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Fas activates NF-κB and induces apoptosis in T-cell lines by signaling pathways distinct from those induced by TNF-α

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Abstract

The p55 tumor necrosis factor (TNF) receptor and the Fas (CD95/APO-1) receptor share an intracellular domain necessary to induce apoptosis, suggesting they utilize common signaling pathways. To define pathways triggered by Fas and TNF-α we utilized human CEM-C7 T-cells. As expected, stimulation of either receptor induced apoptosis and TNF-α-induced signaling included the activation of NF-κB. Surprisingly, Fas-induced signaling also triggered the activation of NF-κB in T cells, yet the kinetics of NF-κB induction by Fas was markedly delayed. NF-κB activation by both pathways was persistent and due to the sequential degradation of IκB-α and IκB-β. However, the kinetics of IκB degradation were different and there were differential effects of protease inhibitors and antioxidants on NF-κB activation. Signaling pathways leading to activation of apoptosis were similarly separable and were also independent of NF-κB activation. Thus, the Fas and TNF receptors utilize distinct signal transduction pathways in T-cells to induce NF-κB and apoptosis.

Keywords: Fas, TNF-α, NF-κB, IκB-α, IκB-β, apoptosis

Abbreviations: TNF, tumor necrosis factor; NGF, nerve growth factor; ROS, reactive oxygen species; IL, interleukin; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; EMSA, electrophoretic mobility shift assay; NAC, N-acetyl-L-cysteine; PDTC, pyrollidine dithiocarbamate; CHX, cycloheximide; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; DIC, 3,4-dichloroisocoumarin; BTEE, N-benzonyl L-tyrosine ethyl ester; APNE, N-acetyl-DL-phenylalanine β-Napthyl Ester; Aniso, Anisomycin; ALLN, calpain inhibitor I/N-Ac-Leu-Leu norleucinal; CAL II, calpain inhibitor II/N-Ac-Leu-Leu norleucinal; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF); PMSF, phenylmethylsulphonyl fluoride

Introduction

The tumor necrosis factor (TNF) receptors and Fas (CD95/APO-1) are members of the TNF/nerve growth factor (NGF) receptor superfamily (Smith *et al*, 1994). Treatment of cells with TNF-α or activation of the Fas receptor induces apoptosis (Itoh *et al*, 1991; Nagata and Golstein, 1995; Tartaglia *et al*, 1993a; Trauth, *et al*, 1989), although some exceptions have been reported (Aggarwal *et al*, 1995; Alderson *et al*, 1993). Cells express two distinct TNF-α receptors and the p55 TNF receptor is thought to be responsible for signaling TNF-α-induced cytotoxicity (Tartaglia *et al*, 1991, 1993a; Thoma *et al*, 1990). Sequence similarity between members of the TNF/NGF receptor family is largely restricted to their cysteine rich extracellular domains, but the p55 TNF-α receptor and the Fas receptor share additional intracellular sequence similarity (Itoh and Nagata, 1993; Tartaglia *et al*, 1993b). This region, coined the 'death domain' (Cleveland and Ihle, 1995; Feinstein *et al*, 1995; Nagata and Golstein, 1995; Tartaglia *et al*, 1993b), is necessary and sufficient for the induction of apoptosis by either receptor (Itoh and Nagata, 1993; Tartaglia *et al*, 1993b) and is required for TNF-α-induced NF-κB activation (Hsu *et al*, 1995).

Potential signal transduction events that occur following stimulation with TNF-α include activation of sphingomyelinases and phospholipases, generation of reactive oxygen species (ROS), phosphorylation, alterations in calcium homeostasis and induction of NF-κB (Bellomo *et al*, 1992; Beg *et al*, 1993; Darnay *et al*, 1994; Dressler *et al*, 1992; Duh *et al*, 1989; Lowenthal *et al*, 1989; Neale *et al*, 1988, Osborne *et al*, 1989; Schutze *et al*, 1992; Schulze-Osthoff *et al*, 1994; Suffys *et al*, 1991; Wiegmann *et al*, 1994). The Rel/NF-κB family of transcription factors bind to κB sequence motifs in the regulatory regions of responsive genes and play a key role in immune and inflammatory responses, as well as viral gene expression (for recent reviews see references Bauerle and Henkel, 1994; Finco and Baldwin, 1995; Kopp and Ghosh, 1995; Siebenlist *et al*, 1994; Thanos and Maniatis, 1995; Verma *et al*, 1995). The prototypical NF-κB complex is a heterodimer of p50 and p65/RelA, yet cells contain other NF-κB family proteins (p52, c-Rel, RelB) which can form homodimeric and heterodimeric complexes. NF-κB is generally present in an inactive cytoplasmic complex that contains a member of

the IκB proteins (IκB-α and/or IκB-β). NF-κB is activated by diverse stimuli through pathways which ultimately result in proteolytic degradation of IκB molecules and the release and subsequent nuclear translocation of 'free' NF-κB (Beg *et al*, 1993; Brown *et al*, 1993; Cordle *et al*, 1993; Henkel *et al*, 1993; Rice and Ernst, 1993; Sun *et al*, 1993, 1994a,b).

The events that trigger TNF-α-mediated degradation of IκB-α are complex. TNF-α induces IκB-α phosphorylation (Beg *et al*, 1993; Henkel *et al*, 1993), yet this modification is not sufficient to trigger release of NF-κB and may be required for targeting IκB-α for subsequent proteolytic degradation (Alkalay *et al*, 1995; Beg *et al*, 1993; DiDonato *et al*, 1995; Finco *et al*, 1994; Finco and Baldwin, 1995; Henkel *et al*, 1993; Lin *et al*, 1995; Miyamoto *et al*, 1994; Traeckner *et al*, 1994). As some protease inhibitors can also block the induction of IκB-α phosphorylation, there may be a role for proteases prior to this step (Finco *et al*, 1994; Mellits *et al*, 1993). Finally, in some cells, NF-κB activation by TNF-α is inhibited by antioxidants, suggesting that the signaling pathways leading to IκB-α degradation also involve ROS or redox-sensitive processes (Schreck *et al*, 1991).

