Nuclear Factor kB-independent Cytoprotective Pathways Originating at Tumor Necrosis Factor Receptor-associated Factor 2*

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Most normal and neoplastic cell types are resistant to tumor necrosis factor (TNF) cytotoxicity unless co-treated with protein or RNA synthesis inhibitors, such as cycloheximide and actinomycin D. Cellular resistance to TNF requires TNF receptor-associated factor 2 (TRAF2), which has been hypothesized to act mainly by mediating activation of the transcription factors nuclear factor kB (NFkB) and activator protein 1 (AP1). NFkB was proposed to switch on transcription of yet unidentified anti-apoptotic genes. To test the possible existence of NFkB-independent cytoprotective pathways, we systematically compared selective trans-dominant inhibitors of the NFkB pathway with inhibitors of TRAF2 signaling for their effect on TNF cytotoxicity. Blockade of TRAF2 function(s) by signaling-deficient oligomerization partners or by molecules affecting TRAF2 recruitment to the TNF receptor 1 complex completely abrogated the cytoprotective response. Conversely, sensitization to TNF cytotoxicity induced by a selective NFkB blockade affected only a fraction of TNF-treated cells in an apparently stochastic manner. No cytoprotective role for c-Jun amino-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), which are activated by TRAF2 and contribute to stimulation of activator protein 1 activity, could be demonstrated in the cellular systems tested. Although required for cytoprotection, TRAF2 is insufficient to protect cells from TNF + cycloheximide cytotoxicity when overexpressed in transfected cells, thus indicating an essential role of additional TNF receptor 1 complex components in the cytoprotective response. Our results indicate that TNF-induced cytoprotection is a complex function requiring the integration of multiple signal transduction pathways.

Intracellular signal transduction from p55 tumor necrosis factor (TNF) receptor 1, which is the main TNF receptor on most cell types, occurs through a controlled series of protein interactions (1–3). Following TNF-induced trimerization of the receptor (4), TNF receptor-associated death domain-containing protein (TRADD) (5) is recruited to a region of TNF-R1 to which the cytoxic function has previously been mapped, namely the death domain (6–8). TRADD acts as an adapter required to recruit to the receptor the downstream transducers Fas-associated death domain-containing protein (FADD) (9–11), TNF receptor-associated factor 2 (TRAF2) (12) and receptor-interacting protein (RIP) (13, 14). Whereas FADD interacts with and activates the apoptotic proteases (15, 16), thus triggering cell death, TRAF2 has been implicated in the activation of two distinct pathways, one of which (17), which requires the recently identified protein kinase NFkB-inducing kinase (NIK) (18), activates a multiprotein catalytic complex (IkB kinase complex) (19–24) that phosphorylates the NFkB inhibitory subunit IkBα at serines 32 and 36. Phospho-IkBα is then degraded, thus liberating NFkB, which enters the nucleus and activates transcription of target genes. The other pathway, which is independent of NIK (25, 26), activates the c-Jun amino-terminal kinases/stress-activated protein kinases (JNKs/SAPKs) (27–29), a family of extracellular signals-regulated Ser/Thr protein kinases that stimulate transcription by phosphorylation and activation of a number of transcription factors, including c-Jun, activating transcription factor 2 (ATF-2), and ternary complex factor (TCF)/Elk2 (30). Although its specific mode of action and function are not completely clear, the protein kinase receptor-interacting protein (14) may represent a critical component of the NFkB signaling apparatus, as suggested by the defective NFkB activation of a receptor-interacting protein-deficient mutant Jurkat cell line (31).

Therefore, although TNF-R1-induced apoptosis requires FADD, stimulation of gene expression mainly occurs through TRAF2-dependent pathways. The first biological role of TNF-stimulated gene expression is to protect cells from TNF cytotoxicity: indeed, most TNF-treated cells are resistant to TNF cytotoxicity unless treated with protein or RNA synthesis inhibitors, such as cycloheximide or actinomycin D, respectively. Moreover, TNF pretreatment usually protects cells from a subsequent challenge with TNF + cycloheximide (32). Evidence for a TRAF2 role in turning on protective genes originally came from experiments with a signaling-deficient TRAF2 mutant, TRAF2(87–501), that acts in a dominant negative fashion (17): unlike wild type cells, those expressing TRAF2(87–501) are TNF receptor, NFkB, nuclear factor kB; AP1, activator protein 1; JNK, c-Jun amino-terminal kinase; SAPK, stress-activated protein kinases; TRADD, TNF receptor-associated death domain-containing protein; FADD, Fas-associated death domain-containing protein; TRAF, TNF receptor-associated factor; NIK, NFkB-inducing kinase; CHX, cycloheximide; PCR, polymerase chain reaction; βgal, β-galactosidase; EF, embryonic fibroblast.

