Cooperation of Both TNF Receptors in Inducing Apoptosis: Involvement of the TNF Receptor-Associated Factor Binding Domain of the TNF Receptor 75

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Cooperation of Both TNF Receptors in Inducing Apoptosis: Involvement of the TNF Receptor-Associated Factor Binding Domain of the TNF Receptor 75

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TNF-R55 is the main receptor mediating TNF-induced cytotoxicity. However, in some cells TNF-R75 also signals cell death. In PC60 cells, the presence of both receptor types is required to induce apoptosis following either specific TNF-R55 or TNF-R75 triggering, pointing to a mechanism of receptor cooperation. In this study, we extend previous observations and show that TNF-R55 and TNF-R75 cooperation in the case of apoptosis in PC60 cells is bidirectional. We also demonstrate ligand-independent TNF-R55-mediated cooperation in TNF-R75-induced granulocyte/macrophage-CSF secretion, but not vice versa. To determine which part of the intracellular TNF-R75 sequence was responsible for the observed receptor cooperation in apoptosis, we introduced different TNF-R75 mutant constructs in PC60 cells already expressing TNF-R55. Our data indicate that an intact TNF-R-associated factors 1 and 2 (TRAF1/TRAF2)-binding domain is required for receptor cooperation. These findings suggest a role for the TRAF complex in TNF-R cooperation in the induction of cell death in PC60 cells. Nevertheless, introduction of a dominant negative (DN) TRAF2 molecule was not able to affect receptor cooperation. Remarkably, TRAF2-DN overexpression, which was found to inhibit the TNF-dependent recruitment of endogenous wild-type TRAF2 to the TNF-R75 signaling complex, could neither block TNF-R55- nor TNF-R75-induced NF-κB activation nor granulocyte/macrophage-CSF secretion. Possibly, additional factors different from TRAF2 are involved in TNF-mediated NF-κB activation. The Journal of Immunology, 1998, 161: 390–399.

The role of TNF-R75 in cell death has long been controversial, and its activity was proposed to be independent of signal transduction (13–18). The ligand-passing model reconciled many seemingly conflicting data and showed that, due to its higher affinity and rapid dissociation kinetics, TNF-R75 could regulate the rate of TNF association with TNF-R55 (17). However, in the past few years it has become clear that TNF-R75-specific triggering has a signaling role for apoptosis in PC60, K5M-1, HeLa, Colo205, and mature T cells (11, 15, 19–22).

In recent years, the signaling events leading to TNF-R-mediated cell death have been partially elucidated (1, 23, 24). Mainly by the use of two-hybrid-based strategies, several intracellular TNF-R55-, TNF-R75-, and Fas/Apo-1-signaling complex-constituting proteins were identified. These molecules belong to four novel families of signal-transducing effectors. First, the TNF-R-associated factors 1 and 2 (TRAF1 and TRAF2) were cloned on the basis of their associating capacity with TNF-R75 (25). TRAFs associate by means of a conserved C-terminal TRAF domain with members of the TNF-R superfamily. A 78-residue C-terminal region of TNF-R75, the TRAF-binding region, interacts with these signal transducers. TRAF2 mediates NF-κB activation by TNF-R55, TNF-R75, and CD40 (26, 27). In general, TRAF family members seem to be involved in cytokine-induced gene activation (26–30). Second, the TNF-R55- and Fas-associated DD proteins TRADD, FADD/MORT1, and RIP, together with the TNF-R family members TNF-R55, Fas, and the DR3–5, constitute a family of molecules containing a DD motif, which is involved in homotypic and heterotypic protein/protein interactions (7–10, 31–34). Ligand-induced receptor aggregation targets these DD proteins to their respective receptors, i.e., TRADD/FADD/RIP to TNF-R55 (although FADD recruitment to the endogenous TNF-R55 has not been shown yet) and FADD to Fas/Apo-1 (27, 35–37). Overexpression of these DD molecules leads to cell death, probably by...
mimicking the formation of an intracellular ligand-induced signaling complex. A FADD molecule lacking its N-terminal region functions as a dominant negative (DN) mutant able to inhibit TNF-R55-, Fas/Apo-1-, and DR3-induced apoptosis (5, 27, 37). TRADD and RIP, besides their presumed role in cell death, are also involved in NF-kB activation by their ability to recruit TRAF2 (27, 35, 38). A third family of TNF-R complex-constituting proteins involves the baculoviral IAP-related inhibitors of apoptosis c-IAP1 and c-IAP2 (39, 40). Although it is known that c-IAP1 and -2 participate in the TNF-R75 complex and that c-IAP1 can be recruited to TNF-R55, in both cases via their affinity for TRAF1 and/or TRAF2, their function in signal transduction is still unclear. A fourth class of proteins links the receptor-associating death-inducing FADD molecule to ICE/CED-3-like cysteine proteases (caspases) involved in the process of apoptosis. The caspase-8 molecule binds with its N-terminal part, by means of a homologous death effector domain, to the N-terminally located death effector domain of FADD (41, 42). Expression of caspase-8 mutants lacking enzymatic activity block both TNF-R55- and Fas/Apo-1-mediated cytotoxicity (41). The mechanism by which procaspase-8 becomes activated after recruitment in the death-inducing signaling complex is still unclear (43); but once activated, caspase-8 is able to activate downstream caspases (44).

