Signal Transduction by DR3, a Death Domain–Containing Receptor Related to TNFR-1 and CD95

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Tumor necrosis factor receptor–1 (TNFR-1) and CD95 (also called Fas or APO-1) are cytokine receptors that engage the apoptosis pathway through a region of intracellular homology, designated the "death domain." Another death domain–containing member of the TNFR family, death receptor 3 (DR3), was identified and was shown to induce both apoptosis and activation of nuclear factor κB. Expression of DR3 appears to be restricted to tissues enriched in lymphocytes. DR3 signal transduction is mediated by a complex of intracellular signaling molecules including TRADD, TRAF2, FADD, and FLICE. Thus, DR3 likely plays a role in regulating lymphocyte homeostasis.

Apoptosis, or programmed cell death, is a physiologic process essential to the normal development and homeostasis of multicellular organisms (*1*). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, neurodegenerative disorders, and acquired immunodeficiency syndrome (*2*). CD95 and TNFR-1 (*3*) are both members of the TNFR family that also includes TNFR-2, low-affinity nerve growth factor receptor (NGFR), CD40, and CD30 (*4*). Family members are defined by the presence of cysteine-rich repeats in their extracellular domains, but CD95 and TNFR-1 also share a region of intracellular homology, designated the "death domain," which is distantly related to the *Drosophila* suicide gene *reaper* (*5*). Activation of CD95 recruits the Fas-associated death domain–containing molecule FADD (also called MORT1) (*6*), which in turn binds and presumably activates the FADD-like ICE (FLICE; also called MACH1), a member of the interleukin 1β-converting enzyme (ICE) family of proapoptotic proteases (*7*). Although the central role of CD95 is to trigger apoptosis, TNFR-1 can signal an array of diverse biological activities, many of which stem from its ability to activate nuclear factor κB (NF-κB) (*8*). Accordingly, TNFR-1 recruits the multivalent adapter molecule TRADD (TNFR-1–associated death domain protein), which like FADD also contains a death domain (*9*, *10*). Through its associations with a number of signaling molecules, including FADD, TRAF2 (TNFR-associated factor–2), and RIP (receptor interacting protein), TRADD can signal both apoptosis and NF-κB activation (*10*, *11*).

To identify additional receptors, we searched an expressed sequence tag (EST) database (*7*, *12*) for clones with homology to both the extracellular cysteine-rich domain and the intracellular death domain of TNFR-1 and CD95. A compilation of two clones fulfilled the search criteria: HTTBN61, from a human testes tumor library, had homology to the cysteine-rich domain, and HSAVO45, from a human anergic T cell library, had homology to the death domain. Clone HSAVO45 contained sequence identical to the 3′ end of HTTBN61, which was not full-length. To obtain a full-length coding sequence, we screened a human umbilical vein endothelial cell (HUVEC) library with a cDNA insert obtained from clone HTTBN61. Two independent clones were obtained containing an open reading frame encoding a protein of 417 amino acids that exhibited a predicted domain structure for a cell surface receptor (Fig. 1). Residues +1 to +201 constitute a cysteine-rich extracellular domain, with 28 cysteine residues and two potential sites for N-glycosylation (Asn-X-Ser/Thr). The middle of the molecule contains a stretch of 23 uncharged amino acids extending from Trp^{202} to Tyr^{224}, then a basic amino acid (Arg^{225}) characteristic of a transmembrane-spanning domain. The putative cytoplasmic domain comprises the remaining 192 amino acids.

Alignment of the predicted amino acid sequence of the clone with TNFR-1 and CD95 (Fig. 1) showed that the three molecules share significant homology in both the extracellular and intracellular domains. Like all members of the TNFR superfamily, the isolated clone contained characteristic cysteine repeats in its extracellular ligand-binding domain. The identity of the cysteine-rich subdomains of the clone with those of TNFR-1 and CD95 was 26% and 22%, respectively, consistent with the 20 to 30% identity reported for the known TNFR

