Oncogene (2002) 21, 4237–4246

www.nature.com/onc

Multiple tristetraprolin sequence domains required to induce apoptosis and modulate responses to TNFα through distinct pathways

Barbra A Johnson[1] and T Keith Blackwell*,[1]

[1]*Center for Blood Research and Department of Pathology, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts, MA 02115, USA*

Expression of the immediate early protein tristetraprolin (TTP) is induced by numerous stimuli, including tumor necrosis factor-alpha (TNFα). Evidence indicates that TTP limits production of TNFα and other cytokines by directly binding and destabilizing their mRNAs. This effect seems to require only the conserved TTP zinc finger region, and is characteristic of the related proteins TIS11b and TIS11d. TTP, TIS11b, and TIS11d each also induce apoptosis through the mitochondrial pathway analogously to certain oncogenes, suggesting that they influence growth or survival signals. Among TTP/TIS11 proteins, TTP alone also promotes apoptosis synergistically with TNFα. Here we show that other regions of TTP along with the zinc fingers are required for TTP to induce apoptosis. We also demonstrate that TTP acts through an additional pathway to sensitize cells to the pro-apoptotic stimulus of TNFα. This modulation of TNFα responses specifically requires the TTP N-terminal region, which is not conserved in TIS11b or TIS11d. We conclude that the physiological functions of TTP depend upon multiple regions of the TTP protein, that TTP has diverged functionally from TIS11b and TIS11d, and that modulation of TNFα responses may be a unique and important aspect of TTP function.
Oncogene (2002) **21,** 4237–4246. doi:10.1038/sj.onc.1205526

Keywords: tristetraprolin; TNFα; immediate-early; apoptosis; zinc finger

Introduction

Tristetraprolin (TTP; also Nup475 and TIS11) is an immediate-early protein that is expressed transiently in response to numerous extracellular agents, including the cytokine TNFα (Carballo *et al*., 1998; DuBois *et al*., 1995; Lai *et al*., 1990; Varnum *et al*., 1989). In mice, disruption of the TTP gene causes a general inflammatory and arthritic syndrome which is mediated largely by TNFα, and is associated with increased levels and half-life of the TNFα mRNA (Carballo *et al*., 1997; Taylor *et al*., 1996a). The localization, stability, and translation of TNFα and other cytokine mRNAs are regulated through an AU-rich element (ARE) that is located within their respective 3′ untranslated regions, and responds to multiple signaling pathways (Chen and Shyu, 1995; Dumitru *et al*., 2000; Kontoyiannis *et al*., 1999, 2001; Kotlyarov *et al*., 1999; Rutault *et al*., 2001; Vasudevan and Peltz, 2001; Winzen *et al*., 1999). TTP binds directly to the AREs of TNFα and other cytokine mRNAs *in vitro*, and in transfection assays TTP acts through the ARE to destabilize these mRNAs (Carballo *et al*., 1998, 2000; Chen *et al*., 2001; Lai *et al*., 1999; Mahtani *et al*., 2001; Ming *et al*., 2001; Raghavan *et al*., 2001). These findings suggest that TTP modulates cytokine production through direct ARE-dependent mRNA destabilization (Carballo *et al*., 1998, 2000; Lai *et al*., 1999).

TTP and the related proteins TIS11b and TIS11d (TTP/TIS11 proteins) consist of two conserved tandem Cys-X_8-Cys-X_5-Cys-X_3-His (CCCH) zinc fingers, along with divergent N- and C-terminal regions (Figure 1a,c). Other CCCH zinc finger proteins have been implicated in control of mRNA transcription, processing, or translation (Bai and Tolias, 1996; Barabino *et al*., 1997; Batchelder *et al*., 1999; Guedes and Priess, 1997; Tabara *et al*., 1999; Tenenhaus *et al*., 2001; Zuo and Maniatis, 1996). In transfection assays, the TNFα mRNA is bound, deadenylated, and apparently destabilized by all three TTP/TIS11 proteins and, surprisingly, by a 77 amino acid TTP fragment that contains the TTP zinc finger region (Lai *et al*., 2000). These last findings raise the question of whether other regions of the TTP protein contribute to essential aspects of its physiological functions.

Other evidence suggests that TTP has additional functions besides influencing cytokine mRNA stability. A wide variety of growth factors and mitogens induce transient TTP expression in numerous cell types, and in mice TTP is expressed in regenerating small intestine, liver, and lung, as well as in hematopoietic tissues (Carballo *et al*., 1998; DuBois *et al*., 1995, 1990; Ehrenfried *et al*., 1995; Heximer and Forsdyke, 1993; Landesberg *et al*., 2001; Nakajima and Wall, 1991; Taylor *et al*., 1996a;

*Correspondence: TK Blackwell;
E-mail: blackwell@cbr.med.harvard.edu
Received 14 December 2001; revised 18 March 2002; accepted 26 March 2002