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1 The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; NFkB, nuclear factor kB; AP1, activator protein 1; JNK, c-Jun amino-terminal kinase; SAPK, stress-activated protein kinases; TRADD, TNF receptor-associated death domain-containing protein; FADD, Fas-associated death domain-containing protein; TRAF, TNF receptor-associated factor; NIK, NFkB-inducing kinase; CHX, cycloheximide; PCR, polymerase chain reaction; βgal, β-galactosidase; EF, embryonic fibroblast.
almost invariably doomed to death upon TNF-R1 cross-linking (27, 28). Moreover, degradation of endogenous TRAF2 by constitutive signaling through CD30, a TNF receptor superfamily member that also signals through TRAF2, is associated with increased cellular sensitivity to TNF (33). One critical downstream effector of TRAF2-dependent cytoprotection is transcription factor NfkB, as suggested by TNFα sensitivity of both cells lacking the p65/RelA NfkB subunit (34) and cells expressing a phosphorylation-defective IkBα mutant (IkBo332A/S36A) (27, 35, 36), that, being unresponsive to extracellular signals, irreversibly sequesters NfkB in the cytoplasm, thereby acting as a “super-repressor” of NfkB function (37–39). Although the experimental evidence for a NfkB role in transcriptional induction of protective genes is strong, it is not yet clear whether additional TNF-R1-originating pathways do exist that contribute to cytoprotection against TNF-induced apoptosis.

In this study, we comparatively analyzed the effect of a selective NfkB inhibition with that of a complete TRAF2 blockade; the experiments were carried out in cellular systems in which TNF-induced new gene expression is both sufficient and required for protection against TNF-induced apoptosis, i.e. cells that are killed by TNF only in the presence of translational inhibitors and that when pretreated with TNF are protected against a subsequent challenge with TNF + cycloheximide (CHX). Our results indicate that NfkB-independent TRAF2-dependent pathways make a significant contribution to the TNF-induced cytoprotective response.

MATERIALS AND METHODS

Expression Vectors—The β-galactosidase expression vector (pcDNA-HisAαZ) was from Invitrogen. pcDNA-FlagA20 was constructed by PCR amplification of full-length human A20 cDNA (a kind gift from David V. Goeddel, Tularkin Inc.) using the primers A20FL (5′-GATATCATGGACATACCAAGGAGACGATGAACGCTGAGC-3′) and A20 antisense (5′-ATTGCCGCCGCTTTGAGGATCATCGAGC-3′) and A20 antisense (5′-ATTGCCGCCGCTTTGAGGATCATCGAGC-3′); the PCR fragment was digested with EcoRV and NotI and inserted in the corresponding sites of pcDNA3. pcDNA-FlagZnA20 was amplified by 357FL (5′-CTCGATATCATGGACTACCAAGGAGACGATGAACGCTGAGC-3′) and A20 antisense (5′-ATTGCCGCCGCTTTGAGGATCATCGAGC-3′) and cloned in the Nhel-NotI sites of pcDNA-Flag A20. The primers used for pcDNA-FlagC4znA20 were 540FL (5′-CTCGATATCATGGACTACCAAGGAGACGATGAACGCTGAGC-3′) and A20 antisense (5′-ATTGCCGCCGCTTTGAGGATCATCGAGC-3′) and 358 (5′-CGGCCGGCTTTGAGGATCATCGAGC-3′) and cloned as above. pcDNA-FlagNlA20 was PCR-amplified using primers A20FL and 358/5′-CGGCCGGCTTTTATTTTTATCATCGAGC-3′ and cloned in the Nhel-NotI sites of pcDNA-Flag A20. The primers used for pcDNA-FlagC4znA20 were 540FL (5′-CTCGATATCATGGACTACCAAGGAGACGATGAACGCTGAGC-3′) and A20 antisense (5′-ATTGCCGCCGCTTTGAGGATCATCGAGC-3′) and A20 antisense (5′-ATTGCCGCCGCTTTGAGGATCATCGAGC-3′) and cloned in the Nhel-NotI sites of pcDNA-Flag A20. The primers used for pcDNA-FlagC4znA20 were 540FL and 358 (5′-CGGCCGGCTTTGAGGATCATCGAGC-3′) and cloned as above. The PCR amplification was carried out by Pfu polymerase (Stratagene); after cloning, the PCR products were subjected to DNA sequencing. pRK-FADD80–205, pcDNA-CrmA, and pRK-FlagTRADD have already been described (Refs. 11, 40, and 5, respectively). pcDNA-HA-p65A PKy has already been described (40). pcDNA-HA-TRAF6 was PCR-amplified using the primers hTf6 sense (5′-CTCGTCTAGCATGAGCTCTGCTAATACGCTG-3′) and hTf6 antisense (5′-TGGCGGCCGCTTACCATGGGATCAG-3′) and digested with Nhel-NotI and cloned in the corresponding sites of pcDNA-HA (25). Prsv-p65 has already been described (37). pcDNA-3XHAIkBαS22A/S26A was constructed by inserting a HindIII-NotI fragment excised from pRexA-3XHAIkBαS22A/S26A (38) (a kind gift from M. Karin and J. Di Donato) into HindIII-NotI-digested pcDNA3.

