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Similar but distinct effects of the tristetraprolin/TIS11 immediate-early proteins on cell survival

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The immediate early protein tristetraprolin (TTP) is required to prevent inappropriate production of the cytokine TNF-α, and is a member of a zinc finger protein family that is associated with RNA binding. TTP expression is induced by TNF-α, and evidence indicates that TTP can bind and destabilize the TNF-α mRNA. TTP and the closely related TIS11b and TIS11d proteins are evolutionarily conserved, however, and induced transiently in various cell types by numerous diverse stimuli, suggesting that they have additional functions. Supporting this idea, continuous expression of each TTP/TIS11 protein at physiological levels causes apoptotic cell death. By various criteria, this cell death appears analogous to apoptosis induced by certain oncoproteins. It is also dependent upon the zinc fingers, suggesting that it involves action on appropriate cellular targets. TTP but not TIS11b or TIS11d also sensitizes cells to induction of apoptosis by TNF-α. The data suggest that the TTP and TIS11 immediate early proteins have similar but distinct effects on growth or survival pathways, and that TTP might influence TNF-α regulation at multiple levels. *Oncogene* (2000) **19,** 1657–1664.

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Introduction

Complex programs of gene expression ensue when quiescent cells are stimulated by growth factors to enter the cell cycle (Iyer *et al*., 1999). The 'immediate-early' genes are induced directly by many stimuli, and during some apoptotic events. Their products include transcription factors associated with proliferation and apoptosis (Hafezi *et al*., 1997; Shi *et al*., 1992; Zhan *et al*., 1997). In addition, during these responses the localization, stability, and translation of specific mRNAs are affected (Brown and Schreiber, 1996; Chen and Shyu, 1995), indicating that post-transcriptional regulatory mechanisms are also involved.

The immediate early protein tristetraprolin (TTP; also known as TIS11, Nup475, and G0S24) is induced transiently in various cell types by diverse stimuli, and during regeneration of certain tissues (DuBois *et al*., 1990; Lai *et al*., 1990; Varnum *et al*., 1989; Worthington *et al*., 1996). TTP is closely related to the TIS11b and TIS11d proteins, particularly within tandem Cys-X_8-Cys-X_5-Cys-X_3-His (Cys_3His) zinc fingers (Varnum *et al*., 1991). Each of these three proteins, which we refer to as the TTP/TIS11 proteins, is induced rapidly by multiple different agents, although they vary with respect to their baseline mRNA levels and induction by particular stimuli (Corps and Brown, 1995; Gomperts *et al*., 1992; Varnum *et al*., 1991). Other Cys_3His zinc finger proteins are involved in mRNA binding, cleavage, or processing, or have been implicated in post-transcriptional gene regulation (Barabino *et al*., 1997; Batchelder *et al*., 1999; Guedes and Priess, 1997; Murray *et al*., 1997; Rudner *et al*., 1998; Tabara *et al*., 1999; Tronchere *et al*., 1997) predicting that TTP/TIS11 proteins also perform mRNA-associated functions.

Although TTP is induced in a variety of contexts, mice in which the TTP gene has been disrupted (TTP −/− mice) have a limited phenotype. They are normal at birth, but later develop a systemic inflammatory, arthritic, and myeloproliferative syndrome which is mediated by the cytokine tumor necrosis factor-alpha (TNF-α), and derives from an abnormality in non-lymphoid hematopoietic cells (Carballo *et al*., 1997, 1998; Taylor *et al*., 1996a). When stimulated *in vitro*, TTP −/− macrophages produce moderately elevated levels of TNF-α protein and mRNA, the half-life of which is prolonged. TNF-α and many cytokine and growth factor-induced mRNAs are regulated post-transcriptionally through AU-rich elements (AREs) in their 3′ untranslated regions (Chen and Shyu, 1995; Ross, 1995; Shaw and Kamen, 1986). When TTP is over-expressed, TNF-α and other cytokine mRNAs are deadenylated and destabilized, and binding of TTP to the TNF-α ARE can be detected readily (Carballo *et al*., 1998; Lai *et al*., 1999). These observations, and the finding that TTP is induced by TNF-α, have suggested the model that TTP normally destabilizes the TNF-α mRNA directly, through a feedback mechanism (Carballo *et al*., 1998; Lai *et al*., 1999). It is intriguing, however, that TNF-α mRNA levels are also decreased by low-level TTP expression but increased by intermediate TTP amounts (Lai *et al*., 1999), suggesting that TTP may have complex and apparently indirect effects on TNF-α expression.

Although TTP/TIS11 proteins are evolutionarily conserved and induced by numerous extracellular stimuli, suggesting a broader role, no other functions of metazoan TTP/TIS11 proteins have been described. TTP is induced during apoptosis, however, in response to the breast cancer susceptibility protein BRCA1 (Harkin *et al*., 1999), and withdrawal of growth factors from neuronal cells (Mesner *et al*., 1995). In *S. pombe*, a related protein is required for effective transmission of a pheromone-induced *ras*/mitogen-activated protein

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kinase signal (Kanoh *et al*., 1995). In addition, a *nim1 cdc25* mutant can be complemented by either the *cdc2* kinase or a TTP/TIS11 gene, suggesting a cell cycle effect (Warbrick and Glover, 1994). A TTP/TIS11-related protein in *S. cerevisiae* is required for normal metabolism, and retards cell growth when over-expressed (Ma and Herschman, 1995; Thompson *et al*., 1996). These observations indicate that TTP/TIS11 proteins might influence pathways regulatory that regulate survival, differentiation, or proliferation.