The biochemical events leading to degradation of IκB-β are likely distinct from those regulating IκB-α. First, IκB-β degradation is triggered by only a subset of activators which target IκB-α (Thompson *et al*, 1995). For example, treatment of 70Z/3 pre-B cells with TNF-α or phorbol 12-myristate 13-acetate (PMA) triggers degradation of IκB-α but not IκB-β, whereas lipopolysaccharide (LPS) or interleukin (IL)-1 trigger degradation of both IκB-α and IκB-β (Thompson *et al*, 1995). Second, the degradation of IκB-β leads to a persistent, rather than transient, activation of NF-κB. For example, TNF-α and PMA trigger IκB-α degradation and transient activation of NF-κB whereas LPS or IL-1 induce IκB-β degradation and long term activation. Although the mechanism for this difference is not totally resolved, it appears that the transient response is regulated by newly synthesized IκB-α, which is itself a NF-κB-regulated gene (LeBail *et al*, 1993; deMartin *et al*, 1993), whereas IκB-β expression is not modulated by NF-κB (Thompson *et al*, 1995).

Signaling events triggered by the Fas receptor are less well characterized, but appear to include activation of kinases, proteases, phospholipases and sphingomyelinases (Cifone *et al*, 1994; Chow *et al*, 1995; Eischen *et al*, 1994; Lahti *et al*, 1995; Nagata and Golstein, 1995; Peter *et al*, 1996; Skowronski *et al*, 1996; Schulze-Osthoff *et al*, 1996). Since Fas and p55 TNF receptor require a conserved sequence motif to signal cell death, it is possible they share signal transduction components (Itoh and Nagata, 1993; Tartaglia *et al*, 1993b). However, several reports have suggested that Fas and TNF receptor signaling pathways are distinct (Aggarwal *et al*, 1995; Grell *et al*, 1994; Hug *et al*, 1994, Nagata and Golstein, 1995; Sato *et al*, 1995, Schulze-Osthoff *et al*, 1994; Wong and Goeddel, 1994; Zimmerman *et al*, 1989). In particular, stimulation of a fibrosarcoma cell line engineered to ectopically express the Fas receptor does not lead to activation of NF-κB, yet NF-κB is induced when these cells are treated with TNF-α (Schulze-Osthoff *et al*, 1994). However, Fas-mediated apoptosis *in vivo* is generally restricted to lymphoid cells (Adachi *et al*, 1995; Nagata and Suda, 1995), as deletion (Adachi *et al*, 1995) or mutation (*lpr*, Watanabe-Fukunaga *et al*, 1992) of the Fas receptor or of the Fas ligand (*gld*, Lynch *et al*, 1994; Takahashi *et al*, 1994) results in inappropriate lymphoproliferation. We have therefore compared Fas and TNF-α-induced signaling events in T cells, which is a physiologically relevant setting. Surprisingly, we demonstrate that similar to TNF-α, Fas receptor-mediated signaling includes activation of NF-κB. However, we demonstrate that pathways leading to apoptosis and NF-κB activation triggered by either receptor are markedly distinct.

Results

TNF-α and anti-Fas induce NF-κB in T cells

To analyze signaling mediated by the TNF-α- and Fas-receptors in a more physiologically relevant setting, we utilized human CEM-C7 T-cells (Bulva *et al*, 1991; Lahti *et al*, 1995). Stimulation of CEM-C7 cells with TNF-α or anti-Fas induces DNA degradation (into oligonucleosomes) and morphological changes characteristic of apoptosis (data not shown). Anti-Fas-induced apoptosis was first apparent 3 h of stimulation. By contrast, TNF-α-induced apoptosis was more protracted and was first evident after 6 h of stimulation (data not shown).

Stimulation of the p55 TNF-α receptor is known to trigger NF-κB activation in several cell types. We therefore analyzed NF-κB activity in CEM-C7 cells treated with TNF-α or anti-Fas. Binding activity in nuclear extracts was determined by electrophoretic mobility shift assay (EMSA) using a double-stranded oligonucleotide containing a canonical κB site as a probe. TNF-α strongly induced a single κB-binding complex within 5 min of treatment (Figure 1a). Unlike NF-κB activation triggered by some stimuli (Thompson *et al*, 1995), inducible κB-binding activity was sustained in cells that had been treated with TNF-α for up to 6 h (Figure 1a). Surprisingly, and in contrast to previous reports (Schulze-Osthoff *et al*, 1994), anti-Fas also induced a similar κB-binding complex (Figure 1a). The effects of the anti-Fas antibody were specific, as treatment of these cells with a control IgM class antibody did not induce κB binding activity or cell death (Figure 1b and data not shown).

Although TNF-α and anti-Fas induced κB-binding activity in CEM-C7 cells to the same extent, the kinetics of activation by the two receptors was distinct. Induction by anti-Fas was delayed relative to TNF-α-induced activation and was first detected 40 min after stimulation, reaching a maximum level after 1 h (Figure 1a). Similar to TNF-α, anti-Fas-induced κB-binding activity was persistent and was detected up to 3 h post-stimulation (Figure 1a, right panel). Anti-Fas-induced κB-binding activity was apparently down regulated after 6 h of treatment (see below, Figure 3a), although this may be due to a non-specific inhibition of DNA-binding activities since a significant number of cells had died by this time. Therefore, stimulation of either the TNF-α or Fas receptors triggers κB-binding activity with distinct kinetics. Although CEM-C7 cells are also sensitive

to glucocorticoid-induced cell death (Norman and Thomson, 1977; Bulva *et al*, 1991; Danel-Moore *et al*, 1993; Lahti *et al*, 1995), we did not observe activation of κB binding complexes at either early (1 and 3 h or late times [i.e. when cells begin to die] after addition of dexamethasone (data not shown). Therefore, activation of the κB-binding complex is not a universal response to inducers of apoptosis in CEM-C7 cells. Activation of κB-binding activity by anti-Fas was not restricted to CEM-C7 cells and was also observed in Molt-4 T cells (Figure 1b).