pcDNA-Flag TRAF2 was constructed by inserting in the Nhel-NotI sites of pCDNAFlag the PCR product obtained with primers TF2 sense (5′-TTGGGA TCCTAGTCAGCATGGTGGTCGAGAACTG-3′) and TF2 antisense (5′-CTCGGCGGCGCTTATGGCTCTTTATGAGTC-3′); pcDNA-FlagTRAF2F (236–501) was constructed as above using primer TF2COOH (5′-TTGGAGATTCTCACGACGATGAGTGGAAGAAC-3′); prk-FlagTRAF2F (37–501) has been already described (17). The dominant negative mutant pcDNA-FlagHA-TRAF6F (27/525–522) was PCR-amplified using hTP6s275 (5′-CTCGGTACGACAGGAATGCTCTCGAGC-3′) and hTf6 antisense, respectively. pcDNA-FlagTRAFL (1–355) and pcDNA-FlagTRAF2F (1–358) were obtained with the sense primer TF2 sense and the antisense primers TF2–355 antisense (5′-TTGGGACGCGTTAGGAAGACCCCAT-3′) or TF2–358 antisense (5′-CTGGGCTTTAGTAGAAGACCCCAT-3′) respectively. pcDNA-FlagHA-TRAF6F (27/525–522) was PCR-amplified using hTP6s275 (5′-CTCGGTACGACAGGAATGCTCTCGAGC-3′) and hTf6 antisense, respectively. pcDNA-FlagTRAFL (1–355) and pcDNA-FlagTRAF2F (1–358) were obtained with the sense primer TF2 sense and the antisense primers TF2–355 antisense (5′-TTGGGACGCGTTAGGAAGACCCCAT-3′) or TF2–358 antisense (5′-CTGGGCTTTAGTAGAAGACCCCAT-3′) respectively.

RESULTS

Selective Inhibition of TNF-induced NfkB Activation by A20

Zinc Finger Protein—A20, a zinc finger protein that is expressed at very low levels in most cell types, is rapidly induced upon NfkB activation by TNF-α, interleukin 1, CD40, phorbol myristate acetate, Epstein-Barr virus latent infection membrane protein 1, and human T-cell lymphotrophic virus 1 tax (48–52). Although originally characterized as an inhibitor of NfkB-induced cell death in stably expressing clones (53), A20 has later been shown to act as a potent inhibitor of NfkB activation by TNF, as well as by phorbol myristate acetate, oxygen radicals, and lipopolysaccharide (54–57). We first characterized A20 effects on TNF-R1 signal transduction pathways in HeLa cells, a human epithelial cell line that undergoes TNF-dependent apoptosis only in the presence of translational inhibitors. Treatment of HeLa cells with TNF + CHX caused massive cell death that was entirely dependent on FADD and on the subsequent activation of cysteine-aspartate proteases (caspases), as shown by the complete inhibitory effect of both a dominant negative FADD mutant (11) and of the cowpox virus-encoded caspase inhibitor CrmA (40) (Fig. 1a). High levels of exogenously overexpressed A20 did not affect apoptosis by either TNF + CHX treatment or by TRADD overexpression (which induces apoptosis in a CHX-independent manner) (Fig. 1a), thus indicating that the pro-apoptotic caspase pathway is fully active in A20-expressing HeLa cells. Conversely, A20 expression strongly inhibited both basal and TNF-stimulated NfkB activity (17) (Fig. 1b). In a similar manner, A20 strongly interfered with NfkB activation by both TRADD and phorbol myristate acetate but not p65/RelA (Fig. 1b). Although the molecular bases for NfkB inhibition by A20 are not clear, the fact that A20 is a broad spectrum (but not general) NfkB

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inhibitor preventing IkBα degradation suggests that it probably acts downstream of TRAF2 and before IkB kinase along the NFkB pathway.