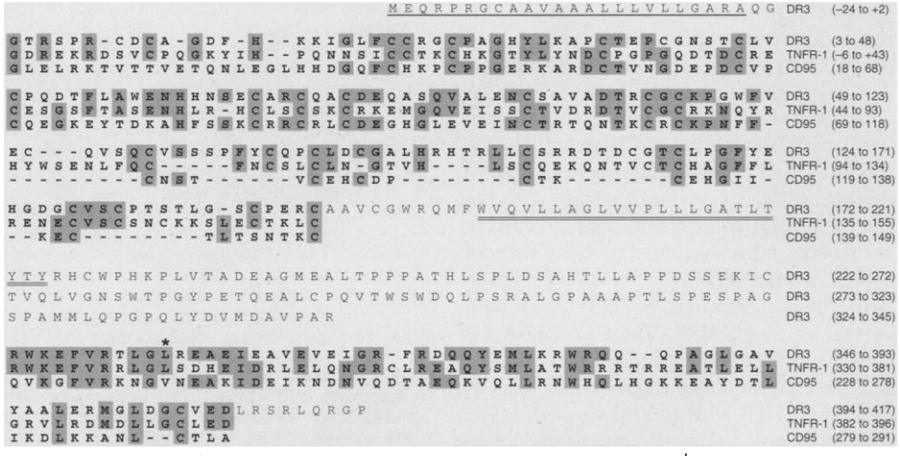

Fig. 1. Sequence analysis of DR3 (nucleotide sequence is available through GenBank accession number U72763) and sequence homology of the DR3 extracellular domain (residues 3 to 191) and intracellular death domain (residues 346 to 408) with the cell death receptors CD95 and TNFR-1. In the deduced amino acid sequence of the DR3 protein product, the putative signal peptide and transmembrane domain are single- and double-underlined, respectively. Alignments were done with Megalign (DNASTAR) software. Shading denotes consensus identical residues. The asterisk indicates a critical amino acid conserved in many death domains, mutation of which leads to inactivation of the receptor and the lpr phenotype in the case of CD95 (*21*). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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family members. The clone contained significant homology to the death domains of TNFR-1 (47% identity) and CD95 (23% identity). Thus, the clone was designated death receptor 3 (DR3).

To determine the distribution of the DR3 transcript, we subjected various tissues to Northern (RNA) blot analysis. Expression of DR3 was detected in tissues enriched in lymphocytes, including peripheral blood leukocytes (PBLs), thymus, spleen, colon, and small intestine (Fig. 2). DR3 was not detected in any of the other tissues (Fig. 2). In contrast, TNFR-1 is ubiquitously expressed and CD95 is expressed in lymphocytes, liver, heart, lung, kidney, and ovary (*13*).

In vitro and in vivo binding studies were undertaken to investigate DR3 signaling pathways. As an initial screen, death domain–containing adapter molecules such as FADD, TRADD, and RIP were in vitro translated and precipitated with various glutathione-S-transferase (GST) fusion proteins immobilized on glutathione-Sepharose beads (Fig. 3A). As predicted (*6*, *9*), FADD associated with the GST-Fas cytoplasmic domain, whereas TRADD associated with the GST–TNFR-1 cytoplasmic domain (Fig. 3A). GST–TNFR-1 also weakly interacted with RIP. GST-DR3 associated specifically with TRADD but not with FADD or RIP (Fig. 3A), and a truncated death domain mutant of DR3 (GST-ΔDR3) did not interact with TRADD. To demonstrate the association of DR3 and TRADD in vivo, we transiently transfected 293 human embryonic kidney cells with plasmids that direct the synthesis of Myc epitope–tagged TRADD (myc-TRADD) and Flag epitope–tagged DR3 (Flag-DR3), Flag–TNFR-1, or mutants (Fig. 3B). In a manner consistent with the in vitro binding results, TRADD specifically coprecipitated with DR3 and TNFR-1, but not with the death domain mutants, ΔDR3 and ΔTNFR-1 (Fig. 3B). Thus, DR3, like TNFR-1, may activate downstream signaling cascades by recruiting the adapter molecule TRADD. This result was expected because the death domains of DR3 and TNFR-1 have high homology (47% identity), whereas other death domain–containing molecules share 20 to 30% identity (*3*).

