Caspase-induced inactivation of the anti-apoptotic TRAF1 during Fas ligand-mediated apoptosis

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Abstract The activation of the transcription factor NF-κB often results in protection against apoptosis. In particular, pro-apoptotic tumor necrosis factor (TNF) signals are blocked by proteins that are induced by NF-κB such as TNFR-associated factor 1 (TRAF1). Here we show that TRAF1 is cleaved after Asp-163 when cells are induced to undergo apoptosis by Fas ligand (FasL). The C-terminal cleavage product blocks the induction of NF-κB by TNF and therefore functions as a dominant negative (DN) form of TRAF1. Our results suggest that the generation of DN-TRAF1 is part of a pro-apoptotic amplification system to assure rapid cell death.

Key words: Apoptosis; Fas; Tumor necrosis factor; NF-κB; Survival; TNFR-associated factor

1. Introduction

The transcription factor NF-κB regulates the expression of a diverse set of genes involved in immune function, differentiation and proliferation [1]. NF-κB activation conveys protection against cell death induced by growth factor withdrawal, tumor necrosis factor (TNF) α and Fas ligand (FasL) [2–6]. However, little is known about the mechanism by which NF-κB provides protection against apoptosis, although the transcriptional activation of survival genes is believed to play a major role [3].

Death induced by TNF is most tightly regulated by NF-κB. Modulation of the response in favor of NF-κB protects cells from TNF-mediated apoptosis, failure to do so results in increased cell death [2,3]. Intriguingly, TNF itself is an activator of NF-κB, indicating the existence of a complex regulatory loop [7]. Upon binding to TNF, TNFR1 binds the adaptor protein TRADD which recruits caspase-8 via FADD leading to apoptosis [8]. Alternatively, TRADD binds to the kinase RIP, TNFR-associated factor 1 (TRAF1) and TRAF2, resulting in the degradation of the inhibitor of NF-κB, I-κB, followed by the translocation of the transcription factor from the cytoplasm into the nucleus. I-κB is degraded by proteasomes upon phosphorylation of two serine residues by specific kinases, named I-κB kinases (IKKs). The importance of these proteins in NF-κB signaling is illustrated by the analysis of IKKβ and NF-κB (RelA subunit) deficient mice, which die at mid-gestation due to extensive apoptosis in the developing liver [1,9,10].

TNF is therefore an interesting example of the activation, by a single cytokine, of two conflicting pathways. It appears that partial activation of the NF-κB pathway suffices to induce a positive modulatory loop which further increases NF-κB signals, thus resulting in a potent blockade of the competing pro-apoptotic pathway. At least four NF-κB responsive genes are involved in this survival amplification loop [3]: IAP-1 and IAP-2 which block caspase activity [11], and TRAF1 and TRAF2 which are implicated in the NF-κB signaling pathway [12]. Whether a similar positive modulatory loop also exists for the pro-apoptotic pathway of TNF (and other death receptors) is not known. It is justifiable that, upon induction of a weak pro-apoptotic signal, silencing of NF-κB inhibitory signals would occur, allowing cell death to proceed with maximal pace. Here we show that apoptosis results in a caspase-dependent cleavage of TRAF1. TRAF1 processing results in the generation of dominant negative (DN) fragments that inhibit further NF-κB signals, thereby blocking anti-apoptotic signals.

2. Materials and methods

2.1. Cell culture

The 293T human embryonic kidney (HEK) cells, BJAB Burkitt lymphoma cells and HT1080 cells (fibrosarcoma cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (100 μg/ml of each) and grown in 5% CO_2 at 37°C. Jurkat and Raji cells were cultured in RPMI supplemented as above.

2.2. Antibodies/reagents

Monoclonal antibodies used in immunoprecipitation and Western blotting include: anti-Flag antibody (Kodak), anti-caspase-3 antibodies (Transduction Laboratories), anti-caspase-8 antibody (PharMingen), anti-TRAF1 antibody (H3, Santa Cruz), anti-VSV antibody (P5D4, Sigma). Human recombinant ligands (TRAIL, FasL, TNFα) were obtained from Alexis, Switzerland. Incubations with TRAIL and FasL were performed in the presence of 1 μg/ml anti-Flag M2 antibody. Caspase inhibitors were purchased from Alexis. Active caspase-3, -6, -7 and -8 were kindly provided by Gay Salveson, San Diego, CA, USA.