Inhibition of NFkB Activation by A20 Correlates with Sensitization to TNF-induced Apoptosis—Because A20 selectively inhibits NFkB activation by TNF-R1/TRAF2 without affecting the apoptotic pathway, and because NFkB has been shown to be required for cytoprotection from TNF-induced cytotoxicity, we would expect an increased rate of TNF-induced apoptosis in A20-expressing cells with respect to vector-transfected cells.

Indeed, transient A20 expression increased the percentage of TNF-induced death about 3-fold over the basal level (from about 10% to 25–30%). However, most transfected cells remained viable even after a prolonged treatment with high TNF concentrations. To evaluate whether a cause-effect relationship between A20-dependent NFkB inhibition and sensitization to TNF-induced death does exist, we comparatively analyzed a number of A20 mutants for their effect on NFkB activation and cellular viability following TNF treatment (Fig. 2a).

3 G. Natoli, A. Costanzo, F. Guido, F. Moretti, A. Bernardo, V. L. Burgio, C. Agresti, and M. Levrero, unpublished observations.
FIG. 2. Sensitization to TNF-induced death by A20 correlates with NFκB inhibition. a, schematic representation of the A20 mutants used in the study. The schematic A20 structure with the seven zinc fingers is shown. Details on plasmids construction are given under “Materials and Methods.” Expression of Flag-tagged A20 mutants in transfected HeLa cells is shown on the left. b, inhibition of TNF-induced NFκB activity by A20 mutants and correlation with sensitization to TNF-induced death. HeLa cells were cotransfected with NFκB-luc reporter (500 ng), pCDNA-HislacZ (200 ng), and the indicated expression vector (1.5 μg). 24 h after transfection, half of the plates were treated with TNF (20 ng/ml) for 16–20 h, and the remaining half were left untreated. NFκB assays and βgal assays were performed as described above. The results are representative of three independent experiments.
NFkB-independent TNF Resistance

Selectivity of NFkB inhibition by transiently expressed IkBo super-repressor (IkBoS32A/S36A) partially sensitizes to TNF-induced death. HeLa cells or Chang liver cells were cotransfected with NFkB-luc (500 ng), pCDNA-HislacZ (200 ng), and pCDNA-IkBoS32A/S36A or empty vector (1.5 μg). 24 h after transfection, half of the plates were treated with TNF (20 ng/ml), and the remaining half were left untreated, as indicated.

NFkB Inhibition by IkBo Super-repressor Partially Sensitizes to TNF-induced Death—To determine whether NFkB inhibition tends to provide the same level of TNF sensitization irrespective of the blocking agent used, we studied the effects of inhibition tends to provide the same level of TNF sensitization.

To determine whether NFkB inhibition tends to provide the same level of TNF sensitization irrespective of the blocking agent used, we tested a number of NFkB mutants interfering with TNF-R1 signaling by alternative mechanisms for their effect on NFkB activity and cell viability following TNF treatment. Deletion of the amino-terminal Ring finger (TRAF2 87-501) or of the most amino-terminal region (TRAF2 226-501) generates signaling-deficient molecules that are capable of TRAF2 homodimerization and interaction with TRADD, thereby acting as dominant negative mutants (17, 59). Despite a similar NFkB inhibition, both mutants sensitized to TNF-induced death much more effectively than IkBoS32A/S36A (up to 85% apoptotic cells) in both HeLa and Chang cells (Fig. 4). Unlike IkBoS32A/S36A, these mutants also block JNK/SAPK activation by TNF (27-29): therefore, their stronger sensitizing effect could be ascribed to concomitant JNK/SAPK inhibition. However, blockade of JNKs/SAPs by two different dominant negative JNK kinase/SAP or ERK kinase) mutants (41, 42) did not alter HeLa or Chang cells sensitivity to TNF (Fig. 5a); more importantly, simultaneous NFkB and JNK/SAPK blockade by coexpression of IkBoS32A/S36A and SEK-AL or SEK-KR did not generate potentiated biological response with respect to IkBoS32A/S36A alone (Fig. 5a). Unlike SEK-AL, a dominant negative c-Jun mutant (c-JunΔ169), which acts as a general inhibitor of AP1-dependent transcription (43, 60, 61) without affecting NFkB activation, sensitized Chang but not HeLa cells to TNF-induced death (Fig. 5b), thus pointing to the existence of NFkB-independent and cell type-specific cytoprotective pathways. The different behavior of SEK-AL and c-JunΔ169 is not surprising, as the latter is a much stronger inhibitor of AP1-dependent transcription than SEK-AL (25).