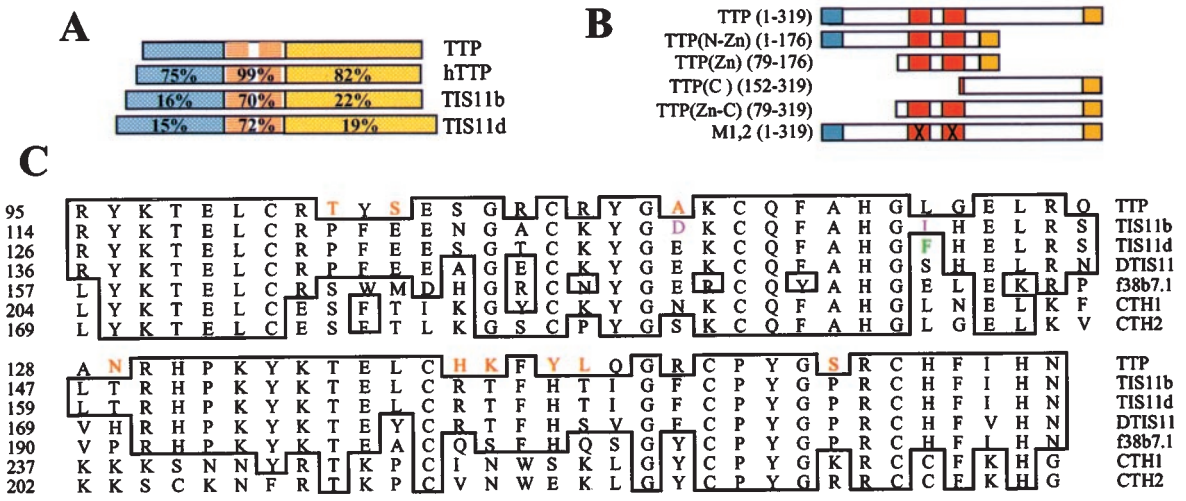

Figure 1 Mutagenesis analysis of TTP. (**a**) Diagram showing the percentage identity between different regions of mouse TTP/TIS11 proteins and human TTP (hTTP, or GOS24 (Heximer and Forsdyke, 1993)). The CCCH zinc finger regions are represented in red. Blue and orange denote the N and C terminal regions respectively. (**b**) TTP mutants. Red boxes indicate the CCCH zinc fingers, and each cross in mutant M1,2 indicates the substitution of a critical zinc finger cysteine with serine (Johnson *et al*., 2000). Blue and orange boxes represent the 23 and 21 amino acids at the TTP N- and C-termini, respectively, against which anti-peptide antibodies were raised (nTTP and cTTP). Amino acids present in each construct are indicated in parentheses, except that TTP(N-Zn) and TTP(Zn-C) also include the 21 C-terminal amino acids to allow detection with cTTP. (**c**) Comparison of the zinc finger regions of mouse TTP/TIS11 proteins (TTP, TIS11b and TIS11d; Genbank accession numbers P22893, P23950 and P23949, respectively) with those of their closest relatives in *D. melanogaster* (DTIS11; P47980), *C. elegans* (F38b7.1; T21955) and *S. cerevisiae* (CTH1; S57977 and CTH2; P47977). Sequence similarities to mouse TIS11b (Clustal method) are boxed. Signature residues that define vertebrate TTP, TIS11b, and TIS11d proteins (Lai *et al*., 2000) are shown in red, purple and green, respectively. Positions are numbered as in the full-length proteins

Varnum *et al*., 1991). TTP expression is also induced during responses to some apoptotic stimuli, including BRCA1 expression and withdrawal of growth factors from neuronal cells (Haas *et al*., 1993; Harkin *et al*., 1999; Mesner *et al*., 1995; Mittelstadt and DeFranco, 1993). We have determined that continuous expression of each TTP/TIS11 protein induces various primary and transformed cell types to undergo apoptosis (Johnson *et al*., 2000). This response depends upon the mitochondrial apoptosis pathway and is enhanced by growth factor deprivation, analogously to cell death induced by c-Myc, E1A, and certain other oncogenes. These observations together suggest that TTP influences growth or survival pathways, and that TTP, like some other immediate early genes, may have a physiological role in apoptosis induction (Johnson *et al*., 2000).

When TTP is expressed at levels that are insufficient to cause widespread cell death, it induces apoptosis synergistically with TNFα (Johnson *et al*., 2000). Synergistic induction of apoptosis in combination with TNFα is also a property of the immediate-early oncoprotein c-Myc, the pro-apoptotic factor E2F-1, and the adenovirus oncogene E1A (Chen *et al*., 1987; Janicke *et al*., 1994; Klefstrom *et al*., 1997; Phillips *et al*., 1999). In striking contrast to TTP however, neither TIS11b nor TIS11d act synergistically with TNFα (Johnson *et al*., 2000). This is the only functional difference that has been identified among TTP/TIS11 proteins, suggesting that this synergy between TTP and TNFα may reflect a physiological function carried out by TTP alone. TNFα causes apoptosis by binding to its Type I receptor and activating caspase 8, an apoptotic stimulus that under certain circumstances can be amplified by activation of the mitochondrial apoptosis pathway (Green, 2000). Simultaneously, TNFα stimulates pathways that promote cell survival, proliferation, and activation (Baker and Reddy, 1996; Green, 2000). To understand how TTP and TNFα act synergistically, it is essential to determine whether TTP and TNFα simply cooperate to activate the mitochondrial apoptosis pathway, or whether TTP influences the balance between the survival and apoptotic stimuli of TNFα.

In this study, we have investigated how different regions of the TTP protein contribute to apoptosis induced solely by TTP expression, and by TTP acting in conjunction with TNFα. We find that the TTP zinc finger region fails to induce apoptosis independently of either the N- or C-terminal regions, suggesting that multiple regions of the TTP protein are critical for its functions *in vivo*. We have also determined that TTP and TNFα induce apoptosis synergistically independently of mitochondrial involvement, indicating that TTP induces apoptosis on its own and modulates responses to TNFα through distinct pathways. Finally, both the zinc finger and unique N-terminal regions of TTP are required to sensitize cells to the apoptotic stimulus of TNFα, suggesting that this activity of TTP reflects a specific functional divergence between TTP and other TTP/TIS11 proteins.

Results

Multiple regions of TTP are required for its induction of apoptosis

The only predicted functional domains within TTP are its tandem zinc fingers, but outside of these elements TTP is highly conserved between mouse and human (Figure 1a). This similarity suggests that other regions of TTP in addition to the zinc fingers are likely to be important for TTP functions *in vivo*, and may be required for TTP to induce apoptosis. To test this model, we constructed a series of TTP truncation mutants by dividing the protein into N-terminal, zinc finger and C-terminal regions, and retaining overlapping sequences to minimize possible disruption of functional elements (Figure 1b). For example, the 98 amino acid TTP(Zn) mutant contained flanking residues on both sides of the two CCCH zinc fingers. We then assayed these TTP mutants for induction of apoptosis by transfecting them into 3T3 cells as described previously (Johnson *et al*., 2000).

When expressed continuously by transfection, TTP induces cell death that is associated with TUNEL positivity and pyknotic nuclei, and is inhibited by Bcl-2 co-expression, indicating apoptosis that is dependent upon the mitochondrial pathway (Figure 2a; not shown) (Johnson *et al*., 2000). The levels at which TTP is expressed under these conditions overlap with levels to which endogenous TTP is induced transiently in response to extracellular stimuli (Johnson *et al*., 2000). In addition, significant apoptosis is associated with low level expression of TTP, suggesting that this cell death derives from continuous presence of TTP and does not require excessive TTP expression (Johnson *et al*., 2000). Induction of apoptosis by TTP was nearly abrogated by point mutation of both zinc fingers (M1,2; Figure 2b), as noted previously (Johnson *et al*., 2000), and was reduced but not eliminated by deletion of either the N- or C- terminus (TTP(N-Zn) and TTP(Zn-C), respectively, Figure 2b). Like TTP, both TTP(N-Zn) and TTP(Zn-C) triggered apoptosis that could be inhibited by Bcl-2, suggesting dependence on the mitochondrial pathway (Figure 2a). As observed previously for TTP (Johnson *et al*., 2000), in each case cell death was also attenuated by the caspase inhibitor CrmA, suggesting that death receptors may be involved. In contrast, the TTP zinc fingers on their own were incapable of inducing apoptosis (TTP(Zn); Figure 2b), even when a 10-fold greater amount of expression vector was introduced (not shown).