These findings indicate that members of the TNF-R family share various intracellular signaling proteins to activate similar transduction pathways. This sheds new light on the possible mechanism of TNF-R55 and TNF-R75 cooperation. Several recent reports have strongly suggested cooperation between TNF-R55 and TNF-R75, especially in the case of induction of cytotoxicity (11, 12, 21, 45, 46).

We developed a thymoma system, based on the introduction of human TNF-R in the rat/mouse T cell hybridoma PC60, to investigate the role of TNF-R55 and TNF-R75 in TNF responses. Previously, we have shown that both receptors are signal-transducing in these cells (11, 12, 47). Remarkably, only PC60 cells expressing both of the receptor types were sensitive to TNF-induced apoptosis. Furthermore, we reported that the intracellular domain of TNF-R75 is required to mediate this receptor cooperation, thereby excluding a role for ligand passing (48). In the present report, we show the existence of a bidirectional TNF-R55 and TNF-R75 cooperation at the level of apoptosis induction and a unidirectional ligand-independent cooperation of TNF-R55 in TNF-R75-induced granulocyte/macrophage-CSF (GM-CSF) secretion. Our studies with TNF-R75 mutants suggest a role for the TNF-R75-associated TRAF1/TRAF2 complex in the observed TNF-R55 cooperation in apoptosis.

Materials and Methods

Cytokines and cytokine assays

Purified E. coli-derived human TNF, the human TNF-R55-specific mutein R32WS86T, and the human TNF-R75-specific mutein D143F were prepared, using the murine GM-CSF preparation of the National Institute for Biological Standards (Potters Bar, U.K.) as a reference, in an FDCp1 proliferation assay (49).

Cells

The hybridoma PC60.21.14.4, provided by Dr. M. Nabholz (Swiss Institute of Experimental Cancer Research, Epalinges, Switzerland), was transfected with cDNAs coding for TNF-R55, TNF-R75, or both, as previously reported (11). The representative cell clones used for each type of transfectant were PC60 R55/8, PC60 R75/24, and PC60 R55R75/5, respectively.

Construction of mutant TNF-R75 and electroporation of PC60 cells

The starting plasmid for TNF-R75 mutagenesis, containing wild-type (wt) TNF-R75 under control of the SV40 early promoter, was previously described (11). TNF-R75 M1 was generated by digestion of the unique BstXI restriction site, removal of the protruding 3' sticky end with T4 polymerase, and ligation to the XhoI linker CCTCTAGAG, generating an in-frame stop codon. Due to the cloning procedure, an additional stretch of five irrelevant amino acids was added. The M3 mutant (∆304–345) resulted from the removal of the sequence between two SacI restriction sites present in the cytoplasmic domain. R75 M4 (∆288–340) was constructed by deleting an XmaI fragment in the intracellular receptor part. M3 (∆403-end) was generated by introducing an in-frame stop codon at the indicated position by PCR, thereby deleting the sequence coding for the 37 C-terminal amino acids. Mutant receptors were verified using dsDNA sequencing. PC60 R55/8, PC60 R75/24, or PC60 R55R75/5 cells were transfected by electroporation as reported (48). Briefly, cells were washed with growth medium (RPMI 1640) supplemented with FCS. Wild-type and mutant TNF-R expression plasmids (20 μg), together with 1 μg of a selection plasmid pSV2Neo (G418 selection), or a murine TRAF2-DN expression plasmid prK5TRAf2D-N (20 μg), a generous gift of Dr. D. V. Goeddel (25), together with the selection plasmid pUTSV1 (Cayla, Toulouse, France; phleomycine selection) were added to the cells, and the mixture was exposed to a single electric pulse (1500 μF, 300 V). Two days later, G418 (1500 μg; Life Technologies, Paisley, U.K.) or phleomycine (200 μg; Cayla) was added, and cells were cloned by limiting dilution. Cell lines derived from transfected PC60 R55/8 or PC60 R75/24 were named PC60 R55NR75 and PC60 R75NR55, respectively.