TRAF2(1-355) is a signaling-deficient mutant that retains the whole amino-terminal region and still interacts with TRAF2 but not with TRADD or NIK (26, 59). We found that TRAF2(1-355), in addition to being unable to activate NFkB, is a powerful dominant negative inhibitor of NFkB activation by TNF (Fig. 4). This mutant also provided a degree of sensitization to TNF cytotoxicity that is comparable to that obtained with the amino-terminal TRAF2-deletion mutants described above.

TRAF6, a TRAF family member that has been implicated in interleukin-1R1 signal transduction (62) is capable of TRAF2 interaction upon overexpression (62). Consistent with its TRAF2-interacting ability, a dominant negative TRAF6 mutant lacking most amino-terminal region (TRAF6 275-522) partially interfered with TNF-induced NFkB activation in both HeLa and Chang cells (Fig. 4). The sensitizing effect of TRAF6AN to TNF cytotoxicity was lower than that of TRAF2(226-501) but much higher than that of IkBoS32A/S36A (Fig. 4); therefore, despite affecting NFkB activation by TNF at a lesser degree than IkBoS32A/S36A, TRAF6AN was more powerful at sensitizing cells to TNF cytotoxicity, thus suggesting an interference with additional NFkB-independent cytoprotective functions of TRAF2.

Effects of NFkB-activating TRAFs on Cellular Sensitivity to TNF-induced Apoptosis—TNF pretreatment renders cells re-
**Fig. 4.** Modulation of TNF sensitivity by signaling-deficient TRAF2-dimerization partners. a, HeLa or Chang cells were transfected with the β-galactosidase expression vector (pCDNA-HislacZ, 200 ng/plate), the NFκB reporter (NFκB luc, 500 ng/plate), and the indicated expression vector. For each assay, half of the plates were left untreated and half were treated with TNF (20 ng/ml). The effects of each mutant on NFκB activation and apoptosis by TNF are shown. The results are representative of six independent experiments. The top panel shows the expression of each mutant. In b, a representative βgal assay in HeLa cells is shown. A and B, vector-transfected cells; C and D, pCDNAIKBa32/36Ala; E and F, TRAF2(87–501); A, C, and E, untreated cells; B, D, and F, TNF-treated cells.
NFκB-independent TNF Resistance

Fig. 5. Effects of simultaneous blockade of NFκB and JNK/SAPK pathways or of NFκB and AP1 on TNF sensitivity. Coexpression of IkBαS32A/S36A and dominant negative JNK kinase/SEK mutants (SEK-AL or SEK-KR) (41, 42) does not augment sensitivity of HeLa or Chang liver cells to TNF-induced apoptosis (a). Conversely, expression of c-JunΔ169, dominant negative inhibitor of AP1-dependent transcription (43, 60, 61) sensitizes Chang but not HeLa to TNF-induced apoptosis (b).

Fig. 6. Effects of NFκB-activating TRAFs on cellular sensitivity to TNF. a, NFκB activation by TRAFs is not sufficient to protect from apoptosis induced by TNF + CHX. HeLa cells were transfected with NFκB-luc, pCDNA-HislacZ, and the indicated expression vectors; 24 h after transfection cells were either tested for NFκB activity or treated with TNF + CHX for 16 h and then stained for βgal. The results are representative of three different experiments. The effect of TNF or interleukin 1 pretreatment, as well as of p65/RelA expression, is shown. b, expression of the NFκB-activating TRAF2 mutant TRAF2(1–358) sensitizes cells to TNF-induced apoptosis. HeLa or Chang liver cells were transfected with pCDNA-HislacZ and with the indicated expression vectors. 24 h after transfection, cells were treated with TNF (20 ng/ml) for 16 h and stained for βgal.