Persistent NF-κB activation by anti-Fas and TNF-α in CEM-C7 cells is due to the activation of p65/p50 heterodimers

The κB-binding complex detected in CEM-C7 cells treated with TNF-α or anti-Fas was characterized using competition and antibody supershift analyses (Figure 2). To identify the components of the induced complex we used antibodies specific for the Rel family of proteins. κB-binding complexes from nuclear extracts of cells treated with TNF-α or anti-Fas for 10 or 60 min, respectively, were supershifted by antibodies specific for p50 and p65, but not by antibodies to either p52 or c-Rel (Figure 2a). The specificity of the induced complex was confirmed by competitions with an excess of unlabeled κB, but not an unrelated oligonucleotide (Figure 2b, data shown for Fas extracts only). Therefore, TNF-α and anti-Fas induce nuclear translocation of p65 : p50 heterodimers. Identical results were obtained using extracts prepared from cells treated with either inducer for 3 h, demonstrating that the same components of the κB-binding complex induced by TNF-α or anti-Fas persist following induction (Figure 2a).

Sustained activation of NF-κB by TNF-α and anti-Fas is mediated by inducible degradation of both IκB-α and IκB-β

Nuclear translocation of NF-κB complexes is triggered by the inducible degradation of IκB molecules, including IκB-α and IκB-β (Bauerle and Henkel, 1994; Beg *et al*, 1993; Thanos and Maniatis, 1995; Thompson *et al*, 1995). We therefore analyzed levels of IκB-α and IκB-β in cytoplasmic extracts prepared from TNF-α or anti-Fas treated CEM-C7 cells. As a control, nuclear extracts from these cells were tested for binding to the κB site. As expected, TNF-α and anti-Fas both induced κB-binding activity with the kinetics previously demonstrated (Figure 3a). Treatment of cells with TNF-α

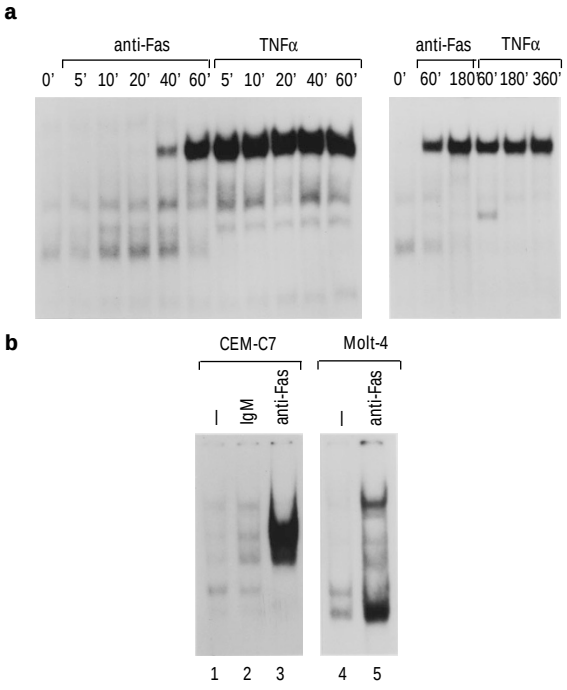

Figure 1 TNF-α and anti-Fas induce κB-binding activity in T cells. (**a**) Nuclear extracts were prepared from CEM-C7 cells treated with anti-Fas or TNF-α for the indicated times and tested by EMSA for binding to a canonical κB site. The free probe is not shown. (**b**) Nuclear extracts were prepared from CEM-C7 (lanes 1–3) and Molt-4 (lanes 4 and 5) cells treated for 1 h with anti-Fas or a control IgM class antibody and bound to the κB probe.

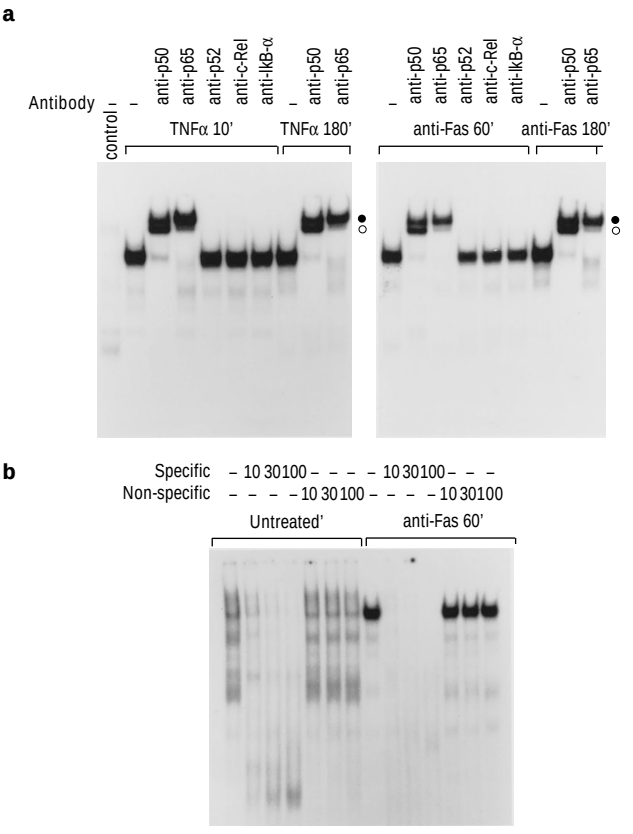

Figure 2 Characterization of κB-binding activities in CEM-C7 cells. (**a**) Supershift analysis of κB-binding activities. Nuclear extracts prepared from control cells, or cells treated with TNF-α for 10 or 180 min or with anti-Fas for 60 and 180 min, were tested for binding to a κB site by EMSA. Where indicated, antibodies specific for p50, p65, p52, c-Rel or IκB-α were added 10 min after initiating the binding reactions. Closed circles indicate specific supershifted complexes, whereas open circle indicates a complex due to non-specific interactions observed using anti-p65 antibody in the absence of any nuclear extract (data not shown). (**b**) Competition analysis to demonstrate specificity of κB-binding activity. A nuclear extract prepared from CEM-C7 cells treated with anti-Fas for 60 min was tested for binding to a κB binding site by EMSA. A 10- to 100-fold molar excess of the oligonucleotide spanning the κB binding site (specific) or of an oligonucleotide spanning an AP-1 binding site (non-specific) was included in the binding reaction, as indicated.