Overexpression of TRADD induces apoptosis and NF-κB activation, two of the most important activities signaled by TNFR-1 (*9*). Upon oligomerization of TNFR-1 by trimeric TNF, TRADD is recruited to the receptor signaling complex (*10*). TRADD can then bind TRAF2 (*14*), RIP (*15*), and FADD (*6*). Thus, we determined whether RIP, TRAF2, and FADD could be coimmunoprecipitated with DR3. In 293 cells expressing DR3 and RIP, only a weak association could be detected between the two molecules (Fig. 3C). However, in the presence of TRADD, RIP association with DR3 was enhanced (Fig. 3C). Likewise, little TRAF2 directly coprecipitated with DR3 in 293 cells. However, when DR3 and TRAF2 were expressed in the presence of both TRADD and RIP (both of which can bind TRAF2), enhanced binding of TRAF2 to DR3 could be detected (Fig. 3D). A similar association between FADD and DR3 was also observed. In the presence of TRADD, FADD efficiently coprecipitated with DR3 (Fig. 3E).

FADD can recruit the ICE-like protease FLICE to the CD95 death-inducing signaling complex (*7*). To determine whether FLICE can associate with TNFR-1 and DR3, we carried out coprecipitation experiments in 293 cells. FLICE formed complexes with TNFR-1 and DR3 (Fig. 3F). Cotransfection of TRADD, FADD, or both failed to enhance the FLICE–TNFR-1 or FLICE-DR3 interaction (*16*), which suggested that endogenous amounts of these adapter molecules were sufficient to maintain association.

Overexpression of CD95 and TNFR-1 in mammalian cells mimics receptor activation (*7*); thus, we used this approach to study the functional role of DR3. Ectopic expression of DR3 in MCF7 breast carcinoma cells and 293 cells induced rapid apoptosis (Fig. 4, A to C). The cells displayed morphological alterations typical of cells undergoing apoptosis, becoming rounded, condensed, and detaching from the dish (Fig. 4A). In MCF7 cells, plasmids encoding full-length DR3 or ΔDR3

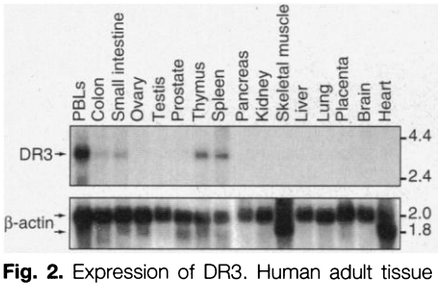

Fig. 2. Expression of DR3. Human adult tissue Northern blots (Clontech) containing 2 μg of polyadenylated RNA were probed with DR3 cDNA. PBLs, peripheral blood leukocytes. The blot was subsequently probed with β-actin cDNA. Heart and skeletal muscle contain two forms of β-actin. Numbers at right denote kilobases.