Assistant to a subsequent challenge with TNF + CHX, thus indicating that the induction of protective genes has occurred (Fig. 6a). We asked whether TRAF2 is sufficient to provide protection from TNF + CHX. Despite activating NFκB more strongly than TNF, overexpressed TRAF2 did not provide protection from TNF + CHX (Fig. 6a). TRAF6 overexpression activated NFκB to a similar extent and did not provide any degree of protection (Fig. 6a). TRAF2(1–358), a TRAF2 deletion mutant that, unlike TRAF2(1–355), retains the ability to bind NIK, activates NFκB much more strongly than TRAF2 or TRAF6 (26, 59) (Fig. 6a); however, unlike full-length TRAF2, this mutant is unable to bind TRADD (59). Similarly to TRAF2, and despite the prominent NFκB induction, this mutant is unable to protect cells from TNF + CHX (Fig. 6a). Conversely, TRAF2(1–358) sensitized cells to TNF cytotoxicity to a maximal extent (Fig. 6b). The simplest interpretation of these results is that TRAF2-induced NFκB activation, although required for a complete cytoprotective response, is itself not sufficient to protect cells from TNF + CHX. Therefore, a cell wherein NFκB has been hyperinduced by TRAF2(1–358) is not
TNF-resistant: upon subsequent challenging with TNF, the TRAF2(1–358) mutant blocks endogenous TRAF2 recruitment to the TNF-R1/TRADD complex, the protective response does not take place, and the cell undergoes apoptosis. Consistent with the inability of NFkB to provide protection from TNF-induced cytotoxicity, neither interleukin 1 pretreatment nor p65/RelA transfection afforded protection from TNF-induced apoptosis (Fig. 6a).

DISCUSSION

Tumor necrosis factor is capable of transducing cytotoxic signals mainly through p55TNF-R1, which is widely and con-
NFκB-independent TNF Resistance

...stutitively expressed (3). Apoptotic signaling occurs through sequential recruitment of the signal transducers TRADD and FADD, which in the end recruits and activates the apoptotic proteases (1, 2). Despite being connected to the caspase enzyme cascade, the engagement of which directly triggers apoptosis, p55TNF-R1 engagement usually does not determine apoptotic cell death. The reason for this is that TNF induces a complex response that protects stimulated cells from apoptosis. The need for such a cytoprotective response arises from the strong inducibility of TNF synthesis and secretion in a very high number of pathological conditions (reviewed in Ref. 64); therefore, a vigorous and prevailing protective mechanisms is absolutely required to avoid unwanted death of cells exposed to TNF.

In most cell types, the cytoprotective response can be completely abrogated by protein or RNA synthesis inhibitors, such as CHX and ActD, thus indicating that transcription and translation of NFκB-induced genes play a required role in cytoprotection. The molecular mechanisms of the cytoprotective response have been only partially elucidated. The signal transducer TRAF2, which is recruited to TNF-R1 via interaction with TRADD (5, 11), transmits signals required for activation of both JNK/SAPK and NFκB (11, 17, 27–29); although the precise role of JNK/SAPK in regulation of programmed cell death is still a matter of debate, NFκB activation has been linked to transcriptional induction of protective genes by a number of experimental evidences. First, embryonic fibroblasts (EFs) from p65/RelA-deficient mice are extremely sensitive to TNF cytotoxicity (34); this result is intriguing because the other transcriptionally active NFκB/Rel subunits are supposed to be still functional in these cells. Therefore, it can be hypothesized that at least in EF cells, only a subset of NFκB-responsive genes specifically requiring p65/RelA to be transcribed carry out a protective function. The second evidence for a NFκB role in protection from TNF cytotoxicity arises from transfection studies performed with a dominant negative IκBα mutant bearing mutations in the amino-terminal amino acids (Ser32 and Ser36), the inducible phosphorylation of which, in response to TNF and other extracellular stimuli, targets IκBα to ubiquitin-dependent degradation (37–39). Both transient and stable expression of this IκB super-repressor (which nearly completely abrogates NFκB-dependent transcription) sensitizes various cell lines to TNF-induced apoptosis (35, 37, 64). In the cellular systems that we tested, we found that NFκB inhibition obtained by expression of either IκBα super-repressor or the zinc finger protein A20, which shares with IκB super-repressor the ability to suppress TNF-induced NFκB activation in an apparently selective manner, significantly impaired the ability of cells to overcome TNF cytotoxicity. However, this sensitizing effect always affected only a fraction of transfected cells, and under no condition tested were we able to completely shut down the protective response. The fact that the cell types tested can be completely sensitized to TNF-induced apoptosis by blocking NFκB-stimulated new gene expression (by use of the protein synthesis inhibitor CHX) but can only partially be sensitized by selectively shutting down NFκB-dependent transcription, suggests that NFκB-independent events occurring at the transcriptional/translational level are required to protect cells from TNF cytotoxicity. One practical implication of this is that it should be possible to identify TNF-induced protective genes in cells with a blocked NFκB-dependent transcription; this would hamper TNF induction of a huge number of NFκB-dependent genes (such as those encoding for cytokines and adhesion molecules) that are not relevant for cytoprotection.