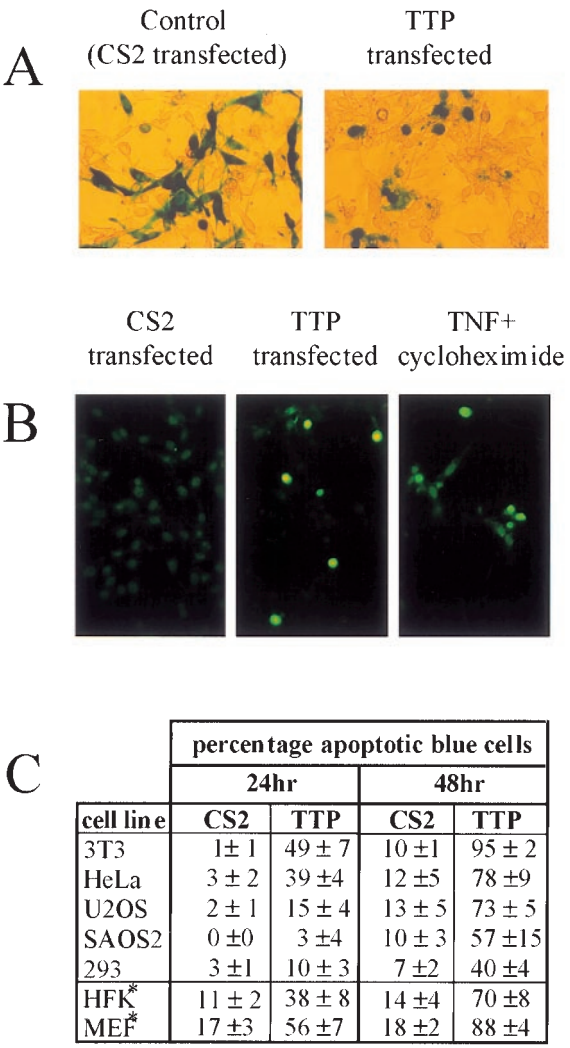
If TTP acts on such pathways when it is induced transiently, its continuous expression might be predicted to affect cell growth or viability. Supporting this idea, we report that continuous TTP expression causes various cell types to undergo apoptotic cell death. This response occurs at TTP expression levels which are comparable to those attained transiently during serum stimulation. Each TTP/TIS11 protein stimulates apoptosis with similar frequency and timing, and by various criteria this cell death appears analogous to apoptosis induced by oncoproteins such as E2F-1, or the immediate early protein c-Myc. In addition, TTP differs from TIS11b and TIS11d in that, like both E2F-1 and c-Myc, it appears to sensitize cells to induction of apoptosis by TNF-α. The data indicate that TTP/TIS11 proteins generally act similarly on growth or survival pathways, but also that TTP may have a distinct influence on responses to TNF-α. They suggest that the role of TTP in TNF-α regulation might be complex, and imply that in TTP −/− mice some TTP functions may be compensated for by other mechanisms.

Results

Programmed cell death in response to TTP

To test whether continuous TTP expression might impair cell viability, we introduced TTP into 3T3 cells by transient transfection. Within 2 days, many TTP-transfected cells appeared apoptotic (Figure 1a), were positive in a TUNEL assay (Figure 1b), and contained pyknotic nuclei (Figure 2b). This cell death increased between 24 and 48 h after transfection (Figure 1c), indicating a relatively slow onset.

Within a transfected population, the frequency of cell death was generally proportional to the amount of expression construct introduced, but in individual cells only modest levels were required (Figure 2). Introduction of either 50 or 200 ng of TTP expression vector triggered apoptosis in a significant fraction of transfected cells (Figure 2c). In these experiments, transfection efficiencies ranged between 25 and 50% (not shown), but TTP protein could not be detected by Western blotting (Figure 2a, lanes 7 and 8). In contrast, endogenous TTP was readily detectable after serum stimulation (Figure 2a, lanes 2 and 3); although by immunofluorescence it was barely apparent above background (not shown). After introduction of 50 ng TTP expression vector, most dead cells (72%) did not express TTP at levels that were detectable by immunofluorescence (Figure 2c). When 200 ng of expression vector was introduced, a moderately higher proportion of apoptotic than non-apoptotic cells expressed TTP at just visible levels (41 versus 25%),



	percentage apoptotic blue cells			
	24hr		48hr	
cell line	CS2	TTP	CS2	TTP
3T3	1± 1	49 ± 7	10 ±1	95 ± 2
HeLa	3 ± 2	39 ±4	12 ±5	78 ±9
U2OS	2 ± 1	15 ± 4	13 ± 5	73 ± 5
SAOS2	0 ±0	3 ±4	10 ± 3	57 ±15
293	3 ±1	10 ± 3	7 ±2	40 ±4
HFK*	11 ± 2	38 ± 8	14 ±4	70 ±8
MEF*	17 ±3	56 ±7	18 ±2	88 ±4