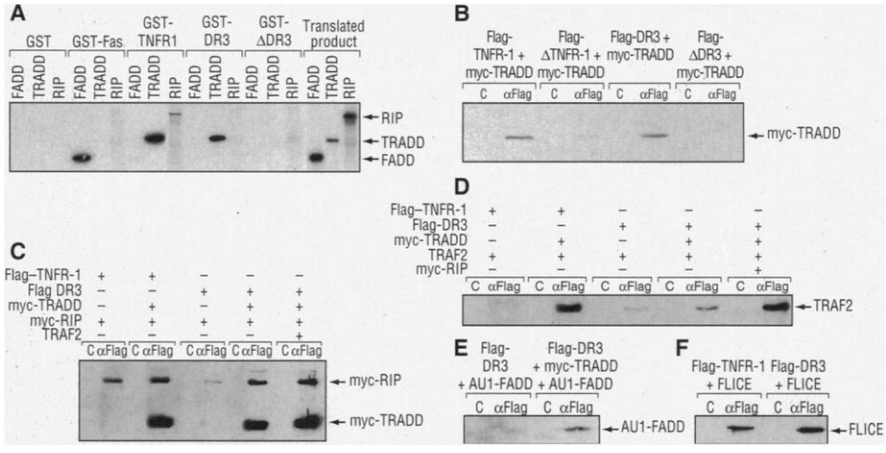

Fig. 3. Intracellular signaling molecules used by DR3. (**A**) In vitro screen for death domain–containing adapter molecules that bind to the cytoplasmic domain of DR3 (*22*). In vitro translated ^{35}S-labeled RIP, TRADD, and FADD were incubated with glutathione beads containing GST alone or GST fusions of the cytoplasmic domain of Fas, TNFR-1, DR3(215–393), or ΔDR3(215–321). After the beads were washed, retained proteins were analyzed by SDS-PAGE and autoradiography. The gel was Coomassie-stained to monitor equivalency of loading. (**B**) TRADD associates with DR3 in vivo. 293 cells were transfected with the indicated expression constructs for Flag-DR3, Flag-ΔDR3(1–321), Flag–TNFR-1, Flag–ΔTNFR-1, or myc-TRADD (*23*). After 24 to 32 hours, extracts were prepared and immunoprecipitated with a control mAb (C) or Flag mAb (αFlag) (IBI Kodak). Protein immunoblot analysis indicated that the amount of myc-TRADD and death receptor expression was similar in all samples. Coprecipitating myc-TRADD was detected by immunoblotting with horseradish peroxidase (HRP)–conjugated anti-Myc (Boehringer Mannheim). (**C**) TRADD enhances association of RIP with DR3. 293 cells were transfected and immunoprecipitated as in (B). Coprecipitating myc-RIP and myc-TRADD were detected by immunoblotting with HRP–conjugated anti-Myc. (**D**) TRADD enhances association of TRAF2 with DR3. 293 cells were transfected and immunoprecipitated as in (B). Coprecipitating TRAF2 was detected by immunoblotting with anti-TRAF2. (**E**) Coimmunoprecipitation of DR3-TRADD-FADD complexes. 293 cells were transfected and immunoprecipitated as in (B). Coprecipitating AU1 epitope–tagged FADD was detected by immunoblotting with anti-FADD. (**F**) FLICE coimmunoprecipitates with DR3 and TNFR-1. As in (B), except immunoblotted with anti–p10 subunit of FLICE.

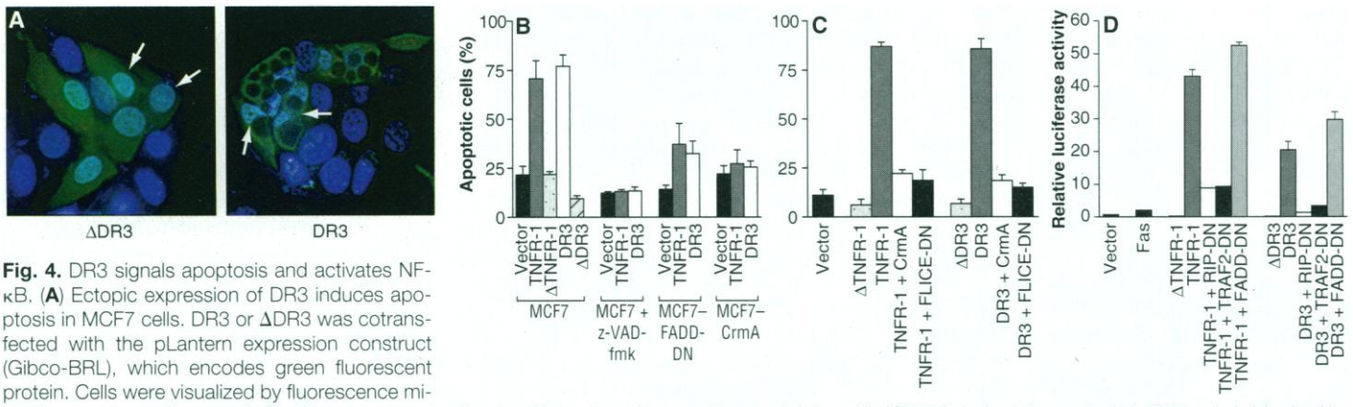

Fig. 4. DR3 signals apoptosis and activates NF-κB. (**A**) Ectopic expression of DR3 induces apoptosis in MCF7 cells. DR3 or ΔDR3 was cotransfected with the pLantern expression construct (Gibco-BRL), which encodes green fluorescent protein. Cells were visualized by fluorescence microscopy using a fluorescein isothiocyanate range barrier filter cube. Nuclei of transfected cells were visualized by DAPI staining and the image overlaid. Arrows indicate representative nuclei. (**B**) Apoptosis in MCF7 cells is inhibited by z-VAD-fmk (10 μM), FADD-DN (dominant negative), and CrmA. MCF7 cells or cells stably expressing CrmA (*17*) or FADD-DN (*18*) were transiently transfected with pCMV–β-galactosidase in the presence of a 10-fold excess of vector alone or expression constructs encoding DR3, ΔDR3, TNFR-1, or ΔTNFR-1 (*24*). The data (mean ± SD) are the percentage of round blue cells as a function of the total number of blue cells counted (n = 3). (**C**) DR3-induced apoptosis in 293 cells is blocked by FLICE-DN/MACHα1(C360S) (*7*) and CrmA. The data (mean ± SD) are the percentage of round blue cells as a function of the total number of blue cells counted (n = 3). (**D**) DR3 induces NF-κB activation, which is inhibitable by RIP-DN (*15*) and TRAF2-DN (*10, 14*). 293 cells were cotransfected with the indicated molecules and an NF-κB luciferase reporter plasmid (*14*), and luciferase activities were then determined (*25*). Results are from a representative experiment performed in duplicate three independent times (mean ± SD).