Immunofluorescence and propidium iodide (PI) exclusion assays

TNF-R75 expression on transfected cells was analyzed by staining for 45 min with 1 μg utr-1/ml, a mAb against TNF-R75 (50), and subsequent incubation with a fluorescein-conjugated goat anti-mouse Ab, followed by fluorocytometric detection with a Coulter Epics 753 (Coulter, Hialeah, FL). The number of apoptotic cells in a given culture was measured by a PI exclusion assay as described (11). PI was added at a final concentration of 30 μM, and cells were analyzed on an Epics 753 fluorometer.

Immunoprecipitation and immunoblotting

PC60 cells (10^7) expressing different TNF-R combinations were washed twice with ice-cold PBS A and lysed (15 min) in 1 ml lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 100 μM PMSF, 0.27 trypsin-inhibitory U/ml aprotinin, 10 μg/ml leupeptin, and 2 mM pefabloc) (27). After centrifugation at 14,000 × g (10 min), 20 μg of the TNF-R75-specific Ab ut-4 (50) was added to the supernatant and left for 1 h at 4°C, then mixed with 40 μl of 1.1 slurry of protein A-Sepharose for 1.5 h. Beads were washed twice with lysis buffer, twice with high salt (1 M) lysis buffer, and again twice with low salt lysis buffer. Proteins were eluted with Laemmli gel-loading buffer containing 4.2% ME-2-50, loaded on a 10% SDS-polyacrylamide gel, and after electrophoresis blotted to a nitrocellulose membrane. TNF-R75 proteins were revealed using a rabbit polyclonal anti-TNF-R75 antiserum (a gift of Dr. W. A. Buurman, University of Maastricht) and enhanced chemiluminescence (ECL; Amersham Life Science, Amersham, U.K.). For immunoprecipitations, the parental PC60 R55R75/5 and PC60 R55R75T2DN cells (10^7) were washed with warm growth medium (37°C) and incubated for different time intervals in the presence or absence of TNF (1 μg/ml) in a 10-ml volume on a rotating wheel, then lysed with 4 ml lysis buffer (150 mM NaCl) further supplemented with 30 mM NaF and 2 mM Na-tyrosophosphate. The lysate was further incubated with a combination of utr-4 Ab (50) and protein A-Trisacryl beads or with protein A-Trisacryl beads alone and further treated as above (27). Immunoblots were developed with anti-murine TRAF2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-TNF-R75 antiserum using ECL.

Results

TNF-R55 and TNF-R75 cooperation in PC60 cells

Previously, we have shown that PC60 cells transfected with both human TNF-R were sensitive to TNF-mediated apoptosis, in contrast to cells expressing only one TNF-R type. Both TNF-R55 and TNF-R75 were able to elicit intermediate apoptosis levels in the double transfecants when separately triggered by agonistic Abs or receptor-specific TNF muteins (11). The percentage of PI-positive cells was taken as a measure for apoptotic death in these cultures.
To further confirm these results, we transfected TNF-R55 and TNF-R75 single transfectant PC60 cells with TNF-R75 or TNF-R55 expression plasmids, respectively. Cell lines expressing only one TNF-R type, i.e., PC60 R55/8 and PC60 R75/24, were transfected with expression plasmids containing TNF-R75 and TNF-R55 cDNAs, resulting in PC60 R55NR75 and PC60 R75NR55 cell lines, respectively (representative clones are used). Cell lines transfected with only the selection plasmid were named PC60 R55/8 Neo and PC60 R75/24 Neo. Cells (5 × 10⁴/well) were cultured in the absence (control) or presence of saturating concentrations of TNF (100 ng/ml) or TNF-R55 (R32WS86T) or TNF-R75-specific (D143F) TNF muteins (500 ng/ml). After 20 h, the percentage of PI-positive cells was determined by cytofluorometric analysis.