Figure 1 Cell death in response to TTP. (**a**) Blue cell assay of TTP-induced death. Cells were transfected with 1.5 μg expression plasmid (either empty vector CS2 or CS2TTP), and 0.5 μg β-gal reporter plasmid, then X-gal stained after 48 h and shown at 40 × magnification. (**b**) TUNEL assay. Cells were stained for TUNEL activity at 24 h after lipofectamine plus transfection (with either 1 μg CS2TTP or CS2 vector control expression plasmid), or after treatment with TNF-α (100 ng/ml) and cycloheximide (30 μg/ml) for 3 h as a positive control. Typical fields are shown. No staining was detected in a parallel experiment that lacked the TUNEL reagent (not shown). (**c**) TTP-induced cell death in cell lines and primary cells (designated by an asterisk). In a similar experiment to (**a**), cells were transfected with 200 ng of CS2 vector or CS2TTP, and 100 ng of β-gal reporter plasmid. After 24 or 48 h they were X-gal stained and the percentage of dead blue cells was determined. Numbers indicate the mean of four transfections ± the standard deviation. Human foreskin keratinocytes are indicated by HFK, and mouse embryo fibroblasts by MEF

but TTP was still undetectable in many apoptotic cells (38%) (Figure 2b,c). In each of these transfections, the proportion of cells expressing TTP at high levels did not appear to be elevated in the apoptotic fraction, perhaps because some of these apoptotic cells had become detached from the plate (not shown). Considering the high transfection efficiency, the observation that a transfected population expressed less TTP than serum-induced cells (Figure 2a, lanes 2, 3, 7 and 8) suggests that many apoptotic transfected cells expressed TTP at levels that were comparable to or lower than those resulting from serum stimulation.

This indicates that excessively high levels of TTP are not required for its induction of cell death.

Various cell types undergo apoptosis in response to TTP, including primary cells (Figure 1c), demonstrating that immortalization is not a prerequisite. In U2OS and SAOS2 cells, death was delayed (Figure 1c) and peaked after 72 h, perhaps because of their slower growth rates (not shown). The lack of functional p53 in SAOS2 cells (Chandar *et al*., 1992) also may have decreased but did not prevent their apoptotic response, indicating that p53 is not required. The frequency of apoptosis was relatively low in 293 cells, particularly at 48 h after transfection (Figure 1c), and was not increased by additional TTP expression vector (not shown). The decreased apoptotic response in this cell line, in which the effects of TTP on the TNF-α mRNA have been studied (Carballo *et al*., 1998; Lai *et al*., 1999) is likely to derive from its expression of adenovirus E1B 19K, which mimics the anti-apoptotic protein Bcl-2 (Han *et al*., 1998). The *C. elegans* POS-1 and PIE-1 proteins each contain two related zinc fingers (Mello *et al*., 1996; Tabara *et al*., 1999) but did not cause significant apoptosis in this assay (not shown), indicating that not all Cys_3His zinc finger proteins trigger cell death when constitutively expressed. Cell death in response to TTP was decreased by mutation of the first Cys_3His zinc finger, and abrogated by alteration of both (Figure 3), supporting the idea that it is caused by TTP acting on appropriate targets.

Similarities between TTP/TIS11- and oncogene-induced apoptosis

When expressed constutively, TIS11b and TIS11d also triggered apoptosis associated with TUNEL-positive nuclei (not shown). Each TTP/TIS11 protein induced cell death with similar frequency and timing over a range of expression vector amounts (Figure 4a,b), and also as fusions with green fluorescent protein (GFP; not shown). We have not developed assays for detecting endogenous TIS11b and TIS11d proteins, nor could we detect the corresponding TTP/TIS11-GFP fusion proteins after introduction of the modest DNA mounts used in Figure 4a,b). When these GFP fusion proteins were overexpressed in Figure 2a (lane 9) or Figure 3b, however, Western blotting with a GFP antibody revealed that they were present at similar levels (not shown). This suggests that the three TTP/TIS11 proteins are also similarly expressed when introduced at low levels in cell death assays, and that they trigger apoptosis comparably.