2.3. Expression vectors

Expression vectors for TRAF1, TRAF1(164–415), TRAF1Asp-163Ala, TRAF1Asp225Ala were generated by PCR amplification/PCR-based mutagenesis, and subcloned into pCR3-derived vectors (Invitrogen) conferring an N-terminal and/or C-terminal Flag-tag.

The following plasmids were obtained from the indicated sources: NF-κB luc, a dominant form of I-κB (I-κBmut) (V. Jongeneel, Epalinges, Switzerland), myc-TRADD (D.V. Goeddel, Tularik, San Francisco, CA, USA).

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2.4. Transfection

293T Cells were seeded at 4×10^5 in a 3.5 cm plate and transfected by the calcium phosphate precipitation method. The precipitate was left for 8 h, cells were collected 24 h after transfection, lysed in sodium dodecyl sulfate (SDS) sample buffer by sonification, and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot. HT1080 cell lines expressing I-κBmut or TRAF1DN were established as described [3].

2.5. Induction of TRAF1 or apoptosis

5×10^5 cells were incubated for the indicated times with TRAIL, FasL and TNF. Incubations with TRAIL and FasL were performed in the presence of 1 μg/ml anti-Flag M2 antibody. Cells were harvested, washed one time with phosphate-buffered saline (PBS), lysed in SDS sample buffer by sonification and analyzed by SDS–PAGE and Western blot analysis. Alternatively, surviving cells were quantified with the Celltiter 96 AQ proliferation assay (Promega) following the manufacturer's instructions.

2.6. In vitro translation/caspase cleavage assay

In vitro translation of the TRAF1 wild-type and mutant forms was carried out with the TNT Coupled Reticulocyte Lysate System (Promega) according to manufacturer's instructions in the presence of [^{35}S]methionine (Amersham). Translations were incubated for 2 h in 20 mM PIPES, 100 mM NaCl, 1% sucrose, 10 mM dithiothreitol, 0.1% CHAPS and 0.1 mM EDTA, pH 7.2, at 37°C in the presence of caspase-3, -6, -7 or -8 as described [13]. Reaction mixtures were subjected to SDS–PAGE, transferred to nitrocellulose and analyzed by autoradiography.

2.7. Luciferase assay

293T Cells were co-transfected with 1 μg NF-κB luciferase reporter plasmid (NF-κB luc), 0.5 μg pCMV β-gal (as an internal efficiency control) and 1 μg of the indicated expression plasmids or empty vector. 24 h post-transfection, the cells were washed one time with PBS and incubated for 5 h with 20 ng/ml TNFα in the absence of FCS. Luciferase activities were determined using luciferase assay reagent (Promega) as previously described [14].

3. Results

3.1. Cleavage of TRAF1 during apoptosis in 293T cells

While studying pro-apoptotic signaling pathways in HEK293T cells, we observed that TRAF1 was cleaved from the intact 51 kDa protein into a 27 kDa N-terminal fragment when co-transfected with an apoptosis-inducing protein (Fig. 1A). This processing of TRAF1 was similarly detected when apoptosis was triggered via the mitochondrial pathway (Bak) or via pathways emanating from the cell surface through death receptors, including Fas, TRAMP/DR-3 and TRAIL-R1, or components of their signaling pathways (TRADD, RIP) which induce cell death in a ligand-independent manner when overexpressed [15]. Identical TRAF1 processing was also observed when 293T cells were treated with recombinant TRAIL and FasL (Fig. 1B). FasL-induced TRAF1 processing was less efficient, which is explained by the fact that 293T cells are only moderately sensitive to FasL. Of the six TRAF proteins identified thus far [12], this processing appeared to be unique to TRAF1 as cleavage was not observed with TRAF2–6 (data not shown). TRAF1 cleavage was effectively blocked by the general caspase inhibitor z-VAD-fmk, but not by other protease inhibitors (data not shown). We therefore hypothesized that TRAF1 cleavage might be caspase-mediated and to this end, we mutated possible caspase cleavage sites in TRAF1 (Fig. 1C). While the Asp225Ala replacement had no effect on TRAF1 processing, the Asp163Ala mutation completely abolished cleavage during apoptosis (Figs. 1D, 2B and data not shown). All TRAFs contain a conserved TRAF domain at