To further dissect the mechanism of TNF-R cooperation in PC60 cells, we characterized the intracellular part of TNF-R75 responsible for cooperation with TNF-R55. Therefore, several TNF-R75 deletion mutants were constructed and expressed in PC60 R55 cells. Comparison of the human and mouse TNF-R75 intracellular sequences revealed two major boxes of homology: a conserved domain containing a sequence of six consecutive serine residues and the TRAF-binding region (Fig. 3A). We decided to make deletion mutants in which the serine-rich region (mutants M3 ([D304 –345]) and M4 ([D288 –340])) or the TRAF-binding domain (M1, [D313-end]) were removed (Fig. 3B). Rothe et al. previously reported that deletion of the 37 C-terminal amino acids resulted in of TNF-R55 and TNF-R75 was as cytotoxic as stimulation with wt-TNF. Specific stimulation of TNF-R75 in PC60 R75 cells induced consistently low, but reproducible GM-CSF levels (Ref. 47; Fig. 2). After introduction of TNF-R55 in these cells, treatment with the TNF-R75-specific agent D143F resulted in considerably enhanced GM-CSF production (Fig. 2). Since single-transfected TNF-R55⁺ cells already produced elevated levels of GM-CSF after specific receptor stimulation and since the levels of induced cytokine varied between different clones, we were not able to conclude unambiguously whether, reciprocally, the presence of TNF-R55 facilitated the R75 GM-CSF-inducing capacity (see below and data not shown). These results confirm and extend our previous observations indicating cooperation by even an unliganded second receptor in PC60 cells (11). Our data show a bidirectional cooperation between both TNF-R in inducing apoptosis and a clear help of the TNF-R55 presence in TNF-R75-signaling to GM-CSF secretion.
FIGURE 3. Expression of mutant TNF-R75 in PC60 R55\(^+\) cells. A, Comparison of the intracellular sequences of human (h) and murine (m) TNF-R75. The TRAF-binding domain is shown in italics. Additionally, the positions of the Ser box, a putative TRAF-binding site, and the positions of the TNF-R75 mutant constructs described here are indicated. B, Schematic representation of the TNF-R75 mutants constructed. TM, transmembrane region; EC, extracellular receptor part; IC, intracellular receptor part. The Ser box contains six consecutive Ser residues. C, PC60 R55/8-derived cell lines expressing different TNF-R75 constructs (see B) were incubated with control (thin line) or TNF-R75-specific utr-1 (thick line) Abs. The second Ab used was FITC-labeled goat anti-mouse Ab. TNF-R75 expression levels were analyzed on a Coulter Epics 753 cytofluorometer. D, PC60 R55/8-derived cell lines expressing different TNF-R75 constructs (see B), were lysed and the cleared lysates were treated with TNF-R75-specific utr-4 Ab. The resulting immunoprecipitates were subjected to PAGE and electroblotted. The blots were developed using a polyclonal rabbit antiserum raised against TNF-R75 and ECL.
Deletion of the serine box-containing domain has no influence on the functionality of TNF-R75

PC60 R55NR75 M3 (Δ304–345) and PC60 R55NR75 M4 (Δ288–340) cells expressing TNF-R75 lacking the Ser box were stimulated with TNF-R-specific muteins or TNF, and the levels of GM-CSF induced were quantified. From Figure 4A it was clear that all cell lines responded to TNF-R55-specific triggering (R32WS86T), thereby showing the presence of TNF-R55 on the transfected PC60 cells. D143F stimulation of the mutant TNF-R75, M3 and M4, stimulated the secretion of similar amounts of GM-CSF as activation of wt-TNF-R75 in PC60 R55R75/S cells. Simultaneous activation of both TNF-R55 and TNF-R75 resulted in a synergistic production of GM-CSF in wt as well as in R75 M3- and R75 M4-coexpressing cells. This indicated that the gene-inducing capacity of TNF-R75 was not affected by removal of the conserved Ser box. When these cultures were analyzed for the percentage of apoptotic cells induced by TNF-R triggering, it was clear that R75 M3 and R75 M4 were still able to cooperate with R55 to render PC60 cells susceptible to TNF-R-mediated cell death, as did wt-R75 (Fig. 4B). Additionally, M3 and M4 R75 mutants induced apoptosis after specific stimulation with D143F. Parental PC60 R55/8 cells and control PC60 R55/8 Neo transfectants showed no apoptotic cells after mutant TNF treatment. Hence, we concluded that the TNF-R75 Ser box region was not involved in the process of receptor cooperation, nor did deletion of this R75 region influence its apoptosis- or gene-inducing capability.