triggered rapid degradation of IκB-α and within 10 min approximately 80% of IκB-α was degraded (Figure 3b). Interestingly, IκB-α levels were restored within 1 h of TNF-α treatment, although inducible κB binding activity persisted in these cells for up to 6 h (Figure 3a). Since persistent induction of NF-κB in pre-B cells correlates with inducible degradation of IκB-β (Thompson *et al*, 1995), we determined whether the persistent induction of NF-κB in TNF-α treated CEM-C7 cells was associated with degradation of IκB-β. Western blot analysis revealed that IκB-β was degraded in TNF-α treated cells, although degradation was delayed relative to IκB-α degradation and was first apparent at 30 min post-stimulation (Figure 3b).

Treatment of cells with anti-Fas also triggered degradation of IκB-α, yet this was delayed relative to IκB-α degradation in TNF-α treated cells and was first evident at 60 min of stimulation (Figure 3b), coincident with the delayed kinetics of NF-κB induction. However, in contrast to TNF-α-treated cells, IκB-α levels were not restored in cells treated with anti-Fas and IκB-α was further degraded at subseqent time points. Similar to the TNF-α-induced activation, however, IκB-β was degraded in anti-Fas treated cells after 3 h of stimulation (Figure 3b). Therefore, activation of NF-κB by the TNF-α and anti-Fas in CEM-C7 cells is mediated by the degradation of both IκB-α and IκB-β and persistent activation is associated with IκB-β degradation.

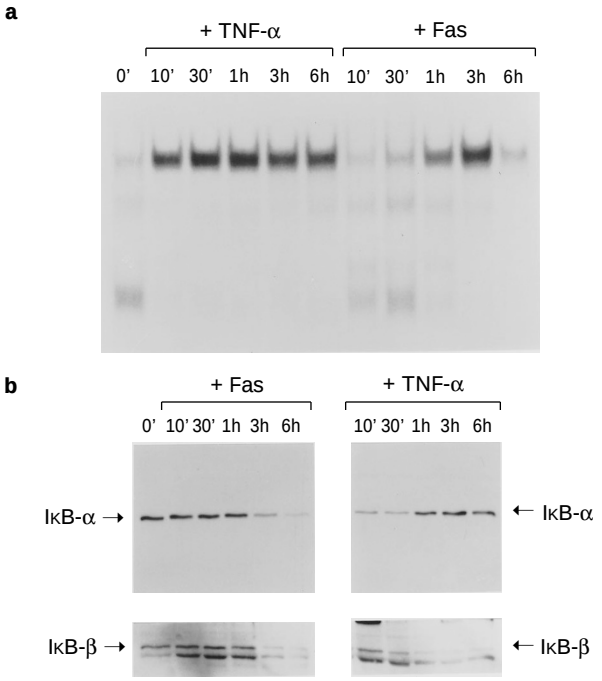

Figure 3 NF-κB activation is associated with degradation of IκB-α and IκB-β. Cytoplasmic and nuclear extracts were prepared in parallel from CEM-C7 cells treated with anti-Fas or TNF-α for the indicated times. (**a**) κB-binding activity present in nuclear extracts was determined by EMSA. (**b**) The cytoplasmic extracts were analyzed by immunoblotting using antibodies specific for IκB-α and IκB-β.

Pathways of activation of NF-κB by TNF-α or anti-Fas are distinct

Activation of NF-κB by TNF-α is mediated by biochemical events which are sensitive to protease inhibitors and anti-oxidants, but not to protein synthesis inhibitors (Bauerle and Henkel, 1994; Finco *et al*, 1994; Kopp and Ghosh, 1995; Schreck *et al*, 1991; Schulze-Osthoff *et al*, 1994; Thanos and Maniatis, 1995). To compare their effects on the stimulation of κB binding activity by TNF-α and anti-Fas, we used a panel of protease inhibitors (TPCK, TLCK, DIC, AEBSF, CAL-II, BTEE, ALLN and APNE), antioxidants (NAC and PDTC) and protein synthesis inhibitors (cycloheximide plus anisomycin) to inhibit protein synthesis. Cells were pre-treated with these compounds for 1 h prior to stimulation with TNF-α or anti-Fas, and NF-κB activation was determined by EMSA analysis after 5 and 60 min of stimulation, respectively (to