Unlike a selective NFκB blockade, knocking out TRAF2 function by alternative strategies results in the complete abrogation of the cytoprotective response. This indicates that in both continuously cultured tumor cells and highly differentiated primary cells (such as primary oligodendrocytes), NFκB-independent cytoprotective signals flow through TRAF2. Indeed, we have found evidence for AP1-dependent and NFκB-independent cytoprotective pathways in Chang but not HeLa cells, thus indicating that some of these cytoprotective responses may be specific for individual cell types. The previous observation that activation of transcription factor AP1 by p55TNF-R1 requires TRAF2 (25) is further evidence of the TRAF2-dependence of at least one of these additional cytoprotective pathways. Whether NFκB-independent TRAF2-elicited signals aimed at protecting cells from TNF cytotoxicity are exclusively transcriptional (i.e. devoted to transcriptional activation of protective genes), however, remains to be determined. Indeed, transcription and translation of protective genes may require some hours; thus, it is not at all clear why cells do not die before this protective protein synthesis has occurred. Activation of the apoptotic caspase pathway by a strictly related receptor, Fas/APO1/CD95, occurs in tens of seconds, with FADD and the first pro tease of the pathway (MACH/Flice) almost instantly recruited to the engaged receptor (65). By analogy, we can hypothesize that to avoid the unwanted propagation of the death signal from TNF-R1 before completion of protective protein synthesis, the death pathway must be kept in a standby status (maybe by mechanisms interfering with FADD recruitment to TRADD or with activation of caspases). Although the experimental systems used did not allow us to probe this hypothesis, the fascinating possibility that TRAF2 is also involved in this transcription-independent protective mechanism do exist.

The recent availability of TRAF2-deficient mice obtained by gene targeting has provided additional relevant information on the physiology of TRAF2, as well as on the mechanisms implicated in protection from TNF-induced apoptosis (66). Several TRAF2−/− cell lineages (including thymocytes and hematopoietic precursors) showed extreme sensitivity to TNF-induced cell death, thus indicating the abrogation of TRAF2-dependent cytoprotection. Extensive characterization of TRAF2−/− EF cells provided some puzzling results. Although TRAF2 deficiency resulted in the absence of TNF-induced JNK/SAPK activation (consistent with the described TRAF2-dependence of this pathway) (27–29), NFκB activation was only delayed and partially reduced but not abolished. TRAF2-independent NFκB activation has also been described in transgenic mice expressing a dominant negative Traf2 mutant in a lymphocyte-specific manner (67). These results are quite surprising in the light of the previous studies addressing TRAF2 (17) and its interacting partner (and downstream effector) NIK (18, 25, 26) as the TNF-R1 signal transducers responsible for the direct activation of the IkB-kinase complex (20–24) and consequently for NFκB activation. The involvement of TRAF2 in NFκB activation by TNF was originally suggested by transfection studies exploiting the signaling-deficient and dominant negative mutant TRAF2(87–501) (17); one possibility is that inhibition of TNF-activation of NFκB by this mutant reflects the titration and sequestering not of endogenous TRAF2 but of other TRAF2-interacting signaling molecules involved in NFκB activation (such as RIP) (14, 31). However, it is also possible that TNF-R1 is capable of activating NFκB through both TRAF2-dependent and TRAF2-independent pathways and that the relative contribution of the two pathways differs in various cell types. In support of this hypothesis, we have found that expression of dominant negative TRAF2 tends to affect NFκB activation by TNF in a cell type-dependent manner; however, most of the cell types tested were sensitive to its inhibitory effect.
cellular sensitivity to TNF, TRAF2−/− EF cells differed from thymocytes and hematopoietic precursors in that they were TNF resistant; interestingly, however, TRAF2−/− cells (unlike wild type EF) were extremely sensitive to TNF in the presence of CHX, thus indicating that TRAF2 is required for a protein synthesis-independent cytoprotective signal.

