranscriptional expression, depending on the activation state of the cells. JNK inhibition resulted in a nonsignificant decrease in TNF-α 3′-UTR-mediated luciferase expression in unstimulated cells, consistent with the low level of JNK activation in unstimulated RAW264.7 cells (53). However, inhibiting JNK followed by LPS stimulation significantly inhibited TNF-α 3′-UTR-mediated luciferase expression (p < 0.01), consistent with JNK involvement in the translational derepression of the TNF-α message in response to activation (20, 44, 50). Finally, ERK inhibition with PD98059 resulted in a slight increase in TNF-α 3′-UTR-mediated luciferase expression. This finding was particularly surprising, given the role of the Tpl2/ERK axis in the regulation of TNF-α mRNA nuclear export (26, 44); we anticipated that ERK inhibition would reduce TNF-α 3′-UTR luciferase expression rather than increasing it. Similar results were obtained when the ERK pathway was inhibited using dominant negative (DN) MEK1, ERK1, or ERK2 (data not shown).

Previous work has established that the loss of Tpl2 results in nuclear retention of about 60% of the TNF-α message, and a similar effect is seen if ERK activation is pharmacologically inhibited in Tpl2 intact macrophages (26). It is well established that the presence of the EJC proteins TAP or Aly bound to a

![Image](image-url)
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message increase the efficiency mRNA nuclear export (for a review, see Refs 54 and 55). Given the link between EJC proteins and nuclear export and the nuclear retention of the TNF-α mRNA in Tpl2−/− animals, we tested the hypothesis that ERK control of TNF-α export requires the deposition of EJC proteins on the mRNA. To do this, we generated a number of TNF-α pGL3 luciferase vector constructs containing introns (Table 1).

The TNF-α gene contains three introns and four exons (Fig. 2A). We generated luciferase constructs containing either the second or third TNF-α introns by amplifying genomic DNA

A

![Diagram](image1)

B

**Fig. 2. Structure of the TNF-α gene and TNF-α constructs.** A, structure of the TNF-α gene. The coding region is indicated in **black**, introns are indicated by **open boxes**, and the 5′- and 3′-UTRs are indicated in **gray**. **B**, structure of the TNF-α vectors employed. **C**, sequence of the two middle exons and location (**boldface type**) of the primers employed to generate the constructs.

(2B). Utilizing the same primer pair and amplifying from mRNA allowed the generation of the constructs containing the same exonic sequence as the genomic constructs but lacking introns. Normally, messages with introns downstream of the stop codon are rapidly targeted for nonsense-mediated decay. The exception is introns located within the first 40–50 nucleotides of the stop codon (for a review, see Ref. 56). Primers were designed to take advantage of this (Fig. 2C), allowing the insertion of an intron downstream of the luciferase stop codon.

We compared the effect of inhibiting either ERK1 or ERK2 on intron containing TNF-α 3′-UTR luciferase vector (Fig. 3). Luciferase expression from the TNF Intron 3 constructs, which contain the third TNF-α intron and the TNF-α 3′-UTR, was significantly reduced (p < 0.01) by the DN ERK1 vector but not by the DN ERK2 vector (Fig. 3A). Luciferase expression from the TNF No Intron 3 vector, which contains the same exonic sequences as the TNF Intron 3 vector but lacks the intron, was comparable with that seen with the TNF 3′-UTR luciferase vector and ERK inhibition in Fig. 1. Luciferase expression from the TNF Intron 2 construct, which contains the second TNF-α intron and the TNF-α 3′-UTR, was significantly reduced (p < 0.01) by the DN ERK1 vector but not by the DN ERK2 vector (Fig. 3B). Expression from the TNF No Intron 2 vector was not reduced. Finally, we demonstrate that the effect is not specific to a TNF-α intron (Fig. 3C). We cloned the TNF-α 3′-UTR into the 3′-UTR of the pRL-CMV Renilla vector. Unlike pGL3, this vector contains an artificial intron in its 5′-UTR. Expression of Renilla luciferase from the backbone pRL vector was not altered by either DN ERK1 or DN ERK2. However, the presence of the TNF-α 3′-UTR in the pRL vector resulted in a significant (p < 0.05) reduction in Renilla luciferase expression that was DN ERK1-specific. These data demonstrate that the presence of an intron confers TNF-α mRNA responsiveness to ERK inhibition but that TNF-α specific intronic sequences are not required. These data also demonstrate that the ERK effect operates specifically through the ERK1 pathway.