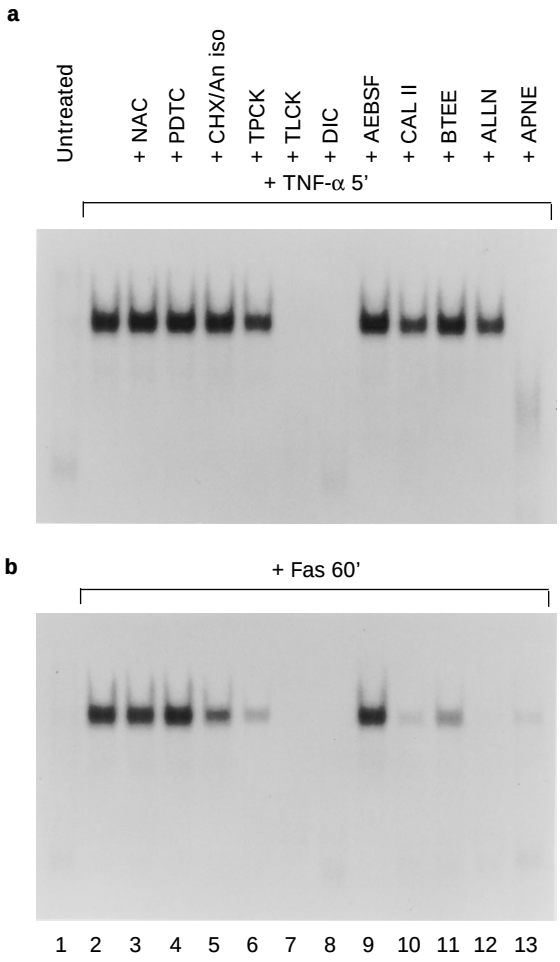

Figure 4 Effects of protease inhibitors and antioxidants on NF-κB activation triggered by TNF-α (**a**) or anti-Fas (**b**). CEM-C7 cells were treated with the indicated compounds at the following concentrations: NAC (200 μM), PDTC (500 μM), CHX (100 μM), Aniso (200 μM), TPCK (100 μM), TLCK (200 μM), DIC (50 μM), AEBSF (250 μg/ml), CAL II (500 μM), BTEE (250 μM), ALLN (500 μM) and APNE (500 μM). After 1 h, cells were stimulated with anti-Fas and TNF-α, or left untreated as control. Nuclear extracts were prepared from anti-Fas- and TNF-α-treated cells at 60 min and 5 min post-stimulation, respectively, and from control untreated cells, and tested for kB-binding activity by EMSA.

allow for maximum NF-κB induction by the two stimuli). Induction of NF-κB by activation of either receptor was resistant to the antioxidants PDTC and NAC (Figure 4). Similarly, activation of NF-κB by either anti-Fas or TNF-α was independent of protein synthesis, as it was resistant to cycloheximide plus anisomycin (Figure 4). However, activation of NF-κB was sensitive to protease inhibitors. TNF-α-induced NF-κB activation was completely blocked by the protease inhibitors TLCK, DIC and APNE (Figure 4a), while others were ineffective. Thus, activation of NF-κB by TNF-α in CEM-C7 cells is apparently dependent upon the activation of specific proteases, although two of these inhibitors, TLCK and DIC, can also block NF-κB activation by direct alkylation (Finco *et al*, 1994). In contrast to TNF-α, activation of NF-κB by anti-Fas was blocked by a broader spectrum of protease inhibitors including TLCK, DIC, CAL II, ALLN and APNE and was partially blocked by BTEE and TPCK (Figure 4b). Thus, pathways coupling the Fas and TNF-α receptors to activation of NF-κB appear to be biochemically distinct and involve distinct proteases.

NF-κB activation is neither necessary or sufficient for TNF-α- and Fas-induced apoptosis

To address whether activation of NF-κB was associated with the induction of apoptosis, we also determined the effects of these protease- and protein synthesis-inhibitors, and antioxidants, on TNF-α and anti-Fas-induced apoptosis of CEM-C7 cells. As above, cells were pre-treated with the various inhibitors for 1 h before stimulation with TNF-α or anti-Fas and after 6 h the extent of apoptosis was determined by TUNEL assays. In contrast to their effects on TNF-α-mediated activation of NF-κB, TNF-α-induced apoptosis of CEM-C7 cells was completely blocked by the protease inhibitor TPCK and by the antioxidant (and Fe^{2+} scavenger) PDTC (Figure 5, Table 1). However, treatment with another antioxidant, NAC, had little effect on cell death. TLCK and DIC, which effectively blocked TNF-α-induced activation of NF-κB, also blocked TNF-α-induced apoptosis, whereas APNE blocked κB activation but not apoptosis (Figure 5 and Table 1). Anti-Fas-induced apoptosis was blocked by the protease inhibitors TPCK, TLCK, and DIC, but, in contrast to TNF-α-induced apoptosis, it was also partially blocked by APNE, and was partially inhibited by both of the antioxidants PDTC and NAC (Figure 5, Table 1). Pre-treatment of TNF-α and anti-Fas treated cells with cycloheximide plus anisomycin (which under these conditions inhibited greater than 95% of protein synthesis within 15 min) did not protect anti-Fas-treated cells from apoptosis and, in agreement with previous studies (Grell *et al*, 1994; Wong and Goeddel, 1994), potentiated rates of death of cells treated with TNF-α. Comparable effects of the panel of compounds on the percent of apoptosis induced by TNF-α or anti-Fas were seen when quantitating the percent of cells expressing apoptosis-specific protein (ASP, Grand *et al*, 1995) (data not shown).

A summary of the effects of the inhibitors on anti-Fas and TNF-α-induced apoptosis and activation of NF-κB is shown in Table 1. Notably, some inhibitors blocked apoptosis without compromising NF-κB activation, e.g. PDTC and TPCK blocked TNF-α-induced death without