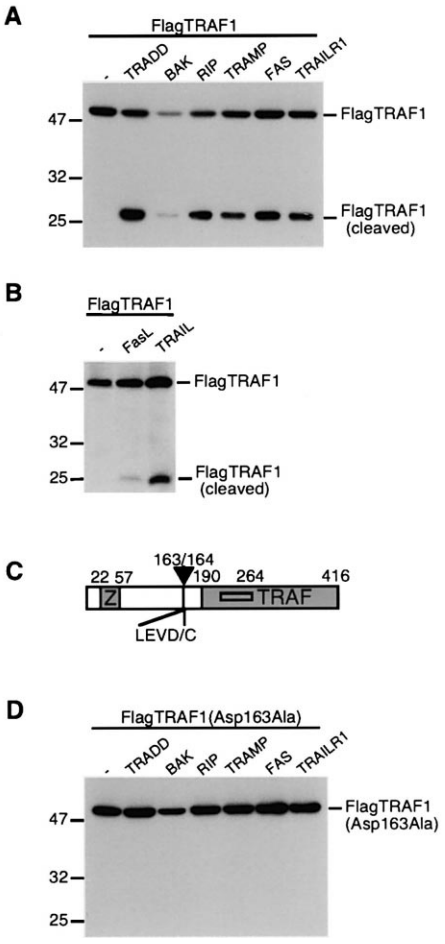

Fig. 1. TRAF1 cleavage during apoptosis. (A) 293T Cells were transfected with 3 μg of a plasmid encoding the indicated apoptosis-inducing proteins together with 0.3 μg FlagTRAF1. Processing of TRAF1 was revealed by immunoblotting using an anti-Flag antibody. (B) 293T Cells were transfected with 0.3 μg FlagTRAF1 during 1 day, treated for an additional 12 h with recombinant cross-linked soluble FasL (FasL, 1 μg/ml) or soluble TRAIL (100 ng/ml) and then analyzed as above. (C) Diagram of human TRAF1, indicating the caspase cleavage site (163/164), the zinc finger region (Z) and the TRAF domain (TRAF) which includes a coiled-coil domain (stippled box). (D) As (A), but 293T cells were transfected with the FlagTRAF1 mutant (Asp163Ala) construct.

their carboxyl-terminus, which is required for homo- and hetero-oligomerization of TRAFs, binding to the receptor and for the interaction with other proteins [12]. The N-terminal segment of most TRAFs is characterized by the presence of a RING finger domain, followed by a stretch of zinc fingers. TRAF1 is unique among the TRAFs in two regards (Fig. 1C). First, it lacks the N-terminal RING finger domain, and second, its expression is not constitutive, but has to be induced [16]. Cleavage at Asp-163 is predicted to separate the N-terminal fragment of TRAF1 containing a zinc finger from the C-terminal TRAF domain (Fig. 1C).

3.2. TRAF1 cleavage by purified caspases in vitro

Comparison of the processing site in TRAF1 (LEVD/C) with the cleavage specificities of various caspases [17] indicated that it resembled the substrate sites of caspase-8 and caspase-6. We therefore performed cleavage experiments using in vitro translated and ^{35}S-labeled TRAF1. Incubation of

TRAF1 with a panel of purified recombinant caspases at different concentrations resulted in the generation of two cleavage products (Fig. 2A). The N-terminal fragment migrated slower than expected (apparent molecular weight: 26 kDa, calculated: 19 kDa), whereas the C-terminal fragment migrated as predicted (29 kDa) and was not further processed by either caspase (Fig. 2B and data not shown). In vitro, caspases-3, -6 and most efficiently caspase-8 cleaved TRAF1, whereas TRAF1 was not a substrate for caspase-7. The mutant TRAF1 (Asp163Ala) was not processed by either caspase (Fig. 2C), again demonstrating that Asp-163 is the only caspase cleavage site present in TRAF1.