Disruption of the TRAF-binding domain in TNF-R75 abrogates its functionality and cooperation in TNF-R55-mediated apoptosis

Complete deletion of the TNF-R75 TRAF-binding domain (M1, Δ313-end) resulted in loss of receptor functionality. TNF-R75-specific triggering of PC60 R55NR75 M1 cells was not able to induce any GM-CSF secretion neither on its own, nor in synergy with TNF-R55 stimulation (Fig. 5A). Moreover, this mutated receptor was no longer capable of transducing a signal required for receptor cooperation in inducing apoptosis by TNF-R55 (Fig. 5B). Removal of the 37 C-terminal amino acids of the intracellular TNF-R75 domain (Δ403-end), which disturbs the TRAF complex binding to this receptor (25), had identical effects. PC60 R55NR75 M5 cells stimulated with D143F did not produce GM-CSF and did not show an apoptotic response even when stimulated with TNF (Fig. 5). These results led us to postulate an involvement of the TRAF-binding domain, and hence the TRAF1/TRAF2 complex, not only in R75-mediated GM-CSF induction but also in the cooperation that allows TNF-R55 to induce apoptosis.

Overexpression of a TRAF2-DN molecule does not inhibit receptor cooperation nor NF-κB activation in PC60 R55R75 cells

Since our results with the TNF-R75 M5 mutant pointed to a role for the TRAF1/TRAF2 complex in receptor cooperation in PC60 R55R75 cells, we transfected these cells with a TRAF2-DN molecule lacking its RING-finger domain. This mutant TRAF2 was previously shown to be able to inhibit TNF-R55-, TNF-R75-, and CD40-mediated NF-κB activation after transient overexpression (26, 27). The function of TRAF1 is still unresolved. PC60 R55R75T2DN clones expressing high levels of the TRAF2-DN protein were selected (Fig. 6A). Functional analysis of TRAF2-DN-expressing PC60 R55R75 cells, however, revealed that there was no influence on the induction of apoptosis by TNF-R55 and/or TNF-R75 (Fig. 6B). PC60 R55R75/S and PC60 R55R75T2DN cells were both equally sensitive to different concentrations of TNF

an inactive receptor due to the inability to bind the TRAF1/TRAF2 complex (25). This deletion mutant was also included in our structure-function studies (M5, Δ403-end).

After selection and isolation of transfected cell lines by limiting dilution, the appropriate PC60 clones were selected on the basis of similar levels of TNF-R75 expression as the original PC60 R55R75/S cells, as determined by flow fluorocytometry (Fig. 3C). Since the levels of transfected TNF-R55 in PC60 cells are extremely low (11), TNF-R55 presence could be evaluated only on the basis of functionality, viz the induction of GM-CSF by R55-specific stimulation (data not shown, see further). To demonstrate that the transfected cell lines expressed the mutant TNF-R75 gene products, the receptors were immunoprecipitated from the respective cell lines using the mAb utr-4 (50), then further analyzed by immunoblotting (Fig. 3D).
was determined in an FDCp1 assay. Supernatants of these cultures were collected, and the amount of secreted GM-CSF (D143F) TNF muteins (500 ng/ml). TNF (100 ng/ml) or TNF-R55 (R32WS86T)- or TNF-R75-specific cultured in the absence (control) or presence of saturating concentrations of TNF-R55-mediated activation of NF-κB shown) and transient overexpression in HEK293 cells could block that of TNF-R55-induced GM-CSF secretion in PC60 cells expressing TNF-R55. A similar approach for TNF-R55 was hampered by the very low expression levels of TNF-R55 in PC60 cells (<50 receptors/cell (11)). This lack of effect was not because we had for some reason been working with an inappropriate TRAF2-DN molecule, since DNA sequence analysis revealed the correct sequence (data not shown). Surprisingly, we were also unable to show any inhibitory effect on the stimulation of GM-CSF secretion or NF-κB activation (Fig. 6C and data not shown), although a strong involvement of NF-κB in GM-CSF promoter activity has been reported (51). This lack of effect was not because we had for some reason been working with an inappropriate TRAF2-DN molecule, since DNA sequence analysis revealed the correct sequence (data not shown) and transient overexpression in HEK293 cells could block TNF-R55-mediated activation of NF-κB determined by a luciferase reporter gene construct (data not shown), as reported previously (27).