A

![Graph](image2)

**Fig. 3. The presence of an intron inhibits TNF posttranscriptional expression in an ERK1-specific manner.** RAW264.7 cells were transfected with TNF-α intron-containing vectors and vectors containing the comparable exonic sequences but lacking introns (Fig. 2). These studies also employed the pRL-CMV Renilla vector, which contains an artificial intron (Table 1). A, transfection of the TNF Intron 3 or TNF No Intron 3 luciferase vectors as well as DN ERK1 or ERK2. DN ERK1 significantly inhibited (p < 0.01) luciferase expression from the TNF Intron 3 but not the TNF No Intron 3. Average RLU was 17,200 from TNF Intron 3 and 18,600 from TNF No Intron 3 in the NT condition. B, transfection of the TNF Intron 2 or TNF No Intron 2 luciferase vectors as well as DN ERK1 or ERK2. DN ERK1 significantly inhibited (p < 0.01) luciferase expression from the TNF Intron 2 construct but not the TNF No Intron 2 construct. Average RLU were 17,400 from TNF Intron 2 and 18,200 from TNF No Intron 2 in the NT condition. C, transfection of the pRL Renilla vector or the pRL TNF 3′-UTR Renilla vector as well as DN ERK1 or ERK2. DN ERK1 significantly inhibited (p < 0.05) Renilla expression from the TNF-α 3′-UTR vector but not the backbone pRL vector. Average RLU was 330,000 from Renilla and 5,700 from Renilla TNF 3′-UTR in the NT condition.
Next, we examined if ERK inhibition in the context of LPS stimulation altered the observations seen in Fig. 3. For these studies, we employed the TNF Intron 3 and TNF No Intron 3 constructs. In addition to the DN ERK1 and ERK2 vectors, we also employed a DN MEK1 vector as well as the MEK1 inhibitor PD98059. Fig. 4A shows the effect of different methods of ERK inhibition in unstimulated RAW264.7 cells on luciferase expression for the TNF Intron 3 and TNF No Intron 3 vectors. ERK inhibition did not reduce luciferase expression from the TNF No Intron 3 vector. Luciferase expression from the TNF Intron 3 vector was significantly inhibited ($p < 0.01$) in all instances of ERK1 inhibition (DN ERK1, DN MEK1, and PD98058 treatment) but not by DN ERK2. LPS stimulation (10 ng/ml, 2 h) (Fig. 4B) significantly increased ($p < 0.001$) luciferase expression from both the TNF Intron 3 and TNF No Intron 3 constructs, and the magnitude of the increase was comparable. Inhibition of ERK1 with DN ERK1 or DN MEK1 or by treatment with PD98059 reduced luciferase expression from the TNF Intron 3 vector but not the TNF No Intron 3 vector. Fig. 4C compares TNF luciferase values from unstimulated and normalized LPS stimulation. These data demonstrate that with regard to the presence of an intron, LPS stimulation was not able to overcome the effect of ERK inhibition in RAW264.7 cells.

Previous work has demonstrated that the TNF ARE is required for ERK-regulated nuclear export of the TNF-α message (26). To confirm this finding in our system, we deleted the ARE from the TNF Intron 3 constructs and compared luciferase expression from the TNF Intron 3 ΔARE vector with that from the TNF Intron 3 vector in unstimulated (Fig. 5A) and LPS-stimulated RAW264.7 cells (Fig. 5B). In unstimulated cells, deletion of the ARE from the TNF Intron 3 construct abolished ERK responsiveness. Deletion of the ARE also abolished the LPS-mediated increase in TNF Intron 3 luciferase expression, although absolute luciferase expression from the TNF Intron 3 ΔARE was about 10 times higher than luciferase expression from the TNF Intron 3 vector. Fig. 5C shows unstimulated and normalized LPS luciferase data as in Fig. 4B. These data confirm previous findings that the TNF-α ARE is required for ERK regulation of TNF-α mRNA nuclear export (26).