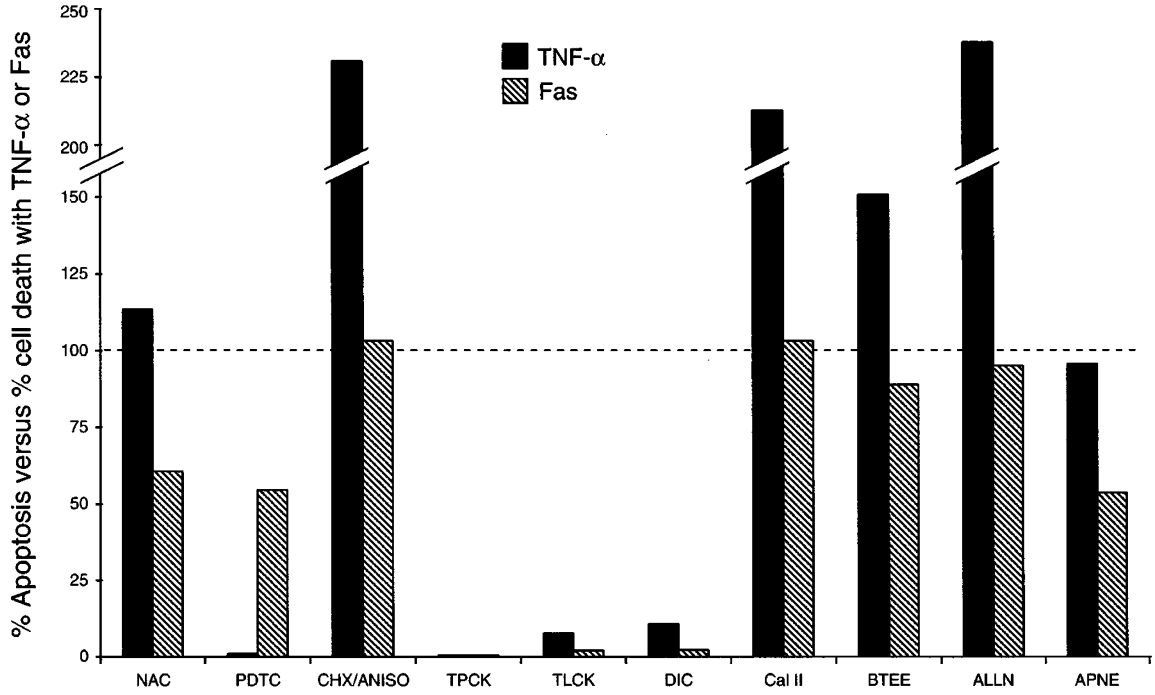

Figure 5 Effect of protease inhibitors and antioxidants on anti-Fas and TNF-α-induced apoptosis of CEM-C7 cells. CEM-C7 cells were treated with the indicated compounds at the concentrations listed in Materials and Methods for 1 h before being treated with anti-Fas or TNF-α. After 6 h the degree of apoptosis was determined by TUNEL assays (Gold *et al*, 1993). Percent apoptosis of cells treated with anti-Fas or TNF-α plus the indicated compounds is shown relative to the percent death observed in cells treated with Fas or TNF-α alone (78% and 30%, respectively).

Table 1 Inhibition of Fas and TNF-a signaling by protease and protein synthesis inhibitors and antioxidants. The relative ability of the indicated compounds to block apoptosis and activation of NK-κB (from dta of Figures 4 and 5) is indicated[a]

	anti-Fas		TNF-α	
Compound	Apoptosis	NF-kB activation	Apoptosis	NF-kB activation
NAC	+	–	–	–
PDTC	+	–	++	–
CHX/Aniso	–	–	– (*)	–
TPCK	++	+	++	–
TLCK	++	++	++	++
DIC	++	++	++	++
Cal II	–	++	– (*)	–
BTEE	–	+	–	–
ALLN	–	++	– (*)	–
APNE	+	++	–	++

[A]Extent of inhibition of apoptosis and NF-kB activation: '++', 50–100%, '+', 20–50%, '–', <20%, (*), Significantly enhanced apoptosis

inhibiting NF-κB induction (Table 1). Conversely, other compounds effectively blocked activation of NF-κB without inhibiting the induction of apoptosis; e.g. APNE in TNF-α-treated cells and CAL-II and ALLN in anti-Fas-treated cells (Table 1). Therefore, the TNF-α and Fas-induced pathways leading to cell death and NF-κB activation are different, and NF-κB is neither necessary or sufficient for TNF-α or Fas-induced apoptosis.

Discussion

The cytoplasmic domains of the Fas and p55 TNF-α receptors share sequence similarity and have been suggested to utilize common signal transduction pathways (reviewed by Cleveland and Ihle, 1995; Nagata and Golstein, 1995; Smith *et al*, 1994). Here we have demonstrated that one shared target of Fas and TNF-α receptor signaling in T cells is the activation of NF-κB. However, the pathways by which the Fas and TNF-α receptors trigger activation of NF-κB are quite different. In addition, Fas and TNF-α-induced pathways leading to apoptosis of T cells are distinct, and NF-κB activation is neither sufficient or necessary for cell death. Thus, although Fas and TNF-α receptors share a 'death domain' (Cleveland and Ihle, 1995; Feinstein *et al*, 1995; Itoh and Nagata, 1993; Nagata and Golstein, 1995; Tartaglia *et al*., 1993b), it is likely their mediators of NF-κB activation and death are unique. In agreement with this concept, the cytoplasmic regions of these two receptors interact with unique proteins (TRADD and FADD with the TNF-R1 receptor and FADD and RIP with Fas) which are all capable of inducing apoptosis (Boldin *et al*, 1995; Chinnaiyan *et al*, 1995; Hsu *et al*, 1995; Stranger *et al*, 1995; Peter *et al*, 1996).

As described in other cell types (Anisowicz *et al*, 1991; Beg *et al*, 1993; Duh *et al*, 1989; Pang *et al*, 1992), TNF-α triggered rapid degradation of IκB-α and nuclear translocation of p50 : p65 NF-κB complexes in CEM-C7 T-cells. Surprisingly, a similar, albeit delayed, NF-κB response was observed in these cells following activation of the Fas receptor. This finding was unexpected, as it has been previously reported that stimulation via the Fas receptor does not trigger activation of NF-κB in L929 murine fibrosarcoma cells ectopically expressing the Fas receptor (Schulze-Osthoff *et al*, 1994). However, in agreement with our findings, it has recently been demonstrated that other Fas-sensitive fibroblasts induce NF-κB in response to Fas (Rensing-Ehl *et al*, 1995) and that NF-κB is also induced by Fas in other cell types (Ponton *et al*, 1996). A possible reason for these discrepancies is that Fas receptor-mediated signaling effectors such as FADD, which may activate IκB degradation, are not expressed in all cell types (such as L929 cells, Schulze-Osthoff *et al*, 1994), yet are coordinately expressed with the receptor in T cells, where Fas normally functions (Adachi *et al*, 1995; Nagata and Golstein 1995a,b; Watanabe-Fukunaga *et al*, 1992). Notably, despite the lack of NF-κB activation, Fas induces apoptosis in L929 cells (Schulze-Osthoff *et al*, 1994). These findings agree with our observations and those of others (Rensing-Ehl *et al*, 1995) that some inhibitors (e.g. CAL-II and ALLN, Table 1) block Fas-induced NF-κB activation but not apoptosis. Thus, NF-κB activation is not essential for Fas-induced apoptosis.