3.3. Cleavage of TRAF1 is an early event during FasL-mediated apoptosis

TRAF1 expression is known to be restricted to certain tissues and to be induced through the activation of NF-κB [16]. We therefore screened several cell lines for TRAF1 expression. TRAF1 was strongly induced in HT1080 cells by TNF, and weakly in HeLa cells (Fig. 3A) as reported previously [3,16]. Burkitt lymphoma-derived Raji and BJAB cells constitutively express TRAF1 (Fig. 3A). As both Raji and BJAB cells are also susceptible to Fas triggering, we chose these cells to study conversion of TRAF1. FasL induces apoptosis by the sequential activation of caspase-8, caspase-3 and other caspases which finally results in the cleavage of a wide range of cellular

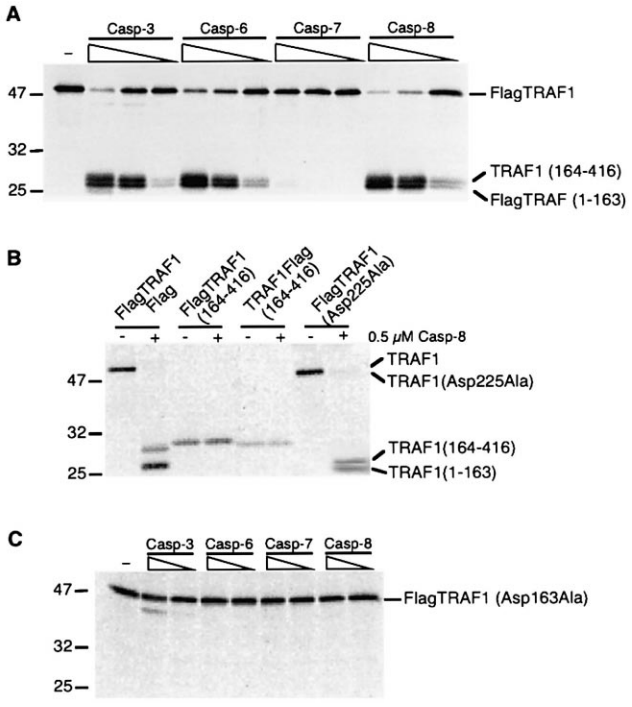

Fig. 2. Processing of TRAF1 by caspases in vitro. (A) In vitro translated FlagTRAF1 was incubated with decreasing concentrations of caspase-3, -6, -7 or -8 (0.5, 0.05 and 0.005 μM). (B) Various in vitro translated TRAF1 constructs (TRAF1 with N- and C-terminal Flag; TRAF1(164–416) with N- or C-terminal Flag; TRAF1(Asp-225Ala)) were incubated in the presence or absence of caspase-8 (0.5 μM). (C) In vitro translated mutant FlagTRAF1(Asp163Ala) was incubated with decreasing concentrations of caspase-3, -6, -7 or -8 (0.5 and 0.05 μM). Reaction mixtures were incubated for 2 h at 37°C, and then analyzed by SDS–PAGE, transferred onto nitrocellulose and subsequently autoradiographed.