We verified expression of these TTP mutants by Western blotting, and by similarly analysing fusion proteins in which TTP mutants were linked at their N-terminus to green fluorescent protein (GFP). Analysis with an antibody against the TTP C-terminus (cTTP) indicated that TTP, TTP(C), TTP(Zn-C), and M1,2 were expressed at comparable levels (Figure 2c, lanes 1 and 4–6). Multiple lines of evidence indicated that the TTP(Zn) and TTP(N-Zn) mutants were also expressed comparably but were not effectively detected by cTTP, presumably because the cTTP epitope is not efficiently recognized when it is present ectopically near the zinc finger region (Figure 2c, lanes 2 and 3). Parallel analysis with an antibody against the TTP N-terminus (nTTP) revealed that TTP(N-Zn) was actually produced at somewhat higher levels than TTP (Figure 2c, lanes 7 and 8). By both Western blotting and immunofluorescence, cTTP detected TTP(Zn) but not TTP(N-Zn) (Figure 2c, lanes 2 and 3; Figure 3b; not shown), indicating that TTP(Zn) was expressed at higher levels. Finally, analysis with a GFP antibody revealed that in this transfection assay, GFP-TTP(Zn) was expressed more robustly than either GFP-TTP or GFP-TTP(Zn-C) (Figure 2e, lanes 1–3; bottom panel). Like their non-tagged counterparts, GFP-TTP(Zn-C) and GFP-TTP each induced significant apoptosis, and GFP-TTP(Zn) failed to induce significant cell death (Figure 2d). Together, our findings demonstrate that the N- and C-terminal regions of TTP, in addition to its zinc fingers, are each important for induction of apoptosis.

The failure of the TTP(Zn) truncation mutant to induce apoptosis could derive from its being mislocalized to a different cellular compartment from TTP mutants that are active in this assay. To address this possibility, we used immunofluorescence to investigate how TTP mutants are localized within transfected 3T3 cells (Figure 3a,b). It has been reported that in hematopoietic cell lines TTP is present primarily in the cytoplasm, and that in quiescent fibroblasts TTP is largely nuclear, but is localized to the cytoplasm upon treatment with growth factors (Taylor *et al*., 1995, 1996b). Consistent with these observations, in nearly all transfected cycling 3T3 cells TTP staining was either predominantly cytoplasmic, or was distributed in a generalized fashion (Figure 3a,b). Mutation of the zinc fingers decreased the fraction of cells in which TTP was localized solely within the cytoplasm (M1,2, Figure 3a,b), indicating that the functional integrity of the zinc fingers is important for the normal distribution of TTP within the cell. TTP(N-Zn), TTP(Zn-C), and TTP(Zn) were each localized predominantly to the nucleus (64, 97, and 96% nuclear respectively; Figure 3a,b), but while TTP(N-Zn) and TTP(Zn-C) induced apoptosis, TTP(Zn) did not (Figure 2b,d). The finding that TTP(Zn) and TTP(Zn-C) differed dramatically in induction of apoptosis but were localized similarly (Figures 2a,d; 3a,b) suggests that the inactivity of TTP(Zn) in this assay does not derive simply from its localization within the cell.

TTP sensitizes cells to TNFα-induced apoptosis through a different mechanism

Among the three TTP/TIS11 proteins, a single functional difference has been identified: only TTP induces apoptosis synergistically with TNFα (Johnson *et al*., 2000). Because this functional synergy is a specific property of TTP, it would be predicted to require sequences that vary among TTP/TIS11 proteins. In vertebrates, TTP, TIS11b, and TIS11d can be

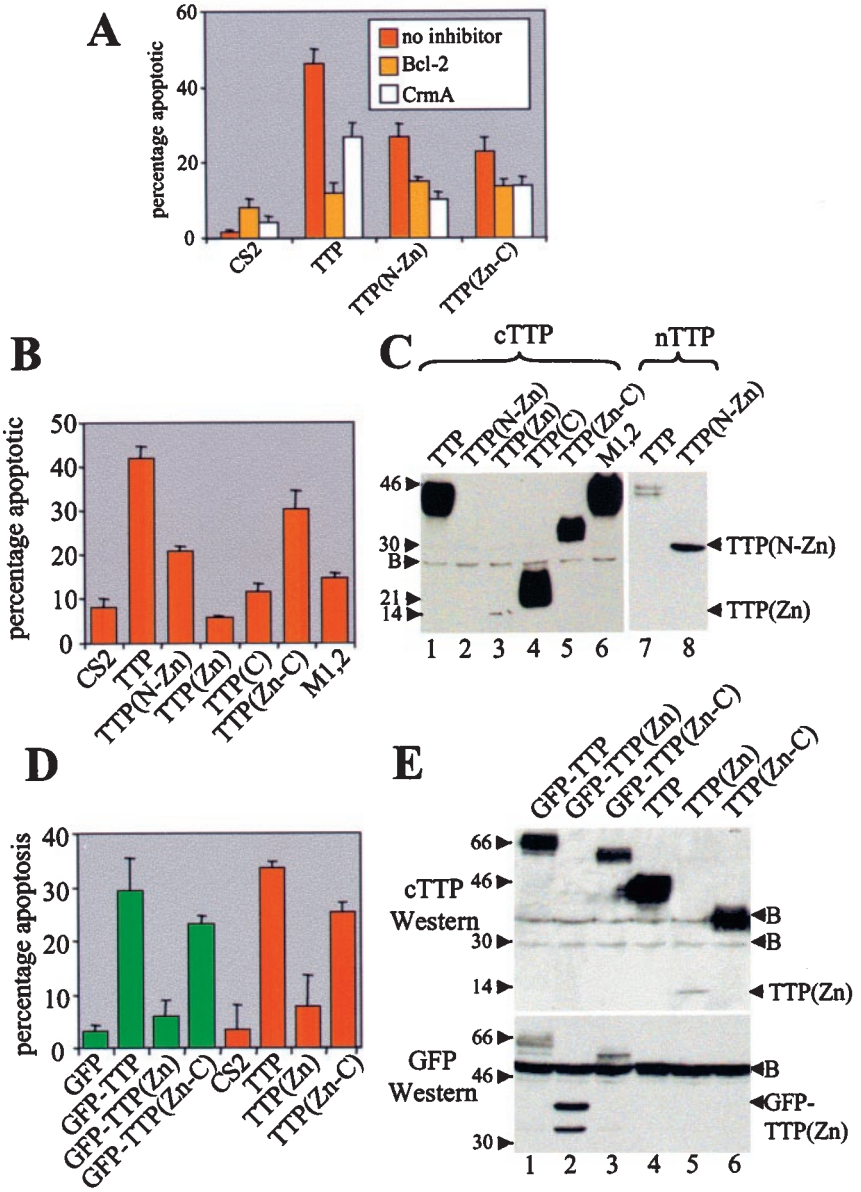

Figure 2 Multiple regions of TTP are necessary to induce apoptosis. (**a**) Inhibition of TTP-induced cell death by Bcl-2 and CrmA. Cells were transfected with 200 ng of constructs that expressed the indicated TTP mutants (Figure 1b), along with 100 ng of β-gal reporter and either 200 ng of Bcl-2 or 1.7 μg of CrmA vector, using Lipofectamine and 2 μg total DNA. After 24 h, cells were X-gal stained and the percentage of apoptotic blue cells was determined. Bars represent the mean of four wells and error bars represent the standard deviation. (**b**) Apoptosis induced by TTP mutants. 3T3 cells were transfected and assayed for apoptosis as in **a**. (**c**) Western blot showing TTP expression levels in a duplicate of the experiment in **b**. Molecular weights and a background band are indicated to the left of the gel. cTTP and nTTP indicate the antibodies used for detection. Lanes 7 and 8 are derived from the same lysates as lanes 1 and 2, respectively. (**d**) Induction of apoptosis by GFP-tagged TTP proteins (green), compared to their untagged counterparts (red), assayed as in **b**. Each bar represents the mean of 2 wells. (**e**) TTP expression in a duplicate of the experiment in **d**, assayed using cTTP and GFP antibodies as in **c**. The lower GFP-TTP(Zn) band in lane 2 (bottom) presumably corresponds to a breakdown product