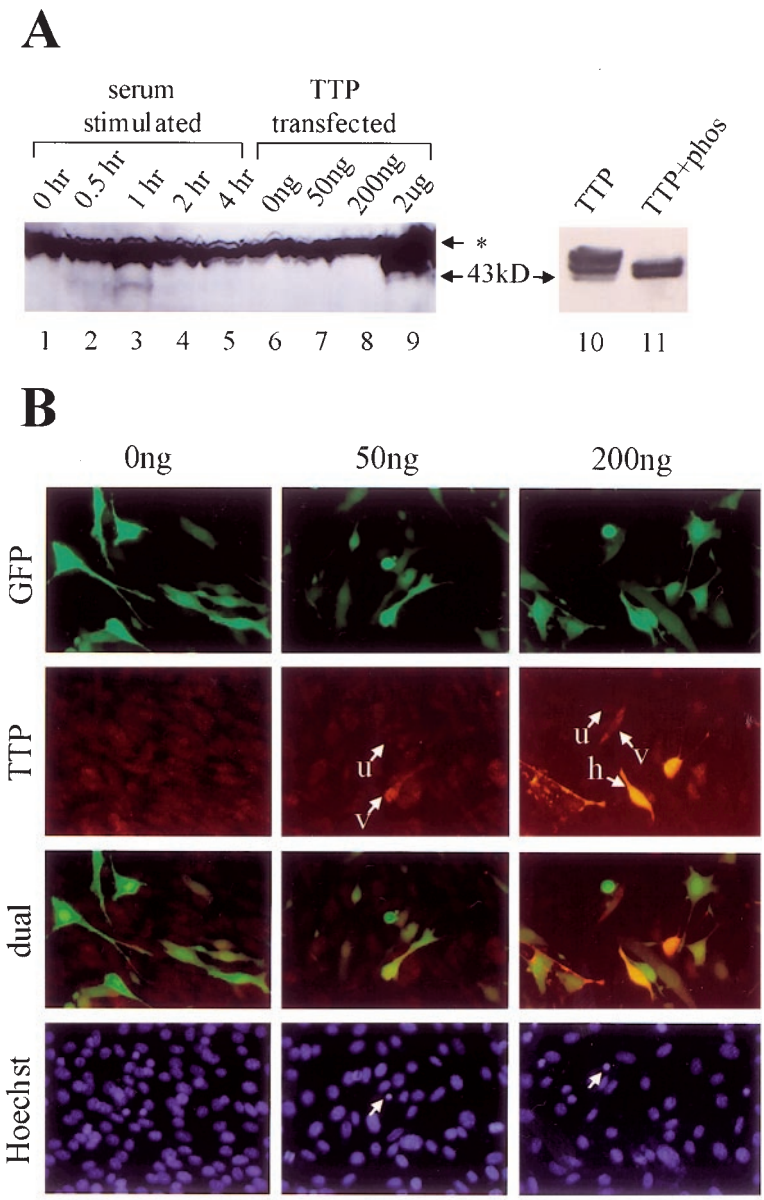
The hypothesis that TTP/TIS11 proteins affect cell growth or survival signals suggests that they might stimulate apoptosis analogously to some oncoproteins. The immediate early protein c-Myc is involved in G1 entry, and possibly in physiological apoptotic events (Shi *et al*., 1992; Zhan *et al*., 1997). Its forced expression under low serum conditions triggers apoptosis over a similar time course as TTP/TIS11 proteins, apparently by stimulating growth or apoptotic pathways in the absence of survival signals (Juin *et al*., 1999). This apoptosis is enhanced when c-Myc is overexpressed, but can be detected when it is expressed constitutively at the levels that are observed following serum induction (Evan *et al*., 1992). It is inhibited when survival signals are restored by treatment with insulin-like growth factor 1 (IGF-1), it requires the mitochondrial death machinery, and it can involve p53 and cell-surface death receptors (Juin *et al*., 1999). In apparent contrast, the S phase transcription factor E2F-1 induces apoptosis in the presence of serum, and can do so independently of p53 when it is expressed at sufficient levels (Hsieh *et al*., 1997; Kowalik *et al*., 1995; Phillips *et al*., 1997).

Cell death in response to TTP/TIS11 proteins appears to be analogous to these apoptotic events in various respects. In each case, it was prevented by co-expression of Bcl-2, which inhibits the mitochondrial death machinery but not direct caspase activation by death receptors (Figure 4c) (Gross *et al*., 1999). It was also partially alleviated by the CrmA protein (Figure 4c), an effective inhibitor of death receptor-activated caspases (Ashkenazi and Dixit, 1998). The latter finding suggests that TTP/TIS11-stimulated apoptosis might involve death receptors, but it is also possible that its inhibition by CrmA derives from effects on caspases activated by the mitochondrial machinery (Gross *et al*., 1999). Serum withdrawal is not a precondition for TTP/TIS11-stimulated apoptosis, but it markedly increased the frequency of death (Figure 4d). This increase was not abrogated by nutrient replacement, and was offset by IGF-1 treatment (Figure 4d), supporting the model that it involves a lack of survival signals. Apparently, constant TTP/TIS11 protein expression can overcome or circumvent the survival signals provided by serum, but also stimulates apoptosis more rapidly when these signals are lacking.

Synergistic induction of apoptosis by TTP and TNF-α

TNF-α stimulates apoptosis by binding to its Type I receptor, an event which triggers caspases directly (Ashkenazi and Dixit, 1998). Simultaneously, however, this binding can activate anti-apoptotic genes, which apparently must remain silent if cell death is to occur (Grumont *et al*., 1999; Wang *et al*., 1998; Wu *et al*., 1998; Zong *et al*., 1999). TNF-α-induced apoptosis is enhanced by expression of the oncoproteins c-Myc, adenovirus E1A, and E2F-1, and by lack of the tumor suppressor Rb, and it is impaired by inhibition of either c-Myc or cell cycle progression (Janicke *et al*., 1994; Klefstrom *et al*., 1994; Meikrantz and Schlegel, 1996; Phillips *et al*., 1999). These findings indicate that pathways which regulate growth or proliferation can influence how a cell responds to TNF-α.