To further investigate the reason for the lack of inhibition of NF-κB activation by TRAF2-DN overexpression, immunoprecipitations of TNF-R75 were conducted to examine whether TRAF2-DN indeed interacted with this receptor in PC60 R55R75 cells. In PC60 R55R75/5 cells, we could show a TNF-dependent recruitment of endogenous wt TRAF2 (Fig. 6D). This recruitment seemed to be an early event in signal transduction, since TRAF2 could already be detected in the TNF-R75 immunoprecipitates after 1 min of TNF treatment. A maximum TRAF2 recruitment was observed after 15 min of TNF treatment. In contrast, PC60 R55R75T2DN cells showed no endogenous wt TRAF2 recruitment to TNF-R75 upon TNF triggering. Instead, the overexpressed TRAF2-DN molecule was recruited to TNF-R75 in a TNF-dependent way. We could even detect a small amount of TRAF-2DN in untreated cells, probably due to an excess of overexpressed TRAF2-DN molecules, which are recruited with low efficiency to TNF-R75 dimerized by means of the immunoprecipitating utr-4 mAb. Immunoprecipitations with beads alone did not reveal TRAF2 or TRAF2-DN molecules. Taken together, these observations show that the level of TRAF2-DN expression in PC60 R55R75T2DN cells was sufficient to completely inhibit the recruitment of functional endogenous TRAF2 to the TNF-R75 signaling complex, in this way blocking TRAF2-dependent signaling pathways. Therefore, it is conceivable that PC60 R55R75 cells might use other receptor-associated molecules leading to NF-κB activation. Considering the clear association of TRAF2-DN with TNF-R75 in PC60 R55R75T2DN cells, we conclude that in PC60 cells no known TRAF2-mediated activities are implicated in receptor cooperation leading to apoptosis and activation of NF-κB.

**Discussion**

We have previously shown a role for unliganded TNF-R cooperation in PC60 cells transfected with TNF-R55 and/or TNF-R75 (11). In these cells, the presence of both receptor types was an absolute requirement for sensitivity to specific TNF-R55- or TNF-R75-mediated apoptosis. Further studies indicated that introduction of only the intracellular domain of TNF-R75 in TNF-R55-expressing cells was sufficient to render these cells susceptible to TNF-R55-induced cell death (48). To further dissect the mechanism of receptor cooperation in apoptosis and to identify the intracellular domains involved, we constructed several TNF-R75 deletion mutants and introduced them into PC60 R55/8 cells, already expressing TNF-R55. A similar approach for TNF-R55 was hampered by the very low expression levels of TNF-R55 in PC60 cells (<50 receptors/cell (11)). This is probably due to regulatory mechanisms that keep the number of TNF-R55 molecules per cell very low in PC60 cells, since elevated TNF-R55 expression levels may cause self-association through their DDs and subsequent activation of the death pathway (52). By transfecting the missing wt-TNF-R in PC60 R55 and PC60 R75 cells and thus making the cells sensitive to TNF killing, we could prove that PC60 R55/8 and PC60 R75/24 cells were not just cell lines coincidentally blocked in their apoptotic capacities. The many different PC60 R55/8 Neo and PC60 R75/24 Neo clones that were tested never showed apoptotic behavior after TNF treatment. Unliganded TNF-R55 not only cooperated with TNF-R75 in the process of apoptosis, but the levels of TNF-R75-induced GM-CSF secretion in PC60 R75NR55 cells were also strongly enhanced as compared with the consistently low, induced GM-CSF levels in PC60 R75 cell lines expressing only human TNF-R75. Nevertheless, TNF-R75-induced NF-κB activation, as measured by gel shift assay, was similar in PC60 cells carrying one or both TNF-R types (G.D., unpublished observation). The molecular basis for this cooperation is still unknown. Most cases of receptor cooperation are a result of simultaneous receptor triggering, although the observation of unliganded receptor cooperation is not restricted to PC60 R55R75 cells. Indeed, it was reported that selective loss of TNF-R75 expression can result...
FIGURE 6. Overexpression of a TRAF2-DN molecule inhibits neither receptor cooperation in apoptosis nor TNF-R55- or TNF-R75-induced GM-CSF secretion in PC60 R55R75 cells. A. PC60 R55R75/5 cells were transfected with the pRK-TRAF2-DN plasmid (24) together with a selection plasmid pUTSV-1 coding for the phleomycin (Phl) resistance gene. Cells were lysed, and 200 μg of protein was subjected to SDS-PAGE and electroblotted. Immunoblots were revealed with a rabbit polyclonal anti-mouse TRAF2 serum and ECL. B. Control cell lines (PC60 R55R75/5 and PC60 R55R75 Phl) and cells expressing the TRAF2-DN molecule (PC60 R55R75T2DN) were cultured in the presence of medium (control), of saturating concentrations of TNF (100 ng/ml), or of TNF-R55 (R52WS86T)- or TNF-R75-specific (D143F) TNF muteins (500 ng/ml). After 20 h of incubation, the percentage of PI-positive cells was determined by cytofluorometric analysis. C. Same as in B, but after 48 h of incubation, supernatants of these cultures were collected and the amount of secreted GM-CSF was determined in an FDCp1 assay. D. Parental PC60 R55R75/5 and PC60 R55R75T2DN cells (10⁶) were washed with growth medium and incubated for different time intervals at 37°C in the presence or absence of TNF (1 μg/ml), then subsequently were lysed. The soluble fraction of the extracts was immunoprecipitated with 20 μg of a nonagonistic utr-4 Ab specific for the TNF-R75 extracellular domain. Immunoblots were developed with anti-mouse TRAF2 using ECL.
in resistance to TNF-mediated cytotoxicity in KYM-1-derived cell lines (45). In this study, a role for ligand passing was excluded by making use of TNF-R-specific Abs.