distinguished from each other by amino acids within their respective zinc finger regions (Lai *et al*., 2000) (Figure 1c), but these proteins are far more divergent within regions that flank the zinc fingers (Figure 1a). We have used the TTP deletion mutants shown in Figure 1b to determine whether these divergent flanking regions are required for TTP to act synergistically with TNFα.

As reported previously, when TTP was expressed at levels that cause only modest apoptosis, treatment with low concentrations of TNFα resulted in a significant increase in cell death (Figure 4a). In contrast, the TTP(Zn) and M1,2 mutants (Figure 1b) each failed to synergize with TNFα to induce apoptosis (Figure 4a). No significant TNFα-dependent apoptosis occurred when TTP(Zn) was expressed at much higher levels

A

	antibody	percentage cytoplasmic	generalized	nuclear
TTP	nTTP	26	71	2
TTP	cTTP	24	76	0
TTP(N-Zn)	nTTP	5	31	64
TTP(Zn)	cTTP	0	4	96
TTP(Zn-C)	cTTP	0	3	97
M1,2	cTTP	2	62	36

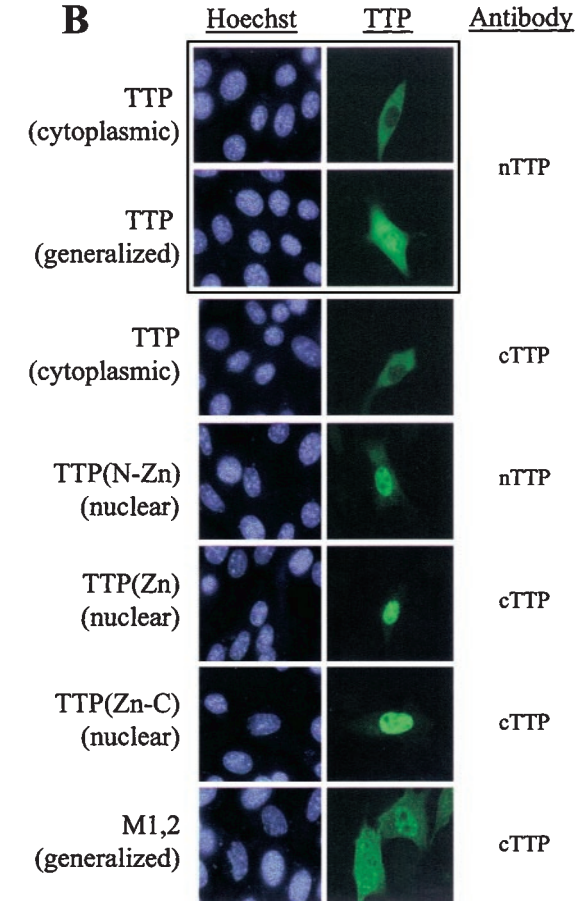

Figure 3 Subcellular localization of TTP mutants. (**a**) 3T3 cells were transfected as in Figure 2a with 200 ng of the indicated TTP mutant expression vector (Figure 1b), along with 200 ng of Bcl-2 vector to prevent apoptosis, then after 24 h were fixed and stained with the indicated TTP antibody. A representative experiment is shown. The subcellular localization of these various TTP mutants was generally comparable over a wide range of expression levels (not shown). (**b**) Representative cells in which TTP mutants were localized as classified in **a**, at 100 × magnification. Antibodies used for staining are indicated to the right, and a box surrounds views of two fields from the same TTP sample. Endogenous TTP is not detectable in these continuously dividing cells (not shown)

(Figure 4c,d), further supporting the idea that the TTP zinc fingers are necessary but not sufficient for this effect. TTP(Zn-C) similarly failed to promote TNFα-dependent apoptosis (Figure 4a), even when it was expressed at levels that caused significant apoptosis in the absence of TNFα (Figure 4c,d). In striking contrast, TTP(N-Zn) was reproducibly comparable to TTP in promoting TNFα-dependent apoptosis (Figure 4a,c,d; not shown). When activation of survival factors by TNFα was suppressed by cycloheximide, low concentrations of TNFα induced rapid apoptosis in TTP-expressing cells (Figure 4b). In the presence of cycloheximide, TNFα treatment similarly caused rapid apoptosis of cells that expressed TTP(N-Zn), but TTP(Zn-C) was only marginally active (Figure 4b). We conclude that the TTP N-terminal and zinc finger regions are together specifically required for TTP to promote apoptosis synergistically with TNFα.

TTP and TNFα could induce apoptosis synergistically by acting together through the mitochondrial apoptosis pathway, which is required for TTP/TIS11 proteins to induce cell death independently of TNFα (Johnson *et al*., 2000). Alternatively, and of greater potential significance, TTP could influence the apoptotic or survival signaling pathways that are activated directly by TNFα. Both models predict that apoptosis derived from the synergistic effects of TTP and TNFα should be attenuated by CrmA, which inhibits cell death that occurs in response to either TNFα or TTP (Figure 2a) (Nakajima and Wall, 1991; Ray *et al*., 1992). Accordingly, CrmA suppressed TNFα-dependent apoptosis in cells that expressed either TTP or TTP(N-Zn) (Figure 5a,b). If the synergistic effects of TTP and TNFα are mediated through the mitochondrial apoptosis pathway, the resulting synergistic increase in cell death should also be inhibited by Bcl-2, which robustly suppresses apoptosis caused by either TTP or TTP(N-Zn) alone (Figure 2a). In striking contrast, Bcl-2 expression failed to inhibit cell death caused by the synergistic effects of TTP or TTP(N-Zn) together with TNFα (Figure 5a,b). This last finding suggests that the mitochondrial pathway is not required for TTP and TNFα to induce apoptosis synergistically, implying that this cell death is caused by direct caspase activation by TNFα. We conclude that TTP sensitizes cells to this apoptotic stimulus, presumably by affecting signaling pathways directly downstream of TNFα (Figure 6).