TNF-α treatment induces TTP mRNA expression (Carballo *et al*., 1998), suggesting the possibility that TTP/TIS11 proteins might also affect responses to TNF-α. To test this idea, we added TNF-α to 3T3 cells that were transfected with TTP/TIS11 expression vectors in amounts that triggered only modest cell death (Figure 5). Administration of TNF-α shortly after transfection dramatically increased apoptosis in cells that expressed TTP but, surprisingly, not TIS11b or TIS11d (Figure 5a). The same trends were observed when TNF-α was added after TTP had been expressed for 24 h after transfection (Figure 5b). In these TTP-expressing cells, death was also increased by incubation with TNF-α for only 4 h in the presence of cyclohex-



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			TTP staining level		
TTP	**morphology**	**Tot.**	**Undetect.**	**Vis.**	**High**
50ng	apoptotic	**21%**	72%	18%	10%
	non-apoptotic	79%	71%	15%	14%
200ng	apoptotic	**58%**	38%	41%	21%
	non-apoptotic	42%	51%	25%	24%

Figure 2 Cell death caused by forced expression of TTP at modest levels. (**a**) Expression of TTP after serum induction or transfection of TTP expression plasmid, assayed by Western blotting with affinity purified TTP antibody. 3T3 cells were serum stimulated (Taylor *et al*., 1996b), or transfected with the indicated amount of TTP expression vector. Each lane contained 200 μg total protein. The 43 kD species present in lanes 2, 3 and 9 corresponds to TTP. A background band similar to that found in all lanes in this gel (indicated by an asterisk) has been detected by a different antiserum against the same peptide (Carballo *et al*., 1998). The more slowly-migrating TTP-specific bands (lanes 9 and 10) can be converted to faster-migrating species by phosphatase treatment (lane 11), suggesting that they represent phosphorylated TTP forms described previously (Taylor *et al*., 1995). The Western blot shown in lanes 10 and 11 was performed using TTP antiserum that had not been affinity purified, and does not detect the background species apparent in lanes 1–9. (**b**) TTP expression in transfected cells. Typical fields are shown. Twenty-four hours after transfection with the indicated amounts of TTP vector and either 100 or 200 ng pN3eGFP, cells were fixed and stained with affinity-purified TTP antibody (1 μg/ml), which was detected with a Cy3-conjugated secondary antibody. Nuclei are revealed by Hoechst staining, and transfected cells by GFP autofluorescence. Additional GFP-positive cells with low fluorescence levels were visible under the microscope. A dual image shows the overlap between GFP fluorescence and TTP staining. Arrows on Hoechst-stained fields indicate apoptotic cells in which TTP staining is indistinguishable from background, and which are labeled in the TTP field with u (undetectable). Cells that have high TTP staining levels are labeled with h, and that have just visible TTP with v. (**c**)

imide (Figure 5c), which blocks its induction of anti-apoptotic proteins. The rapidity of this last effect is more consistent with apoptosis induced by death receptors (Ashkenazi and Dixit, 1998) than with the slower time course of TTP-stimulated cell death (Figure 1c), suggesting that TTP has sensitized these cells to the apoptotic stimulus of TNF-α. Similar results were obtained in parallel experiments performed in HeLa cells (not shown). Supporting the idea that this effect is specific to TTP, expression of TIS11b and TIS11d at higher levels increased the overall level of cell death, but did not promote induction of additional apoptosis by TNF-α (not shown).

Discussion

When expressed constitutively at approximately physiological levels, the TTP/TIS11 immediate early proteins each trigger apoptosis with comparable frequency and timing (Figure 4a,b), suggesting that they act similarly to each other on related or overlapping regulatory pathways. Various findings suggest that they induce cell death analogously to oncoproteins such as c-Myc, E1A, and E2F-1 (Han *et al*., 1998; Hsieh *et al*., 1997; Juin *et al*., 1999; Kowalik *et al*., 1995; Phillips *et al*., 1997, 1999). For example, in contrast to the rapid stimulus associated with death receptor triggering (Figure 5c), TTP/TIS11 proteins cause apoptosis over 24–48 h (Figure 4a,b). This apoptosis is accelerated when survival signals are absent (Figure 4d) and is dependent upon the mitochondrial machinery (Figure 4c), which is responsive to abnormal growth regulation, survival signals, and stress (Gross *et al*., 1999). TTP in particular also appears to be analogous to these oncoproteins in that it can sensitize cells to induction of apoptosis by TNF-α (Figure 5). Unlike c-Myc, however, TTP/TIS11 proteins can trigger apoptosis in the presence of serum, and in this respect may be comparable to E2F-1, which stimulates DNA replication and cell cycle progression (Nevins, 1998; Phillips *et al*, 1999). In some experiments, TTP expression increased the number of cells present at 24 h after transfection (not shown), suggesting that it might also promote proliferation.