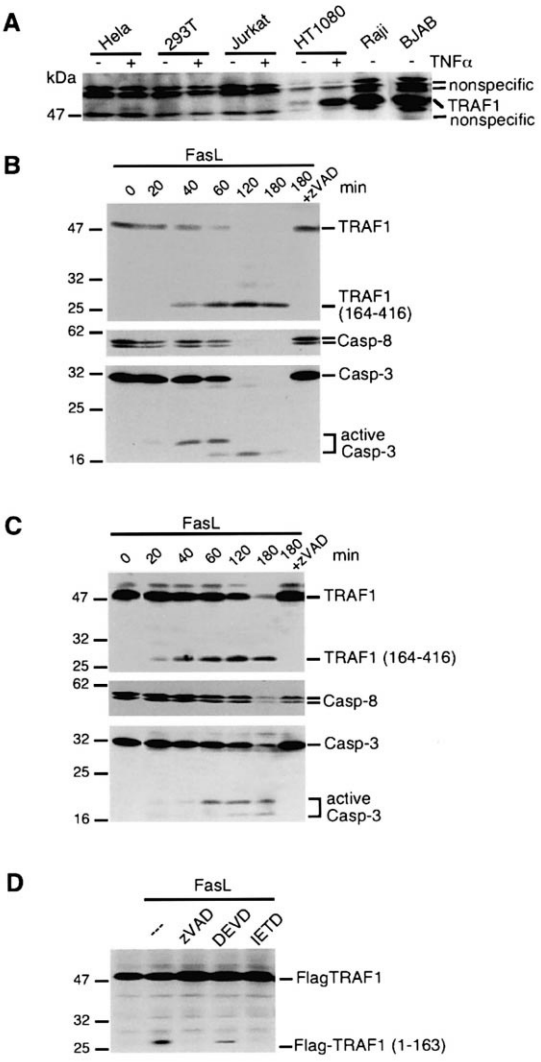

Fig. 3. Expression of TRAF1 in various cell lines and time course of TRAF1 cleavage during FasL-induced apoptosis. (A) Various cell lines (10^6 cells) were incubated for 6 h in the presence or absence of TNF (30 ng/ml) as indicated and assayed for TRAF1 expression by immunoblotting. (B) BJAB cells and (C) Raji cells were treated with cross-linked sFasL (300 ng/ml) for the indicated times. The general caspase inhibitor z-VAD-fmk was added as indicated. Processing of TRAF1, caspase-3 and caspase-8 was assessed by Western blot analysis of cell lysates using the appropriate antibodies. (D) Flag-TRAF1-transfected 293T cells were treated with cross-linked FasL for 12 h (see Fig. 1B) in the presence of various caspase inhibitors. TRAF1 was detected using the anti-Flag M2 antibody.

substrates. The induction of apoptosis in BJAB cells led to the activation of caspase-8 following 40–60 min as shown by the disappearance of the caspase-8 precursor (Fig. 3B). The TRAF1 cleavage product was already detected following 20–40 min, at the same time, when the subunits of active caspase-3 appeared. In Raji cells, FasL-induced TRAF1 cleavage was observed before caspase-3 activation (Fig. 3C), suggesting that TRAF1 cleavage occurs early during signal transduction by activated caspase-8. This is supported by the observation (Fig. 3D) that the addition of either the broad range caspase inhibitor z-VAD-fmk or the caspase-8 inhibitor IETD-fmk completely blocked TRAF1 conversion in 293T

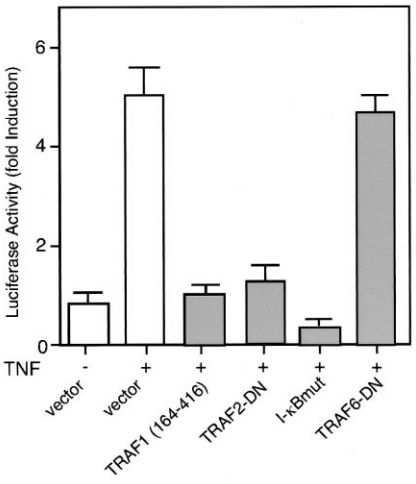

Fig. 4. Transcription factor NF-κB is blocked by the C-terminal TRAF1 cleavage product. 293T Cells were transfected with Flag-TRAF1(164–416) or with DN forms of other proteins implicated in the NF-κB pathway. 24 h after transfection, cells were treated with TNF (10 ng/ml) for 6 h and NF-κB activity was measured by a luciferase reporter assay. Values shown are averages of four representative experiments in which each transfection was carried out in duplicate.

cells, whereas only partial inhibition was observed when the caspase-3 inhibitor DEVD-fmk was included.