Discussion

Multiple TTP domains are required for its functions

Dose-response transfection studies suggest that the TTP zinc finger region can independently bind and destabilize the TNFα mRNA (Lai *et al*., 2000). This indicates that the remainder of the TTP protein might not be essential for its functional activity, but may instead play regulatory roles. In apparent contrast, we have observed that the isolated TTP zinc finger region fails to induce apoptosis or to sensitize cells to the apoptotic stimulus of TNFα (Figures 2 and 4). Multiple lines of evidence indicate that the inactivity of the TTP zinc finger region fragment that we analysed (TTP(Zn)) did not derive from inappropriate

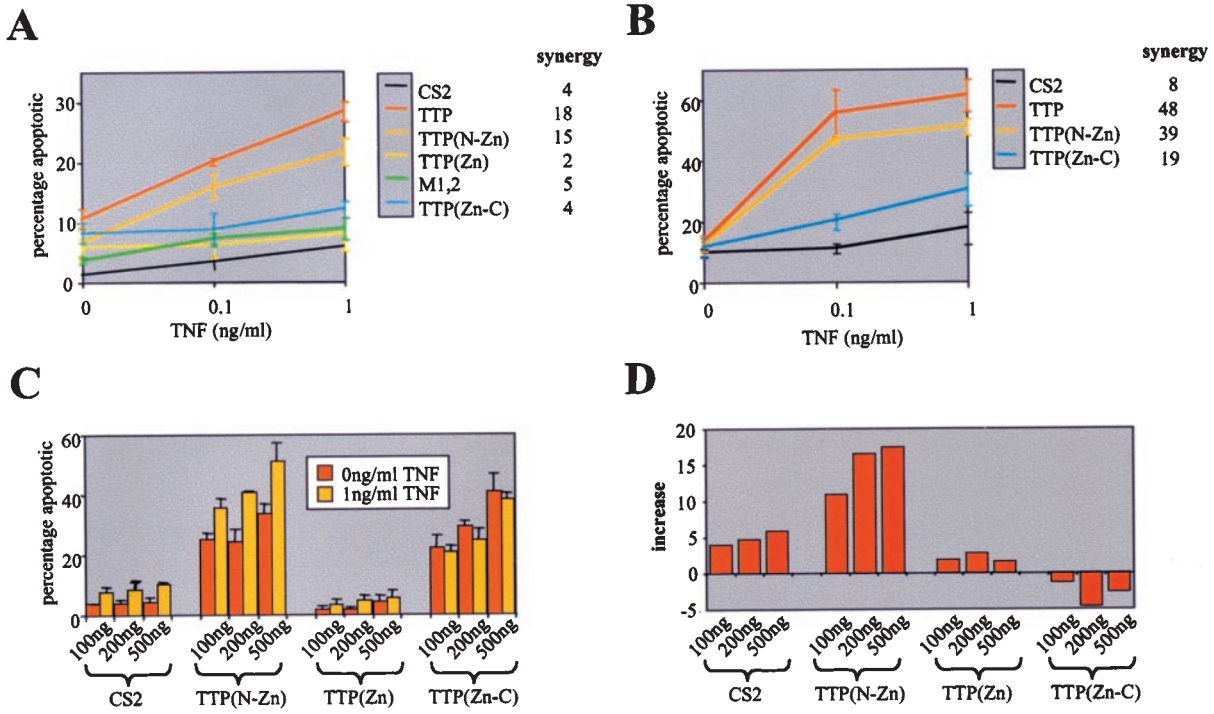

Figure 4 The TTP N-terminal and zinc finger regions are required to induce apoptosis synergistically with TNFα. (**a**) Both TTP and TTP(N-Zn) act synergistically with TNFα. A representative experiment in which 3T3 cells were transfected with 25 ng of the indicated TTP expression or CS2 control vector along with 100 ng of β-gal reporter plasmid, using Lipofectamine Plus and 1 μg total DNA. TNFα was added to the indicated concentrations after 3 h, then after an additional 21 h cell death was assayed as in Figure 2a. Each point is the mean of four wells in a typical experiment. The increase in mean percentage apoptosis associated with exposure to 1 ng ml^{-1} TNFα is defined as 'synergy,' and is indicated on the right for each transfected protein. For TTP, this apoptosis synergy varied between 15 and 33% over several experiments (not shown). (**b**) Rapid synergistic induction of apoptosis by TTP and TNFα when protein synthesis is inhibited. Twenty-four hours after cells were transfected as described in **a**, they were incubated in TNFα and cycloheximide (10 μg ml^{-1}) for 4 h, then assayed for apoptosis. (**c**) TTP(Zn) and TTP(Zn-C) do not promote apoptosis synergistically with TNFα, even when expressed at higher levels. Transfections were performed and analysed as in **a**, but with the indicated amounts of expression plasmid. (**d**) Increase in apoptosis associated with addition of TNFα, determined as in **a**, for each transfection shown in **c**

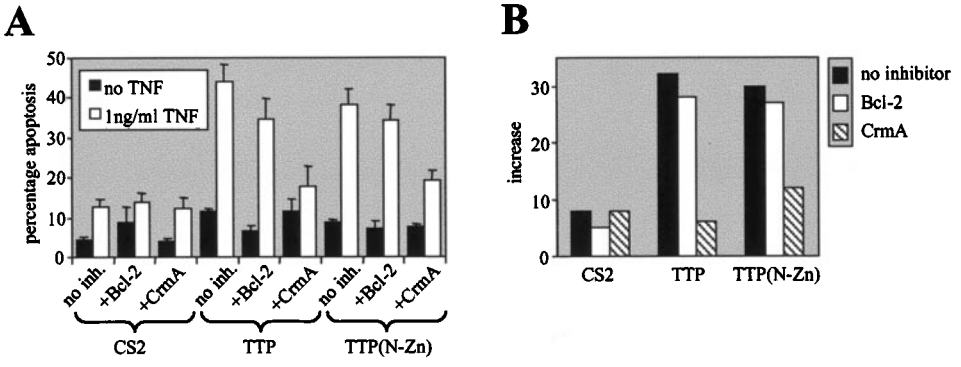

Figure 5 TTP sensitizes cells to the apoptotic stimulus of TNFα independently of the mitochondrial pathway. (**a**) TTP promotes Bcl-2-resistant apoptosis in response to TNFα. Transfections were performed and assayed for cell death as in Figure 4a, except that either 100 ng of Bcl-2 or 875 ng of CrmA expression vector was added as indicated. (**b**) Apoptotic synergy between TNFα and TTP is inhibited by CrmA, but not Bcl-2. The percentage increase in apoptosis associated with TNFα addition is graphed for each data set described in **a** (CS2, TTP, TTP(N-Zn))

folding or other artifactual effects. Firstly, TTP(Zn) included the entire 77 amino acid TTP fragment that binds and acts on the TNFα mRNA (Lai *et al*., 2000). In addition, the presence of the TTP N- or C-terminal region along with the zinc fingers partially restored apoptotic activity (Figure 2b,d). Finally, the TTP(Zn-C) mutant robustly induced apoptosis, indicating that it is functional, but it did not sensitize cells to TNFα-