In order to conclusively demonstrate that the reduction in TNF Intron 3 luciferase expression seen with ERK inhibition is a consequence of nuclear retention of the TNF Intron 3 message, we performed nuclear/cytoplasmic fractionation of transfected RAW264.7 cells, isolated RNA, and performed Northern blotting for luciferase mRNA. These studies employed the TNF Intron 3 vector, the TNF Intron 3 ΔARE vector, and the TNF No Intron 3 vector. The cells were treated with either Me$_2$SO or PD98059 (Fig. 6A). Me$_2$SO-treated RAW264.7 cells transfected with the TNF Intron 3 RNA had a cytoplasmic to nuclear distribution of 81% cytoplasmic, 19% nuclear (Fig. 6B). Treatment with PD98059 shifted this distribution to 41% cytoplasmic, 59% nuclear. These distribution are comparable with those seen with endogenous TNF-α message in macrophages from the
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TPL2Δ/Δ animals as well as from macrophages treated with PD98059 (26). Blotting the same membrane for histone H2AX as a loading control demonstrates that this message does not shift into the nucleus with PD98059 treatment. The TNF ΔARE RNA had a cytoplasmic distribution of 89% cytoplasm, 11% nuclear, and this did not change with ERK inhibition (87% cytoplasm, 13% nuclear). Similarly, the TNF No Intron 3 RNA had a cytoplasmic distribution of 89% cytoplasm, 11% nuclear, and this did not change with ERK inhibition (87% cytoplasm, 13% nuclear). The percentage decrease was also nearly identical with the control RNAi (23%) and HuR RNAi (22%).

Next we sought to determine the trans-acting proteins involved in regulating TNF-α nuclear export. First, we examined the role of two established TNF-α ARE-binding proteins, HuR and TTP, in ERK-regulated TNF-α nuclear export (Fig. 7). The percentage decrease was also nearly identical with the control RNAi (23%) and HuR RNAi (22%).

Finally, we tested the effect of tethering four EJC proteins previously established as involved in the export of mRNA (for a review, see Refs. 54 and 55): UAP56, Aly, TAP, and NxT1. These proteins were tethered to the TNF-α 3′-UTR containing luciferase message via a heterologous RNA-binding domain, the bacteriophage P22 N-peptide, as described by Wiegand et al. (34) (Fig. 8A). The N-peptide specifically binds to the B-box sequence, four copies of which were cloned into the 5′-UTR of the pGL3 control and TNF-α 3′-UTR luciferase construct. Neither of these constructs contains an intron. Co-transfecting different N-peptide fusion EJC proteins with B-box sequence-containing luciferase vectors results in the binding of the N-fusion protein to the mRNA through the interaction of the N}

FIGURE 5. Deletion of the TNF-α ARE abolishes the effect of ERK inhibition on TNF Intron 3 luciferase expression. A, comparison of the TNF Intron 3 and TNF Intron 3 ΔARE luciferase vectors in RAW264.7 cells with ERK inhibition. Loss of the ARE eliminated ERK responsiveness. Average RLU were 17,200 from TNF Intron 3 and 160,000 from TNF Intron 3 ΔARE in the NT condition. B, comparison of the TNF Intron 3 and TNF Intron 3 ΔARE luciferase vectors in LPS-stimulated cells with ERK inhibition. Loss of the ARE eliminated ERK responsiveness and abolished the increase in luciferase expression seen with LPS stimulation. Average RLU were 32,900 from TNF Intron 3 and 157,000 from TNF Intron 3 ΔARE vectors. There is no significant difference in the effect of ERK inhibition in resting and activated cells.
peptide with the B-box sequence. Thus individual EJC proteins are tethered to the luciferase mRNA in the absence of an intron.

Using the tethering assay, we compared the effect of co-transfected N-peptide proteins with and without PD98059 treatment (Fig. 8B). These studies demonstrate that tethering either TAP of NxT1 to the message results in a significant (p < 0.05) decrease in TNF-α 3′-UTR luciferase expression. Tethering UAP56 or Aly and treating with PD98059 did not result in a significant decrease in TNF-α 3′-UTR luciferase expression. There was no effect of PD98059 treatment on luciferase expression of the B-box control luciferase vector with any of the N-fusion proteins (data not shown). NxT1 binds to both TAP and nucleoporin, serving as a bridge between the EJC complex and nuclear pore. We conclude that the interaction of the TAP-NxT1 complex with the message is necessary for ERK1 regulation in TNF-α mRNA nuclear export.