3.4. The C-terminal cleavage product functions as a DN form to inhibit TNF-induced activation of NF-κB

Cleavage of TRAF1 occurs at the Asp-163 residue, which is located approximately 30 amino acids upstream of the TRAF domain. The C-terminal cleavage product of TRAF1 therefore closely resembles the DN versions of TRAF1 and other TRAFs, which only contain the C-terminal TRAF domain [16]. We therefore reasoned that cleavage of TRAF1 could lead to a block of TRAF1-dependent activation of NF-κB, and thus to the triggering of a pro-apoptotic loop. We transfected 293T cells with the truncated TRAF1, corresponding to the fragment generated in apoptotic cells. The treatment with TNF of vector-transfected cells induced a strong activation of NF-κB which was blocked by the expression of TRAF1(164–516) in a similar manner to other inhibitors of TNF-mediated NF-κB activation, including TRAF2DN and a DN form of I-κB (Fig. 4).

3.5. NF-κB activation in HT1080 results in TRAF1 induction and protection from FasL-induced apoptosis which is reverted when NF-κB signals are blocked

HT1080 fibrosarcoma cells have been widely used to study the regulation of TNF-induced cell death. The parental cell line is resistant to TNF-induced apoptosis and instead activates signals leading to NF-κB activation, whereas cell death ensues if NF-κB activation is inhibited [3]. We therefore chose this cell line to study the influence of NF-κB signals on FasL-induced apoptosis and to investigate the significance of TRAF1 cleavage.

In contrast to TNF [3], the parental cell line was not resistant to FasL (Fig. 5). Activation of caspase-3 was observed 3 h after addition of the ligand. However, preincubation of the HT1080 cells with TNF for 6 h, which resulted in a massive NF-κB activation as evidenced by TRAF1 upregulation, conveyed resistance to FasL (Fig. 5A). Nevertheless, Fas resistance remained only partial, due to caspase-3 activation and as a consequence TRAF1 cleavage which was clearly observed after 24 h.

Having established that HT1080 cell death by Fas can be modulated by NF-κB, we investigated whether the complete blockade of the NF-κB signals would also lead to increased death as has been shown for TNF [3]. In agreement with the published results, HT1080 cells stably expressing a non-de-