The recent cloning of *IκB-β* and generation of specific antibodies (Thompson *et al*, 1995) allowed analysis of both IκB-α and IκB-β during activation of NF-κB in TNF-α and anti-Fas stimulated cells. The initial induction of NF-κB triggered by either receptor correlated well with degradation of IκB-α. Thus, the first phase of NF-κB activation is apparently mediated by the inducible degradation of IκB-α and the release of p65 : p50 complexes. The subsequent, persistant, phase of NF-κB activation appears to be mediated by delayed degradation of IκB-β. While it is possible that changes in at least IκB-β levels during Fas signaling are due to non-specific degradation coincident with cell death, the differences we observe in Fas- versus TNF-α-mediated changes in IκB-α levels are prior to overt apoptosis and are clearly distinct. Interestingly, the sustained activation of κB complexes in TNF-α treated cells occurred in the presence of IκB-α, whose levels were re-established within 1 h post-stimulation (Figure 3b). As induced IκB-α can enter the nucleus to bind NF-κB (Arezana-Seisdedos *et al*, 1995), it is not clear why re-induced IκB-α is ineffective at curtailing NF-κB activation by TNF-α. The subunit composition of the κB-binding complex does not change during the course of the TNF-α-induced response (Figure 2a), suggesting that persistent κB binding activity is not due to the release of complexes with altered composition that are resistant to inhibition by IκB-α. However, it is possible that modifications of the p65 : p50 complexes, or of the reinduced IκB-α, not detected by our supershift or immunoblotting analyses, may prevent association and allow for sustained activation of NF-κB.

As both the induction of apoptosis and NF-κB are sensitive to protease inhibitors and antioxidants in many cell types (Chow *et al*, 1995; Henkel *et al*, 1993; Lahti *et al*, 1995; Mellits *et al*, 1993; Palombella *et al*, 1994; Schreck *et al*, 1991; Schulze-Osthoff *et al*, 1994), it was possible that pathways leading to NF-κB activation and apoptosis shared common components. However, our experiments with protease inhibitors clearly separated activation of NF-κB

from apoptosis and indicated that signaling pathways induced by each receptor are unique. For example, TPCK blocks apoptosis but not NF-κB activation in TNF-α-treated cells and ALLN and APNE block NF-κB activation but not apoptosis in anti-Fas-treated cells. Thus, NF-κB activation is neither required or sufficient for induction of apoptosis and the induction of NF-κB is not simply a response to initiation of the apoptotic pathway. Thus, NF-κB is unlikely to be directly involved in the apoptotic pathway and its activation may rather reflect, similar to the induction of manganese superoxide dismutase by TNF-α (Pang *et al*, 1992), an abortive, protective response.

The effects of protease inhibitors and antioxidants on blocking activation of NF-κB and apoptosis also demonstrated the TNF-α and Fas receptor signaling pathways are unique. For example activation of cell death by TNF-α was blocked by PDTC, whereas apoptosis induced by anti-Fas was only marginally affected. Similarly, pathways leading to activation of NF-κB activation are also biochemically distinct. For example, anti-Fas-induced NF-κB activation was blocked by ALLN and TPCK whereas TNF-α-induced activation of NF-κB was refractory to these compounds. Although we cannot rule out subtle effects of these inhibitors on the kinetics of Fas-versus TNF-α-mediated κB activation, the use of these inhibitors does discriminate the pathways coupling each receptor to IκB-α degradation, as effects were analyzed at early time points when IκB-β is not targeted.

ALLN inhibits the proteasome and has previously been shown to block TNF-α-induced IκB-α degradation (Palombella *et al*, 1994; Traeckner *et al*, 1994). It is not clear why TNF-α-induced NF-κB activation in CEM-C7 cells is resistant to ALLN as it clearly involves, at least initially, IκB-α degradation. A possible explanation is that sensitivity to these compounds is cell context specific. In this regard, although antioxidants have been reported to block NF-κB activation in some cell types (Schreck *et al*, 1991), they clearly do not in CEM-C7 cells and in other cell lines (Siebenlist *et al*, 1994; Thanos and Maniatis, 1995).

Although Fas and TNF-α receptors share some common signaling events in T cells (e.g. NF-κB activation), our analyses argue that unique signal effectors are triggered by each receptor to induce apoptosis. This concept is consistent with the recent cloning and biologic effects of potential signaling molecules which associate with the death domains of the p55 TNF-α receptor or the Fas receptor (Boldin *et al*, 1995, 1996; Chinnaiyan *et al*, 1995; Hsu *et al*, 1995; Muzio *et al*, 1996; Stanger *et al*, 1995).

Materials and Methods

Cell culture

Human CEM-C7 cells (Bulva *et al*, 1991), a subclone of the acute lymphoblastic leukemia CEM cell line (Foley *et al*, 1965) and Molt-4 cells (Minowda *et al*, 1972), were maintained in RPMI-1640 (Bio-Whitaker) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Media Tech). Cells were grown to a density of 1.5 to 2.0×10^6 cells per ml and treated with recombinant human TNF-α (Promega) at 10 ng per ml, or a monoclonal antibody specific for human Fas (Upstate Biotechnology Inc) or a control IgM class antibody (Pharmacia) at 100 ng per ml. N-acetyl-L-cysteine (NAC), pyrollidine dithiocarbamate (PDTC), cycloheximide (CHX), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), N-p-tosyl-L-lysine chloromethyl ketone (TLCK), 3,4-dichloroisocoumarin (DIC), N-benzonyl L-tyrosine ethyl ester (BTEE) and N-acetyl-DL-phenylalanine β-Napthyl Ester (APNE) were from Sigma. Anisomycin (Aniso), calpain inhibitor I/N-Ac-Leu-Leu norleucinal (ALLN) and calpain inhibitor II/N-Ac-Leu-Leu normethional (CAL II) were from Boehringer Mannheim. 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) was from Calbiochem. The concentration of protease inhibitors and antioxidants used in these experiments was determined empirically by titration. In all cases, the maximal dose which did not result in decreased CEM-C7 cell viability over a 5–7 h incubation period was selected.