Precedents set by various other Cys_3His zinc finger proteins predict that TTP/TIS11 proteins are likely to have RNA-associated functions (Barabino *et al*., 1997; Guedes and Priess, 1997; Murray *et al*., 1997; Rudner *et al*., 1998; Tabara *et al*., 1999; Tronchere *et al*., 1997), and evidence indicates that TTP can bind and influence the stability of TNF-α and other ARE-containing cytokine mRNAs (Carballo *et al*., 1998; Lai *et al*., 1999). We have determined that TTP/TIS11 proteins influence cell growth and survival mechanisms, suggesting that they may also act on mRNAs that are involved in those pathways. During growth factor responses, the localization, stability, and translation of various mRNAs are regulated through AREs that are distinct from but related to those of cytokine mRNAs (Chen and Shyu, 1995; Ross, 1995). Such mRNAs might be candidate TTP/TIS11 protein targets, but it will be important to discriminate among the direct and indirect effects of these proteins because an extremely large number of genes are regulated during these responses (Iyer *et al*., 1999).

The apoptotic effect of expressing TTP/TIS11 proteins constitutively might derive simply from their being present at inappropriate phases of the cell cycle. However, other immediate-early proteins that are associated with growth or proliferation are involved in apoptosis (Hafezi *et al*., 1997; Shi *et al*., 1992; Zhan *et al*., 1997), and TTP is expressed during apoptotic events (Harkin *et al*., 1999; Mesner *et al*., 1995), suggesting that TTP/TIS11 proteins might normally promote apoptosis in some contexts. The observation that TTP can sensitize cells to the apoptotic effects of TNF-α is consistent with this idea. The apparently similar sensitization by c-Myc has been proposed to involve the mitochondrial death machinery (Juin *et al*., 1999), and the analogous effects of E2F-1 have been linked to down-regulation of anti-apoptotic mechanisms (Phillips *et al*., 1999). TIS11b and TIS11d can stimulate the mitochondrial death machinery (Figure 4c) but do not sensitize cells to TNF-α-induced apoptosis (Figure 5), suggesting that TTP acts on additional pathways. TTP might influence TNF-α-induced apoptosis by acting on growth related path-

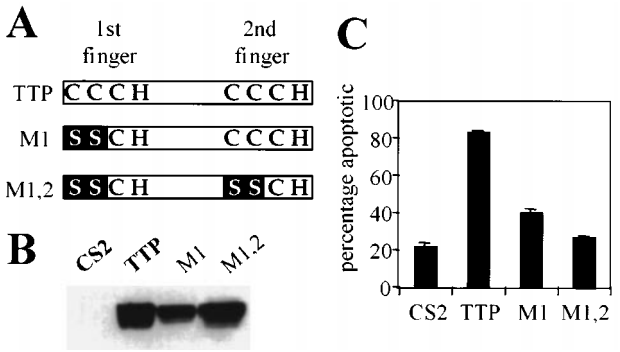

Figure 3 The TTP zinc fingers are required for apoptosis. (**a**) The M1 and M1,2 mutants, in which zinc finger residues that were substituted within full-length TTP are highlighted. (**b**) TTP mutant expression, assayed by Western blotting following transfection of 2 μg of the indicated plasmids. Each lane of the gel, which was not run as far as that shown in Figure 2a, contained 100 μg total protein. TTP was detected using serum that had not been affinity purified (Figure 2a). (**c**) Cell death caused by TTP mutants. Cells transfected with 200 ng of the indicated constructs were assayed for cell death 48 h after transfection as in Figure 1c

TTP staining levels compared with apoptosis. Cells were transfected with the indicated amounts of TTP vector, then 24 h later the percentage of cell death was determined as in Figure 1c. In a control transfection lacking TTP expression vector, 5% of the cells were apoptotic, all of which were GFP-negative (not shown). In a parallel experiment, cells were transfected on coverslips with the same amounts of TTP expression vector and the GFP reporter plasmid as in (**b**). After staining, TTP expression was scored as in (**b**) (u, v, or h) in approximately 50 apoptotic and 300 non-apoptotic GFP positive cells. The percentages indicated in the table refer to the proportion of each group (apoptotic or non-apoptotic) within each staining category