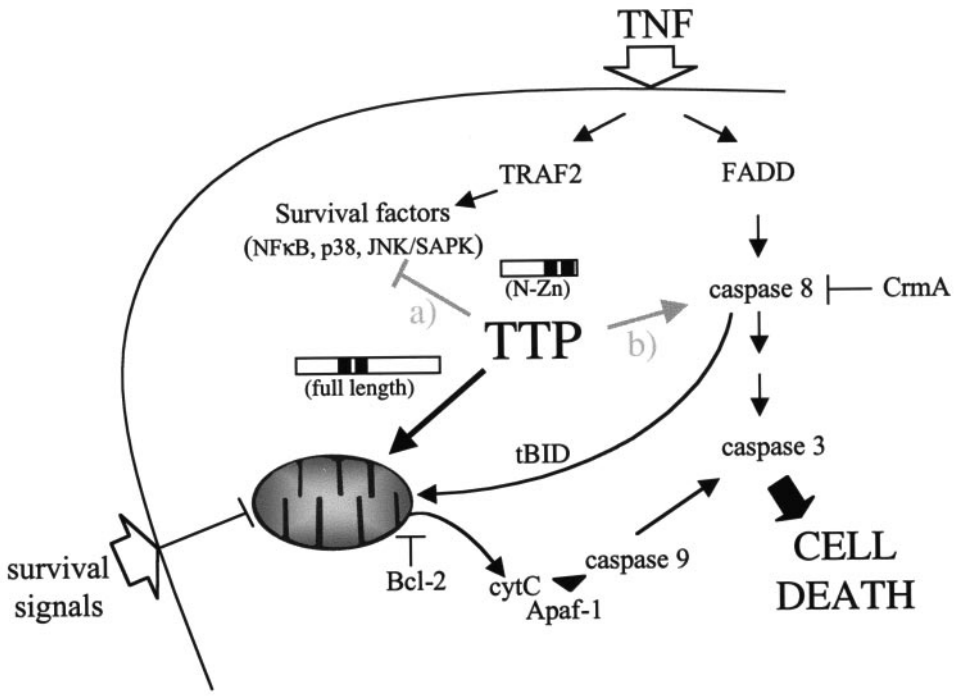

Figure 6 Diagram illustrating how TTP influences apoptosis signaling pathways. TTP induces apoptosis through the mitochondrial pathway, which is inhibited by Bcl-2 (Johnson *et al*., 2000). This effect requires that either the TTP N- or C-terminal regions be present in addition to the zinc fingers, and is reduced by deletion of either of these regions (Figure 2). When TTP is expressed at lower levels, treatment with modest concentrations of TNFα induces apoptosis that is resistant to Bcl-2 but sensitive to CrmA (Figure 5), implying that this cell death derives from direct apoptotic signaling by TNFα (Green, 2000). This effect of TTP on the response to TNFα specifically requires the TTP N-terminal region along with the zinc fingers (N-Zn; Figure 4). Grey lines indicate that TTP may act downstream of TNFα either to (a) inhibit survival signals, or (b) enhance direct apoptotic signals

induced apoptosis (Figure 2a,b, 4c,d). Our findings strongly suggest that regions of TTP outside of the zinc fingers are likely to be important for its functions *in vivo* (Figure 1a).

Both the N- and C-terminal regions of TTP influence its localization within the cell. TTP(Zn) was present predominantly in the nucleus, and the N- and C-terminal regions were each required for normal localization of TTP to the cytoplasm (Figure 3). After submission of this manuscript, it was reported that the entire C-terminal region of TTP is dispensable for its localization to the cytoplasm (Phillips *et al*., 2002). One possible explanation for this discrepancy is that the latter finding was obtained using GFP-TTP fusion proteins. We have observed that fusion of GFP to its amino terminus abrogates the effects of TTP on TNFα responses, and generally enhances the cytoplasmic localization of the various TTP mutants we have analysed (not shown). Apparently, the presence of GFP at its amino terminus interferes with or masks effects of some functional elements within TTP. Because we did not observe a strict correlation between induction of apoptosis and subcellular localization of TTP (Figures 2b and 3a), we conclude that the TTP N- and C-terminal regions are not required simply to localize the TTP zinc fingers, but are involved in interactions or signals that are important for TTP activity. Consistent with this view, expression of TTP zinc finger mutants that lack RNA binding activity stabilizes ARE-containing mRNAs, suggesting that these mutants may act as dominant negatives (Lai *et al*., 2002). In addition, phosphorylation of TTP within regions outside of the zinc fingers not only influences TTP subcellular localization (Johnson *et al*., 2002), but also may inhibit its activity in the cell (Carballo *et al*., 2001; Zhu *et al*., 2001). The related *C. elegans* germline protein PIE-1, which also contains two CCCH zinc fingers, provides a precedent for this apparent functional complexity. Distinct but overlapping regions of PIE-1 are required for its subcellular localization, stability, and functions, which include acting in the nucleus to inhibit transcription, and in the cytoplasm to promote translation of particular mRNAs (Batchelder *et al*., 1999; Reese *et al*., 2000; Tenenhaus *et al*., 2001).

The involvement of multiple regions of the TTP protein in its pro-apoptotic effects is reassuring, because it indicates that these effects do not derive simply from forced expression of its RNA binding zinc finger region, and may reflect aspects of its physiological functions. Our findings support the hypothesis that TTP/TIS11 proteins have additional functions besides cytokine mRNA destabilization, even though TTP −/− mice lack obvious defects other than their

characteristic inflammatory syndrome (Taylor *et al*., 1996a). One possible model is that TTP influences cell survival through mechanisms that are largely redundant with other regulatory networks. How might TTP/TIS11 proteins induce apoptosis? Various other CCCH zinc finger proteins also regulate genes post-transcriptionally. In *C. elegans*, for example, multiple proteins with two characteristic CCCH zinc fingers either promote or inhibit translation (Guedes and Priess, 1997; Schubert *et al*., 2000; Tabara *et al*., 1999; Tenenhaus *et al*., 2001). TTP/TIS11 proteins may similarly influence either stability or translation of mRNAs that are critical for cell growth or survival. In addition, in transfection assays TTP appears to decrease transcription of numerous different genes (Carballo *et al*., 1998; Zhu *et al*., 2001). This effect is significantly less prominent in 293 cells however (Carballo *et al*., 1998), which are resistant to TTP-induced apoptosis (Johnson *et al*., 2000), raising the question of whether this apparent broad 'squelching' of gene expression might be a cause or a consequence of TTP-induced apoptosis.