Our structure/function studies with TNF-R75 deletion mutants focused on two conserved regions in the intracellular part of the receptor, namely a conserved region upstream of the TRAF complex-binding domain containing a Ser box of six consecutive serine residues, and the TRAF1/TRAF2-binding domain itself. No role for the Ser box region in receptor cooperation was found. These data are consistent with the earlier observation that this region is not involved in TNF-R75-mediated NF-κB activation (25). Disruption of the TNF-R75 TRAF complex-binding domain (amino acids 346–423) resulted in loss of receptor functionality. Complete deletion of this region (PC60 R55NR75 M1 cells, Δ313-end) or removal of the TNF-R75 C-terminal amino acids (PC60 R55NR75 M5) abrogated TNF-R75-mediated induction of GM-CSF and apoptosis, in addition to TNF-R55-induced apoptosis. Rothe et al. (25) showed that this TNF-R75 mutant could no longer signal for proliferation and NF-κB activation in C6 T cells, presumably due to the absence of TRAF1/TRAF2 binding. This TNF-R75 M5 mutation deletes part of a putative TRAF interaction site (53). Hence, our results with PC60 R55NR75 M1 and R55NR75 M5 cells strongly point to a role for the TRAF1/TRAF2 complex in apoptosis, both by induction of apoptosis by TNF-R75 itself and by receptor cooperation in TNF-R55. The function of TRAF1 is still unclear, while TRAF2, a molecule involved in both TNF-R55 and TNF-R75 signal transduction (26, 27), might be a likely candidate for the molecule involved in receptor cooperation in PC60 R55R75 cells. Furthermore, recent data suggest a link between TRAF molecules and death signaling. First, CD40, which binds directly to TRAF2, TRAF3, and TRAF5 (26, 28, 29), but presumably not to the DD-containing proteins TRADD, FADD, or RIP (29), is also capable of inducing apoptosis in transformed cells (26, 28, 29, 54). Second, signals mediated by CD30 in combination with signals transduced by the TCR induce Fas-independent cell death in T cell hybridomas (56). Deletion analysis shows that the 66 C-terminal amino acids of the CD30 cytoplasmic domain, necessary for binding TRAF1 and TRAF2, are required to induce cell death. Third, overexpression of an N-terminal truncated TRAF3 molecule abrogated LTβR-induced cell death, but not activation of NF-κB, in HT29 cells (57). Fourth, transient transfection of a TRAF2-DN mutant in HeLa cells renders these cells more susceptible to TNF-mediated apoptosis, pointing to a protective role of TRAF2 in apoptosis (58). Fifth, the TNF-R75 TRAF2 association site seems to be required for TNF-R75-dependent enhancement of TNF-R55-mediated cytotoxicity in HeLa cells (46).