Apoptosis assays

To determine rates of death induced by anti-Fas versus TNF-α, DNA fragmentation was measured by flow cytometry after cell permeabilization and staining with fluorescein-12-dUTP, as described by Gold *et al* (1993). Briefly, TNF-α or anti-Fas treated cells were fixed with 4% paraformaldehyde and permeabilized with 0.025% Nonidet-P40 (Sigma). They were then incubated with nick translation buffer (500 μM Tris-HCl, 100 μM $MgCl_2$, 100 μM β-mercaptoethanol; pH 7.5), containing dTTP, dATP, dCTP, dGTP (Sigma); FITC-12-dUTP and DNA polymerase (5 U; Boehringer Mannheim). After 18 h of incubation at 37°C, the reaction was stopped with 0.5 M EDTA. Cells were then resuspended in 0.5% paraformaldehyde and fluorescein content analyzed with a FACScan flow cytometer (Becton Dickinson).

To measure the effects of protease inhibitors and anti-oxidants on anti-Fas and TNF-α-induced death, fragmented DNA in apoptotic cell was detected by *in situ* labeling of DNA strand breaks using an *In situ* Cell Death Detection Kit (TUNEL assay) as described by the manufacturer (Boehringer Mannheim). To permit enumeration of the cells, the nuclei were counterstained with 4′,6-diamidine-2-phenylindole-dihydrochloride (DAPI). The slides were examined with an Olympus BMX2 fluorescent microscope. At least 500 individual cells, from a minimum of 10 different fields, were counted for each sample. Control labeling reactions indicated that less than 0.5% of the cells were stained when the terminal deoxynucleotidyl transferase was not added to the labeling reaction. The percentage of apoptotic cells in the protease inhibitor/anti-oxidant treated control samples was less than 10% and did not differ significantly from the untreated CEM-C7 cells, indicating that the inhibtors alone were not inducing apoptosis.

As an alternative measure of apoptosis, we also determined the reactivity of the rabbit polyclonal antibody c-*jun*/AP-1 (Ab-2) (a gift of Dr. B. Sanger, Oncogene Science) which recognizes a cytoskeletal component, named apoptosis-specific protein (ASP), that becomes accessible when cells undergo apoptosis (Grand *et al*, 1995). Briefly, cells were permeabilized and fixed with Ortho Permeafix (Ortho, Raritan, NJ). After incubation with the antibody for 10 min at 20°C, cells were washed in PBS with 5% FCS, 1.5% bovine serum albumin and 0.0055% (w/v) EDTA, and incubated with goat anti-rabbit Ig conjugated to phycoerythrin (Southern Biotechnology Assoc., Birmingham, AL). After two further washes, cells were resuspended in 0.5% paraformaldehyde and anaylzed with a FACScan flow cytometer (Becton Dickinson).

Preparation of nuclear and cytoplasmic extracts and electrophoretic mobility shift assays (EMSA)

Cells were stimulated with TNF-α or anti-Fas or left untreated as a control. After the indicated times, cells were collected and washed

twice in ice-cold phosphate buffered saline (PBS) and once in ice-cold buffer A (10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (Hepes) pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulphonyl fluoride [PMSF]), and then resuspended in buffer A supplemented with 0.1% (v/v) NP-40. Ater incubation on ice for 5 min, the lysates were centrifuged briefly. The supernatants were collected and cleared by centrifugation for 10 min at 14,000 r.p.m. at 4°C in a microcentrifuge. The cleared supernatants were used as cytoplasmic extracts. The pellets containing nuclei from the first spin were resuspended in buffer C (20 mM Hepes pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 10% [v/v] glycerol) and incubated on ice. After 15 min the lysates were cleared by centrifugation for 10 min at 14,000 r.p.m. at 4°C. The supernatants (nuclear extracts) were collected, diluted with 4 volumes of buffer D (20 mM Hepes pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 20% (v/v) glycerol) and dialyzed for 8 to 16 h against NF-κB binding buffer (10 mM Tris-HCl pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 4% [v/v] glycerol). Protein concentrations of extracts were determined using the BioRad assay with bovine serum albumin (BSA) as a standard.

EMSA were essentially performed as previously described (Zabel *et al*, 1991). Equal amounts of nuclear proteins (3–5 μg) were incubated for 30 min at room temperature with a ^{32}P-labeled double-stranded κB oligonucleotide in 20 μl of NF-κB binding buffer additionally supplemented with 1 mg/ml BSA and 0.1 mg/ml poly (dI-dC). When necessary, antibodies for Rel family members (kindly provided by Dr. John Hiscott) were added 10 min after initiating the binding reaction. DNA binding complexes were resolved on 4% polyacrylamide gels in 0.25× TBE buffer (20 mM Tris-borate, 1 mM EDTA) at 4°C. Gels were fixed, dried and exposed to film. Double-stranded oligonucleotides were purchased from Promega. The sequence of the κB oligonucleotide was AGTTGAGGGGACTTTCC-CAGG (top strand shown) and an AP-1 oligonucleotide (CGCTTGAT-GAGTCAGCCGGAA) was used as a non-specific competitor.

Immunoblotting

Cytoplasmic extracts were subjected to immunoblot analysis as previously described using IκB-α and IκB-β specific antibodies (Thompson *et al*, 1995).

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