TTP induces apoptosis and modulates responses to TNFα through distinct mechanisms

TTP is unique among TTP/TIS11 proteins in that it induces apoptosis synergistically with TNFα (Johnson *et al*., 2000). Here we have determined that this synergistic induction of cell death is not inhibited by Bcl-2, which blocks induction of apoptosis by TTP/TIS11 proteins in the absence of TNFα (Johnson *et al*., 2000) (Figure 2a). This finding suggests that TTP and TNFα induce apoptosis synergistically independently of the mitochondrial pathway, and implies that TTP sensitizes cells to the direct apoptotic stimulus of TNFα (Figure 6). Further supporting the model that TTP induces apoptosis and sensitizes cells to TNFα through different mechanisms, although both the TTP(N-Zn) and TTP(Zn-C) mutants can induce apoptosis through the mitochondrial pathway, only TTP(N-Zn) acts synergistically with TNFα (Figures 2a, 4 and 6). Since the zinc fingers are relatively conserved among TTP/TIS11 proteins (Figure 1a,c), it appears likely that the more divergent 92 residue N-terminal region of TTP mediates interactions that are essential for its unique influence on TNFα responses. We conclude that TTP (but not TIS11b or TIS11d) alters the balance between the survival and death signals induced by TNFα (Figure 6), most likely by affecting expression of a component or end-product of these signaling pathways.

Transfection and *in vitro* studies strongly support the model that TTP limits production of TNFα and other cytokines by directly binding and destabilizing their mRNAs (Carballo *et al*., 1998, 2000; Chen *et al*., 2001; Lai and Blackshear, 2001; Lai *et al*., 1999, 2000). TTP may act on the TNFα mRNA through more than one mechanism however, because at some expression levels TTP appears to stabilize particular TNFα mRNA species (Lai *et al*., 1999). In addition, although both TIS11b and TIS11d robustly bind and destabilize cytokine mRNAs in transfection assays (Lai *et al*., 2000), they each fail to sensitize cells to the apoptotic stimulus of TNFα (Johnson *et al*., 2000), indicating that its influence on TNFα responses is a highly specific characteristic of TTP.

Individual orthologs of each TTP/TIS11 protein appear to be conserved in vertebrates (Lai *et al*., 2000), but not in *C. elegans* and *Drosophila*, which each encode a single related protein that at some zinc finger positions is more similar to TIS11b or TIS11d (Figure 1c). The two most closely related genes in *S. cerevisiae* similarly do not each correspond directly to an individual vertebrate TTP/TIS11 gene (Figure 1c). These sequence relationships suggest that the three TTP/TIS11 genes may have arisen from a common ancestor specifically in vertebrates, and that TTP may have diverged in tandem with specialized functions that are reflected by its influence on TNFα responses.

The unique effect of TTP on TNFα responses could be important *in vivo* in multiple ways. Maturing activated monocytes face a choice between NF-κB-dependent survival and differentiation, and death by TNFα-induced apoptosis (Figure 6) (Pennington *et al*., 2001). If TTP were involved in this decision in certain hematopoietic lineages, its absence could result in some inflammatory responses not being regulated appropriately. In addition, certain effects of TTP are consistent with the speculative but intriguing possibility that TTP might inhibit stress-activated protein kinase pathways. These signaling pathways are induced by TNFα to promote cell survival (Figure 6), and they enhance TNFα mRNA translation and stability through the ARE (Kontoyiannis *et al*., 2001; Rutault *et al*., 2001; Vasudevan and Peltz, 2001; Winzen *et al*., 1999). By inhibiting these stress-activated signals, TTP could both enhance the apoptotic stimulus of TNFα, and decrease TNFα mRNA stability or translation indirectly. In addition, it may be significant that the stress-activated p38 pathway is required for induction of TTP expression, but also phosphorylates TTP (Mahtani *et al*., 2001), and thereby appears to decrease its RNA binding affinity and inhibit its activity in the cell (Carballo *et al*., 2001; Zhu *et al*., 2001). These complex relationships suggest that by acting post-transcriptionally on different target mRNAs, TTP may influence multiple interconnected feedback loops. Further elucidation of the mechanisms through which TTP induces apoptosis and modulates TNFα responses may yield important insights into how TTP influences inflammatory events *in vivo*.

Materials and methods

Constructs

TTP regions shown in Figure 1b were introduced into the CMV-based expression vector CS2+(Turner and Tjian, 1989) by PCR (Pfu, Stratagene), with a Kozak consensus and ATG added where appropriate. An N-terminal GFP fusion was made by restriction cloning full length TTP from CS2TTP

into C2eGFP (Clontech). N-terminal fusions of TTP deletion mutants were restriction cloned into C3eGFP.

Transfections and cell death assays

Transfections were carried out as described (Johnson *et al*., 2000), in 35 mm plates using Lipofectamine or Lipofectamine Plus (Life Technologies) as indicated. Cell death was assayed by co-transfection of a β-galactosidase reporter plasmid, and examination of cell morphology after X-gal staining at 24 h post-transfection (Johnson *et al*., 2000). Under a variety of experimental conditions, the numbers of apoptotic cells identified by this method were reproducibly comparable to those detected by scoring Hoechst-stained pyknotic nuclei (not shown) (Johnson *et al*., 2000).

Western blotting, antibody production and immunofluorescence

Cells were lysed in 1% TritonX-100, 50 mM Tris pH 8, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 10% glycerol, Complete^{TM} protease inhibitors (Roche Pharmaceuticals), 1 mM Na Vanadate and 50 mM NaF (cell lysis buffer). For Western blotting, 100 μg of protein was used per lane (Johnson *et al*., 2000). Mouse monoclonal anti-GFP (Zymed) was used according to manufacturers directions. The nTTP antibody was previously described as anti-TTP antibody (Johnson *et al*., 2000). cTTP was raised (Cocalico Biologicals, PA, USA) and affinity purified similarly, using a peptide corresponding to the 21 C-terminal amino acids of TTP, with a cysteine added N-terminally to allow conjugation. These antisera were used at 1/5000 dilution. Immunofluorescence was carried out as described (Johnson *et al*., 2000), with 200–300 cells per slide counted for determination of TTP localization.

Acknowledgments
For helpful discussions and critical reading of the manuscript we thank Karl Munger, Grace Gill, Azad Bonni, Valerie Zacny, Michael Yaffe, and Blackwell lab members. Supported by a grant from the NIH to TK Blackwell. (1 R01 CA84418), and an Arthritis Foundation postdoctoral fellowship to BA Johnson.