To test the possible involvement of TRAF2 in receptor cooperation in PC60 R55R75 cells, we transfected this cell line stably with an expression vector coding for a murine TRAF2 (87–501) mutant, which having lost its biologic functions acts as a DN molecule. This mutant is still capable of binding the TRAF domain of R75, while it blocks NF-κB, JNK, and p38 MAPK activation (25, 26, 58). Although a clear inhibition of the TNF-dependent recruitment of functional, endogenous wt TRAF2 to the TNF-R75 signaling complex by overexpression of a TRAF2-DN mutant could be shown, PC60 R55R75T2DN cells were neither impaired in TNF-R75-mediated apoptosis nor in cooperation involved in TNF-R55-dependent apoptosis. The observation that PC60 R55R75T2DN cells were as sensitive to TNF as PC60 R55R75/5 cells indicated that TRAF2 was not involved in providing anti-apoptotic signals in these cells. The kinetics of TRAF2 recruitment to the TNF-R75 were comparable with these of TRADD recruitment to the TNF-R55 signaling complex (27). Additionally, the rapid ligand-dependent association of TRAF2 with TNF-R75 within 1 min following TNF stimulation is also seen in the case of FADD or TRAF3 targeting to the primed Fas receptor or LTβ-R, respectively (36, 57). The low level of TNF-independent TRAF2-DN recruitment to TNF-R75 in PC60 R55R75T2DN cells is probably caused by the bivalent immunoprecipitating Ab and indicates that TRAF2 also can bind to dimerized TNF-R75. This phenomenon is also observed in experiments showing ligand-dependent TRAF3 association with the LTβ-R (57). However, endogenous TRAF2 recruitment in PC60 R55R75/5 control immunoprecipitations could not be observed, even after overexposition of the ECL Western blot. Previously, TRAF2 was postulated as an essential molecule in TNF-R-mediated NF-κB activation by means of transient overexpression experiments (26, 27). Recently, reports on the basis of TRAF2-DN transgene and TRAF2−/− mice indicated the existence of TRAF2-independent pathways leading to the activation of NF-κB (59, 60). Our results confirm these observations. Hence, TNF-R complex-associated proteins that overrule the need for TRAF2 in NF-κB activation remain to be identified. RIP, a signaling molecule that interacts with TRADD and TRAF2 (35), seems to be a good candidate to fulfill this function, since a RIP-deficient Jurkat cell line is defective in TNF-induced NF-κB activation (38). This model can be true for TNF-R55-induced NF-κB activation, since TRADD can recruit RIP to this signaling complex. On the other hand, we were unable to show RIP association to the TNF-R75 by its affinity for TRAF1 and/or TRAF2 in overexpression experiments (W.D., unpublished observation). Alternatively, TNF may activate sphingomyelinases, leading to ceramide generation and subsequent NF-κB activation (61), although acidic sphingomyelinases do not seem to be involved in TNF-induced NF-κB activation (62). In addition, stable overexpression of mutant TRAF family members TRAF3 and TRAF5 block CD40-mediated induction of CD23 expression (28, 29), although experiments with TRAF3−/− cells could not confirm a role for TRAF3 in CD40 signaling (63).

The observation that both TNF-R55 and TNF-R75 share the same intracellular signaling molecules brings us back to the question of why the presence of both TNF-R types is required for induction of apoptosis in PC60 cells. A possible explanation could be the formation of heteromeric receptor complexes, which need to contain both TNF-R55 and TNF-R75 to be fully active. Since we were able to induce apoptosis in PC60 R55R75/5 cells making use of TNF-R-specific mutants or TNF-R-specific Abs (11), such a receptor complex could not be formed by simultaneous interaction of both TNF-R with the same ligand molecule. Possibly, mixed TNF-R complex formation might be due to bridging between associated molecules. However, ligand-induced conformational changes, enabling TNF-Rs to interact with each other, cannot be excluded. Alternatively, a low level of spontaneous homomeric TNF-R aggregation resulting in low level signal transduction sufficient for receptor cooperation in PC60 cells is another possible explanation. This synergizing process would be abrogated if one of the receptors was nonfunctional. We should stress that the necessity for both TNF-R types to allow TNF-R75-induced apoptosis seems not to be a general mechanism, since TNF-R75 has been shown able to induce apoptosis in a subset of T cells in TNF-R55−/− mice (22). However, a similar mechanism of receptor cooperation probably exists in HeLa cells (46).

In summary, our data indicate the existence of bidirectional TNF-R55 and TNF-R75 cooperation in PC60 cells in the case of apoptosis. We were also able to demonstrate ligand-independent TNF-R55-mediated cooperation in TNF-R75-induced GM-CSF secretion, but not the reverse. Mutational analysis of the TNF-R75 intracellular domain revealed the involvement of the TRAF1/
TRAF2-binding region in TNF-R75-mediated receptor cooperation in apoptosis. However, (over)expression of a TRAF2-DN molecule did not block receptor cooperation; nor did it result in inhibition of TNF-R55- or TNF-R75-mediated apoptosis, GM-CSF induction, or NF-κB activation, despite the fact that TNF-dependent recruitment of endogenous wt TRAF2 was prevented by the TRAF2-DN mutant.

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