**DISCUSSION**

The MAP kinase pathways are critical regulators of TNF-α expression. We sought to examine the effect of combined MAP kinase inhibition on TNF-α posttranscriptional expression. Consistent with previous findings, our data confirm the importance and dominance of the p38 pathway in TNF-α posttranscriptional regulation. Additionally, our studies demonstrate that JNK inhibition has little effect on TNF-α posttranscriptional expression in unstimulated cells but results in a significant reduction in TNF-α posttranscriptional production in LPS-stimulated macrophages. More importantly, our studies demonstrate that nuclear export of the TNF-α message requires the interaction of the EJC protein complex TAP-NxT1 with the message (Fig. 9).

In metazoans, the presence of an intron catalyzes the deposition of the EJC (for a review, see Refs. 60 and 61). Following splicing, the EJC...
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is reorganized, and proteins that regulate mRNA nuclear export are recruited (33). Importantly, it has been demonstrated that the yeast homologues of Aly and Tap, Yra1 and Mex67, each associate with distinct and overlapping subsets of messages during export from the nucleus (62). This finding indicates that export of specific messages is a regulated process that differs for different genes. Our data add an additional level of complexity by establishing that the TAP-NxT1 complex and ARE-bound proteins are both involved in regulating mRNA export.

Although the final effect of ERK1 inhibition of TNF luciferase expression, a 25–30% reduction, may appear modest, these data are physiologically appropriate given the complex posttranscriptional regulation controlling TNF-α expression. For example, we have demonstrated that inhibition of TTP function by LPS stimulation increases TNF-α mRNA half-life by about 40% (63). The complete loss of TTP in TTP−/− animals does not even double the half-life of the TNF-α mRNA (64), but the consequence is inflammatory arthritis and cachexia (65). Thus, modest changes in the posttranscriptional control of TNF-α expression can result in dramatic downstream effects. These data also illustrate the lack of correlation between mRNA and protein level within a cell (66), particularly for messages subject to extensive posttranscriptional regulation.

Our data add to the growing complexity and linkage between what have previously been viewed as distinct events. Examining the series of events leading to the production and export of a mature TNF-α message reveals a number of elegant links. Extensive work has established that LPS or cytokine stimulation increases TNF-α mRNA transcription via the NFκB pathway (7, 9, 11). However, NFκB is also critically involved in the control of TNF-α mRNA nuclear export. In resting macrophages, Tp2 is bound by NFκB p105, which prevents both Tp2 degradation and phosphorylation of MEK1 (67, 68). Activating macrophages with TLR ligands or inflammatory cytokines results in IKK activation, and IKKB phosphorylates both NFκB p105 and Tp2, resulting in the release and activation of Tp2 (28, 69, 70). NFκB p105 is cleaved to p50, and the p50-Rel complex translocates to the nucleus and activates TNF-α gene transcription (for a review, see Ref. 19). The released Tp2 activates the ERK pathway, which is required for TNF-α mRNA nuclear export. In our studies, LPS activation of ERK was not required, since we observed the same effect of ERK inhibition in LPS-stimulated and -unstimulated cells. Our data indicate the stimulus independence of ERK activity, since ERK inhibition was equally effective in LPS-stimulated and -unstimulated cells. However, it is possible that the basal level of ERK activation in RAW264.7 cells (53) or the higher level of mRNA expression from the reporter construct as compared with endogenous TNF-α mRNA are responsible for this effect.

In conclusion, our data establish that it is specifically ERK1 that is responsible for regulation of TNF-α nuclear export. Further, we demonstrate that the tethering of TAP or NxT1 to a message containing the TNF-α 3′-UTR is sufficient to confer ERK regulation of TNF-α mRNA nuclear export. The simplest explanation for our data is that the TAP-NxT1 complex interacts with one or more AU-rich element-binding proteins to control export. This model seems the most likely, since there is an extensive literature regarding the regulation of ARE-binding proteins by the MAP kinase pathways, whereas there are no reports of MAP kinase regulation of the TAP-NxT1 complex. However, we cannot exclude the possibility that each complex operates independently or that each is independently targeted by ERK1. Regardless, it is clear from our data and that of previous work (26) that TNF-α mRNA export operates through two separate mechanisms, one regulated by ERK1 and one independent of ERK1.

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