References

Bai C and Tolias PP. (1996). *Mol. Cell. Biol.,* **16,** 6661–6667.
Baker SJ and Reddy EP. (1996). *Oncogene,* **12,** 1–9.
Barabino SM, Hubner W, Jenny A, Minvielle-Sebastia L and Keller W. (1997). *Genes Dev.,* **11,** 1703–1716.
Batchelder C, Dunn MA, Choy B, Suh Y, Cassie C, Shim EY, Shin TH, Mello C, Seydoux G and Blackwell TK. (1999). *Genes Dev.,* **13,** 202–212.
Carballo E, Cao H, Lai WS, Kennington EA, Campbell D and Blackshear PJ. (2001). *J. Biol. Chem.,* **276,** 42580–42587.
Carballo E, Gilkeson GS and Blackshear PJ. (1997). *J. Clin. Invest.,* **100,** 986–995.
Carballo E, Lai WS and Blackshear PJ. (1998). *Science,* **281,** 1001–1005.
Carballo E, Lai WS and Blackshear PJ. (2000). *Blood,* **95,** 1891–1899.
Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M and Karin M. (2001). *Cell,* **107,** 451–464.
Chen CY and Shyu AB. (1995). *Trends Biochem. Sci.,* **20,** 465–470.
Chen MJ, Holskin B, Strickler J, Gorniak J, Clark MA, Johnson PJ, Mitcho M and Shalloway D. (1987). *Nature,* **330,** 581–583.
DuBois RN, Bishop PR, Graves-Deal R and Coffey RJ. (1995). *Cell. Growth Differ.,* **6,** 523–529.
DuBois RN, McLane MW, Ryder K, Lau LF and Nathans D. (1990). *J. Biol. Chem.,* **265,** 19185–19191.
Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G and Tsichlis PN. (2000). *Cell,* **103,** 1071–1083.
Ehrenfried JA, Townsend Jr CM, Thompson JC and Evers BM. (1995). *Ann. Surg.,* **222,** 51–56.
Green DR. (2000). *Cell,* **102,** 1–4.
Guedes S and Priess J. (1997). *Development,* **124,** 731–739.
Haas CA, Donath C and Kreutzberg GW. (1993). *Neuroscience,* **53,** 91–99.
Harkin DP, Bean JM, Miklos D, Song YH, Truong VB, Englert C, Christians FC, Ellisen LW, Maheswaran S, Oliner JD and Haber DA. (1999). *Cell,* **97,** 575–586.
Heximer SP and Forsdyke DR. (1993). *DNA Cell. Biol.,* **12,** 73–88.
Janicke RU, Lee FH and Porter AG. (1994). *Mol. Cell. Biol.,* **14,** 5661–5670.
Johnson BA, Geha M and Blackwell TK. (2000). *Oncogene,* **19,** 1657–1664.
Johnson BA, Stehn JR, Yaffe MB and Blackwell TK. (2002). *J. Biol. Chem.,* Published on line.
Klefstrom J, Arighi E, Littlewood T, Jaattela M, Saksela E, Evan GI and Alitalo K. (1997). *EMBO J.,* **16,** 7382–7392.
Kontoyiannis D, Kotlyarov A, Carballo E, Alexopoulou L, Blackshear PJ, Gaestel M, Davis R, Flavell R and Kollias G. (2001). *EMBO J.,* **20,** 3760–3770.
Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F and Kollias G. (1999). *Immunity,* **10,** 387–398.
Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C, Volk HD and Gaestel M. (1999). *Nat. Cell. Biol.,* **1,** 94–97.
Lai WS and Blackshear PJ. (2001). *J. Biol. Chem.,* **276,** 23144–23154.
Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS and Blackshear PJ. (1999). *Mol. Cell. Biol.,* **19,** 4311–4323.
Lai WS, Carballo E, Thorn JM, Kennington EA and Blackshear PJ. (2000). *J. Biol. Chem.,* **275,** 17827–17837.
Lai WS, Kennington EA and Blackshear PJ. (2002). *J. Biol. Chem.,* **277,** 9606–9613.
Lai WS, Stumpo DJ and Blackshear PJ. (1990). *J. Biol. Chem.,* **265,** 16556–16563.
Landesberg LJ, Ramalingam R, Lee K, Rosengart TK and Crystal RG. (2001). *Am. J. Physiol. Lung Cell. Mol. Physiol.,* **281,** L1138–L1149.
Mahtani KR, Brook M, Dean JL, Sully G, Saklatvala J and Clark AR. (2001). *Mol. Cell. Biol.,* **21,** 6461–6469.
Mesner PW, Epting CL, Hegarty JL and Green SH. (1995). *J. Neurosci.,* **15,** 7357–7366.
Ming XF, Stoecklin G, Lu M, Looser R and Moroni C. (2001). *Mol. Cell. Biol.,* **21,** 5778–5789.
Mittelstadt PR and DeFranco AL. (1993). *J. Immunol.,* **150,** 4822–4832.

Nakajima K and Wall R. (1991). *Mol. Cell. Biol.,* **11,** 1409–1418.
Pennington KN, Taylor JA, Bren GD and Paya CV. (2001). *Mol. Cell. Biol.,* **21,** 1930–1941.
Phillips AC, Ernst MK, Bates S, Rice NR and Vousden KH. (1999). *Mol. Cell.,* **4,** 771–781.
Phillips R, Ramos S and Blackshear PJ. (2002). *J. Biol. Chem.,* **277,** 11606–11613.
Raghavan A, Robison RL, McNabb J, Miller CR, Williams DA and Bohjanen PR. (2001). *J. Biol. Chem.,* **276,** 47958–47965.
Ray CA, Black RA, Kronheim SR, Greenstreet TA, Sleath PR, Salvesen GS and Pickup DJ. (1992). *Cell,* **69,** 597–604.
Reese KJ, Dunn MA, Waddle JA and Seydoux G. (2000). *Mol. Cell.,* **6,** 445–455.
Rutault K, Hazzalin CA and Mahadevan LC. (2001). *J. Biol. Chem.,* **276,** 6666–6674.
Schubert CM, Lin R, de Vries CJ, Plasterk RH and Priess JR. (2000). *Mol. Cell.,* **5,** 671–682.
Tabara H, Hill RJ, Mello CC, Priess JR and Kohara Y. (1999). *Development,* **126,** 1–11.
Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, Schenkman DI, Gilkeson GS, Broxmeyer HE, Haynes BF and Blackshear PJ. (1996a). *Immunity,* **4,** 445–454.
Taylor GA, Thompson MJ, Lai WS and Blackshear PJ. (1995). *J. Biol. Chem.,* **270,** 13341–13347.
Taylor GA, Thompson MJ, Lai WS and Blackshear PJ. (1996b). *Mol. Endocrinol.,* **10,** 140–146.
Tenenhaus C, Subramaniam K, Dunn MA and Seydoux G. (2001). *Genes Dev.,* **15,** 1031–1040.
Turner R and Tjian R. (1989). *Science,* **243,** 1689–1694.
Varnum BC, Lim RW, Sukhatme VP and Herschman HR. (1989). *Oncogene,* **4,** 119–120.
Varnum BC, Ma QF, Chi TH, Fletcher B and Herschman HR. (1991). *Mol. Cell. Biol.,* **11,** 1754–1758.
Vasudevan S and Peltz SW. (2001). *Mol. Cell.,* **7,** 1191–1200.
Winzen R, Kracht M, Ritter B, Wilhelm A, Chen CY, Shyu AB, Muller M, Gaestel M, Resch K and Holtmann H. (1999). *EMBO J.,* **18,** 4969–4980.
Zhu W, Brauchle MA, Di Padova F, Gram H, New L, Ono K, Downey JS and Han J. (2001). *Am. J. Physiol. Lung Cell. Mol. Physiol.,* **281,** L499–L508.
Zuo P and Maniatis T. (1996). *Genes Dev.,* **10,** 1356–1368.