Our results also indicate that, although required for TNF-induced cytoprotection and despite the prominent NFκB induction, TRAF2 is not sufficient to protect cells from a subsequent challenge with TNF + CHX. This would suggest that additional TNF-R1-complex components provide a key contribution to the cytoprotective response. Consequently, NFκB activation through the TRAF2 pathway, although required for a complete cytoprotective response, is not itself sufficient to provide any kind of protection. The fact that NFκB activation is not sufficient to switch on the synthesis of protective genes makes sense; indeed, being responsive to a huge number of stimuli, NFκB is able to signal the occurrence of many different extracellular events. Consistent with this, the target genes of NFκB are numerous and are implicated in different cellular functions. Therefore, there must be mechanisms (such as the interaction of NFκB subunits with additional transcription factors that are activated in a stimulus-specific manner) allowing a restricted and specific program of gene expression to be activated in response to a particular inducing agent. Conversely, NFκB activation is usually required for the induction of NFκB-containing promoters (58, 68, 69) (including those of putative protective genes), and this would explain the sensitizing effect of both IκB super-repressor and A20.

In this context, the effects of the TRAF2(1–358) mutant on cell viability are extremely interesting. This mutant is a stronger NFκB activator than full-length TRAF2, however, having its TrafC domain detected, it is unable to interact with TRADD (59). When overexpressed in transfected cells, TRAF2(1–358) is unable to provide protection from TNF + CHX and conversely sensitizes cells to a subsequent challenge with TNF. Our interpretation of these results is that the strong NFκB activation provided by TRAF2(1–358) is not sufficient to switch on the synthesis of cytoprotective genes; on the other hand, this mutant may either interfere with TNF-dependent Traf2 recruitment to TNF-R1/TRADD or titrate TRAF2-bound protective proteins (such as the inhibitors of apoptosis cIAP1 and cIAP2) (47), thus blocking the activation of the cytoprotective response. Irrespective of its mechanism of action, this mutant shows that TRAF2-dependent NFκB activation and cytoprotection can be dissociated and that a cell in which NFκB has been activated can remain exclusively sensitive to TNF cytotoxicity if the TNF-R1/TRAF2 complex is inappropriate disturbed. Moreover, the observation that both NH2-terminal and COOH-terminal TRAF2 deletion mutants are capable of sensitizing cells to TNF-induced apoptosis indicates that both deleted regions are required for activation and maintenance of the cytoprotective response.

In conclusion, the results reported in this paper indicate that TNF-inducible resistance to TNF cytotoxicity is a complex function and NFκB is only one component of the cytoprotective apparatus. Although cellular systems have been described in which NFκB blockade causes a maximal degree of TNF sensitivity (27, 34–36), in most cell types we have tested so far, NFκB blockade usually did not provide a complete sensitization to TNF cytotoxicity, but always negatively affected cellular resistance to TNF in a variable percentage of cells. Conversely, interfering with TRAF2 function(s) nearly completely abrogated the cytoprotective response, thus suggesting that NFκB-independent protective genes are switched on upon TNF-R1 cross-linking in a TRAF2-dependent manner. The required but not sufficient role of TRAF2 in mounting the protective response points to a requirement for additional TNF-R1 complex components, and once more suggests that the integration of multiple signals must occur. We propose the existence of both generally acting (such as NFκB) and cell type-specific (such as AP1 in some cell lines, including Chang cells) cytoprotective components differentially contributing to the high threshold of TNF resistance of most cell types (Fig. 8). This highly integrated system would both provide multiple potential checkpoints and a high security level toward the unwanted elimination of cells exposed to TNF.

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**REFERENCES**

1. Baker, S. J., and Reddy, E. P. (1996) Oncogene 12, 1–9
2. Tewary, M., and Dixit, V. M. (1996) Curr. Opin. Genet. Dev. 6, 39–44
3. Vandenabeele, P., Declerq, W., Beyaert, R., and Fiers, W. (1995) Trends Cell Biol. 5, 392–399
4. Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H.-J., Breger, C., Loetscher, H., and Lienhard, W. (1993) Cell 73, 431–445
5. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) Cell 81, 495–504
6. Cleveland, J. L., and Ile, J. N. (1995) Cell 81, 479–482
7. Feinstein, E., Kinchi, A., Wallach, D., Boldin, M., and Varfolomeev, E. (1995) Trends Biochem. Sci. 20, 342–344
8. Tartaglia, L. A., Ayres, T. M., Wong, G. H. W., and Goeddel, D. V. (1993) Cell 74, 845–851
9. Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Miett, I. L., Camonis, J. H., and Wallach, D. (1994) J. Biol. Chem. 270, 7795–7798
10. Chinnaiyan, A. M., O'Rourke, K., Tewary, M., and Dixit, V. M. (1995) Cell 81, 565–572
11. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) Cell 84, 299–308
12. Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994) Cell 78, 681–692