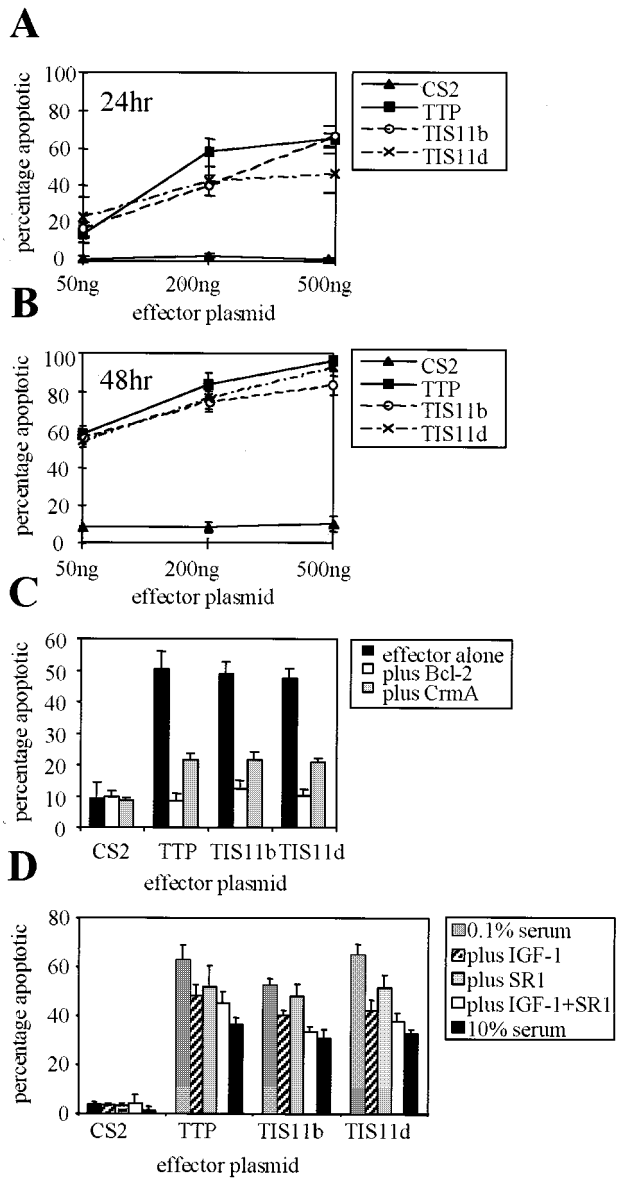

Figure 4 Cell death in response to each TTP/TIS11 protein. In (**a**) and (**b**), 3T3 cells were transfected with the indicated amounts of TTP, TIS11b, TIS11d, or CS2 control vectors along with 100 ng of β-gal reporter, then cell death was assayed as in Figure 1c after either 24 (**a**), or 48 h (**b**). (**c**) Suppression of TTP/TIS11-induced apoptosis by Bcl-2 and CrmA. 200 ng of the indicated expression vector and 100 ng of β-gal reporter were co-transfected into HeLa cells. For Bcl-2 inhibition (white bars), 200 ng of CMV-Bcl-2 were added, and for CrmA inhibition (shaded bars), 1.7 μg of CMV-CrmA were added. After 24 h, death was assayed as in Figure 1c. (**d**) Enhancement of TTP/TIS11-induced apoptosis by serum deprivation. 3T3 cells were lipofectamine plus transfected with 100 ng of effector plasmid as in (**c**). After 3 h, they were incubated in medium containing either 0.1% serum (dark gray bars), 0.1% serum plus 100 ng/ml IGF-1 (Sigma; hatched bars), 0.1% serum plus serum replacement (SR) medium 1 (Sigma; mid-gray bars), 0.1% serum plus IGF-1 and SR1 (white bars) or 10% serum (black bars). SR1 medium provides nutrient replacement but not growth factors. Cell death was assayed 21 h later

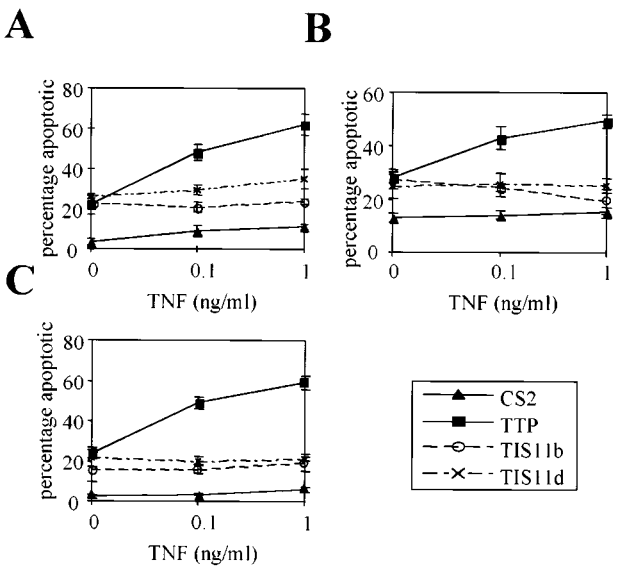

Figure 5 Synergistic induction of cell death by TTP and TNF-α. (**a**) Effects of TNF-α treatment shortly after transfection. 3T3 cells were transfected with 25 ng of the indicated expression vector and 100 ng of β-gal reporter, using lipofectamine plus (Gibco BRL). Recombinant mouse TNF-α (R&D Systems) was added to the indicated concentration after 3 h, and 19 h later cell death was assayed as in Figure 1c. (**b**) Addition of TNF-α after 24 h of TTP expression. This experiment was conducted as in (**a**), except that TNF-α was added 24 h after transfection, and cell death assayed 24 h later. (**c**) Addition of TNF-α and cycloheximide after 24 h of TTP expression. This experiment was conducted as in (**b**), except that cycloheximide was added to 10 μg/ml along with TNF-α, and cell death was assayed 4 h later

ways distinct from those affected by TIS11b and TIS11d, or by interfering with anti-apoptotic gene expression.