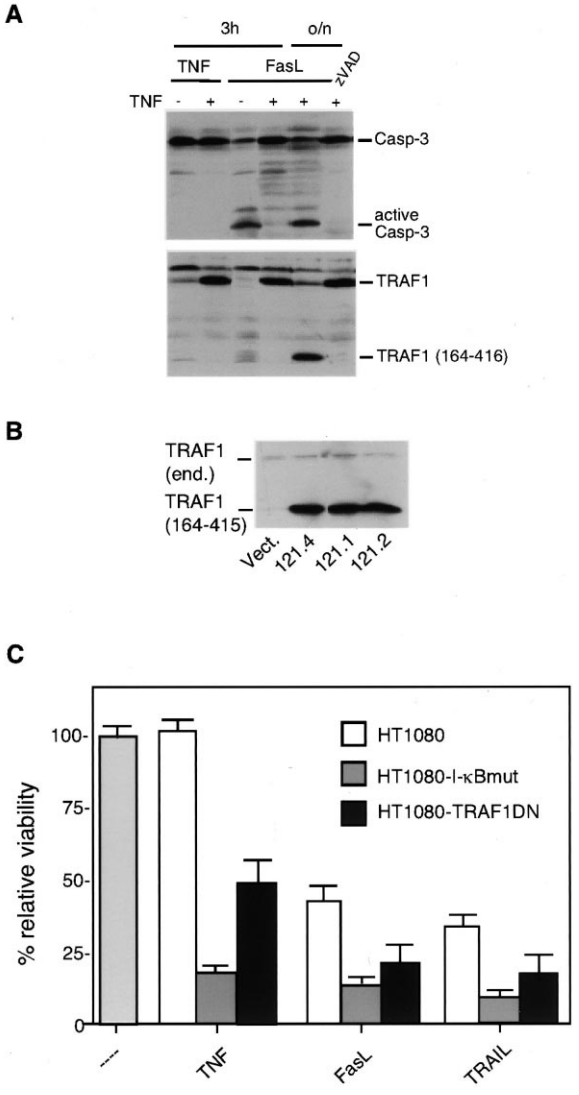

Fig. 5. (A) Activation of NF-κB signaling pathway prevents rapid Fas-mediated apoptosis. HT1080 cells were pretreated or not pretreated with TNF (30 ng/ml) for 6 h and then incubated with cross-linked FasL (300 ng/ml) for 3 h or overnight (o/n) in the presence or absence of z-VAD-fmk. Cell lysates were analyzed for processing of caspase-3 and TRAF1 as described in Fig. 3. (B) DN-TRAF1(164–415) promotes death ligand-induced apoptosis. HT1080 fibrosarcoma cells were stably transfected with the FlagTRAF1(164–415) expression plasmid and expression of FlagTRAF1(164–415) was analyzed in several clones by immunoblotting using the M2 anti-FLAG antibody. (C) A representative HT1080-TRAF1DN clone, and as controls parental HT1080 cells and HT1080 cells expressing a DN mutant form of I-κB (I-κBmut), were incubated with TNF (100 ng/ml), FasL (100 ng/ml) or TRAIL (100 ng/ml), respectively, and the viability of cells was analyzed. Other clones analyzed showed similar resistance to death ligands.

gradable inhibitor of NF-κB (I-κBmut), thereby blocking NF-κB function, were sensitive to TNF killing. The same mutant cells also showed increased sensitivity to FasL and TRAIL (Fig. 5C). Thus, complete suppression of NF-κB activity renders HT1080 cells more sensitive to several death ligands.

To determine whether the TRAF1(164–415) fragment, which in transient overexpression experiments blocked NF-κB activation by TNF almost as efficiently as I-κBmut (see Fig. 4), would also sensitize HT1080 cells, several clones were established that stably express TRAF1(164–415) (Fig. 5B). Indeed, all HT1080-TRAF1DN cells analyzed were sensitive to TNF, and increased sensitivity to FasL and TRAIL was also observed (Fig. 5C). However, if compared to the HT1080-I-κBmut cells, sensitization by TRAF1DN was less efficient.

4. Discussion

The involvement of NF-κB activation in regulating apoptosis has been documented by several groups. Inhibition of NF-κB nuclear translocation increases the susceptibility of cells to undergo apoptosis by TNF, FasL, ionizing radiation, cancer therapeutic drugs and growth factor withdrawal [2–4,6].

Here we show that at least one of the proteins that is crucial in the TNF anti-apoptotic response is inactivated during Fas-mediated apoptosis, i.e. TRAF1. TRAF1 has a direct anti-apoptotic effect. TRAF1 was shown to directly associate with TNFR2, CD30, 4-1BB [18], Ox40[18], HVEM [19] and LMP-1 [20]. In addition to forming homodimers and heterodimers with TRAF2, TRAF1 has been shown to directly interact with TRADD [21], I-TRAF [22], TRIP [23], A20 [24], c-IAP-1, c-IAP-2 [25], FLIP [26], RIP [27], CARMEN/Bcl-10 [28], CARDIAK/RIP2 [29,30] and NIK [31]. The significance of these interactions is not clear in many cases, but the anti-apoptotic nature of most interaction partners is in accordance with published data on TRAF1 function. TRAF1 transgenic mice show a defect in antigen-induced apoptosis of CD8 T cells [32] and TRAF1 is part of the NF-κB-induced anti-apoptotic response [3].

Thus, the generation of a DN form of TRAF1 will lead to the impairment of an anti-apoptotic pathway controlled by NF-κB and will ultimately result in a rapid cell death, thus avoiding the situation that a cell is half dead and half alive.

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