Our findings raise the question of why, in mice, lack of TTP causes a specific defect in TNF-α regulation. Although changes in overall TIS11b and TIS11d mRNA levels have not been detected in various TTP $-/-$ mouse tissues (Taylor *et al*., 1996a), it remains possible that TTP/TIS11 proteins could have partially redundant functions. Alternatively, TTP might function in growth regulatory pathways that are largely redundant with other mechanisms. TNF-α expression is regulated by a complex array of transcriptional and post-transcriptional mechanisms that respond to numerous inputs (Beutler *et al*., 1992). By suggesting that TTP expression influences cell growth or survival pathways, and sensitizes cells to TNF-α induced apoptosis, our experiments have identified avenues through which it might influence TNF-α expression or responses indirectly. They indicate that elucidation of how TTP/TIS11 proteins act on their targets not only will reveal critical aspects of TNF-α regulation, but also may uncover post-transcriptional gene regulation mechanisms that are involved in cellular responses to multiple stimuli.

Materials and methods

Plasmid construction

The murine TTP cDNA was amplified by PCR using *Taq* polymerase and subcloned into the CMV-based expression vector pCS2+ (Turner and Weintraub, 1994). Its coding region was replaced with unamplified DNA. Murine TIS11b and TIS11d cDNAs were amplified using *Pfu* polymerase and also subcloned into CS2+. TTP zinc finger mutants were synthesized by the QuickChange method (Stratagene), then sequenced.

Cell culture and transfections

3T3, HeLa, U2OS and HEK 293 cells were cultured in DMEM plus 10% fetal bovine serum, and SAOS2 cells in DMEM plus 15% serum. MEF cells were grown in DMEM with 15% serum and 0.75 μl/100 ml β-mercaptoethanol, and HFK cells in serum-free keratinocyte medium (Gibco BRL). Transfections were routinely carried out in 6-well plates according to the manufacturer's instructions. Approximately 2×10^5 cells were plated 18–24 h prior to transfection, which used 2 μg of total DNA (including pBluescript carrier) and 6 μl of Lipofectamine (Gibco BRL). Transfection efficiency, which generally ranged between 25 and 50%, was assayed by introducing 200 ng of pN3eGFP (Stratagene), and Hoechst staining 24 h later to determine the proportion of fluorescent cells to total nuclei. Where indicated, transfections were performed using Lipofectamine Plus (Gibco BRL), to increase efficiency (to >80%; not shown). Effector plasmid concentrations were then halved, the β-galactosidase (β-gal) plasmid was kept constant, and each 6-well transfection included 1 μg of total DNA, 4 μl of Lipofectamine, and 6 μl of Plus reagent. All results were confirmed with multiple DNA preps, and each data set shown represents multiple experiments. EcR-3T3 cells (Invitrogen) were generally used because of their higher transfection efficiency, but each TTP/TIS11 protein induced apoptosis comparably in NIH3T3 cells (not shown).

Cell death assays and immunocytochemistry

In blue cell assays, cells were washed twice with PBS at either 24 or 48 h after transfection, then fixed with 0.05% glutaraldehyde in PBS for 15 min and X-gal stained after two additional washes. For each data point, live and dead cells were counted in five fields of 50–100 transfected cells each. Cells were scored as dead if they were rounded or shriveled, or consisted of contiguous lumps of blue debris. This method may have underestimated apoptosis in samples where death was extensive, because it did not score detached cells. For Hoechst staining, GFP visualization, and immunocytochemistry, transfections were performed on Biocoat coverslips (Becton Dickinson) in 6-well plates. Cells were fixed and antibody-stained as described in (Srinivasan *et al*., 1998). Hoechst staining (33258; Sigma) was performed at 1 μg/ml for 5 min. TdT-mediated dUTP nick end labeling (TUNEL) assays were performed using a fluorescein *in situ* cell death detection kit (Boehringer Mannheim) after transfection with lipofectamine plus.

Antibody production and Western blotting

A rabbit polyclonal antiserum was raised against a peptide corresponding to the 24 amino-terminal residues of TTP, and affinity purified using the Sulfolink kit (Pierce). For Western blotting, cells were lysed in 50 mM Tris, pH8, 1% Nonidet p40, 150 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 1 mM DTT, to which a complete protease inhibitor cocktail (Boehringer Mannheim) had been added. Samples were resolved on a sodium dodecyl sulfate-polyacylamide gel (SDS–PAGE), then transferred electrophoretically to a Protran nitrocellulose membrane (Schleicher and Schuell). Blots were stained with Ponceau S to monitor loading. Membranes were blocked using 5% nonfat dry milk prior to addition of either anti-TTP antiserum (1–10 000) or affinity purified antibody (1 μg/ml) for more sensitive detection. Bound antibody was detected using an anti-rabbit IgG secondary (Promega) and an enhanced chemi-luminescence kit (Amersham). For dephosphorylation of extracts, lysates were incubated at 30°C with 1 unit/10 μg protein of Calf Intestinal Phosphatase (New England Biolabs) for 30 min. Negative controls were incubated under the same conditions, in the absence of enzyme, and the presence of phosphatase inhibitors (1 mM sodium orthovanadate, 25 mM sodium fluoride).

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