were cotransfected with the pLantern reporter construct encoding green fluorescent protein (Fig. 4A). Nuclei of cells transfected with DR3, but not with ΔDR3, exhibited apoptotic morphology as assessed by 4′,6′-diamidino-2-phenylindole (DAPI) staining (Fig. 4A). DR3-induced apoptosis was blocked by two inhibitors of ICE-like proteases, CrmA and z-VAD-fmk (Fig. 4, A to C), that also block apoptosis induced by TNFR-1 and CD95 (*7, 17*). Apoptosis induced by DR3 was also blocked by dominant negative versions of FADD (FADD-DN) or FLICE (FLICE-DN), which inhibit death signaling by CD95 and TNFR-1 (*7, 10, 18*). Thus, FADD and the ICE-like protease FLICE are likely necessary components of DR3-induced apoptosis.

Because DR3 activation recruits three molecules implicated in TNF-induced NF-κB activation, we examined whether DR3 could activate NF-κB. Transfection of a control vector or expression of CD95 did not induce NF-κB activation. In contrast, NF-κB was activated by ectopic expression of DR3 or TNFR-1, but not by the inactive signaling mutants ΔDR3 or ΔTNFR-1 (Fig. 4B). DR3-induced NF-κB activation was blocked by dominant negative derivatives of RIP (RIP-DN) and TRAF2 (TRAF2-DN), which block TNF-induced NF-κB activation (*10, 11*). As expected, FADD-DN did not interfere with DR3-mediated NF-κB activation (*10, 18*).

The identification of DR3 has added a third cell death receptor to the TNFR family. Its expression pattern suggests that DR3 may participate in lymphocyte homeostasis. None of the currently known ligands for these receptors [FasL, TNF, and TRAIL (TNF-related apoptosis-inducing ligand) (*19*)] bind to DR3 (*16*). In addition, it is unlikely that TRAIL serves as the ligand for DR3; TRAIL-induced apoptosis is not blocked by FADD-DN (*20*), which suggests that it uses an alternate death signaling pathway. It is also unclear whether TRAIL activates NF-κB. The identification of the DR3 ligand and targeted disruption of the DR3 gene will allow for a greater understanding of the physiologic role of DR3.

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22. In vitro binding experiments were done as described in (*6*).
23. The constructs encoding Flag–TNFR-1 and Flag–ΔTNFR-1 were described elsewhere (*18*). To facilitate epitope tagging, we cloned DR3 and ΔDR3(1–321) into the FLAG plasmid pCMV1FLAG (IBI Kodak) with the use of the signal peptide provided by the vector. Transfection of 293 cells was done by calcium phosphate precipitation with the constructs encoding the indicated proteins in combination with pcDNA3-CrmA (*17*) to prevent cell death and thus maintain protein expression. Cells were lysed in 1 ml of lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and a protease inhibitor cocktail). Lysates were immunoprecipitated with a control monoclonal antibody (mAb) or Flag mAb for at least 4 hours at 4°C, as described (*18*). The beads were washed three times with lysis buffer, but in the case of TRADD binding, the NaCl concentration was adjusted to 1 M. The precipitates were fractionated by 12.5% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Subsequent protein immunoblotting was performed as described (*10, 18*).
24. Cell death assays were done as described (*6, 18*). The data (mean ± SD) are the percentage of round blue cells among the total number of blue cells counted. Data were obtained from at least three independent experiments.
25. NF-κB luciferase assays were performed as described (*11, 12, 14, 18*). The amounts of dominant negative inhibitors used were four times the amounts of the death receptors. Total DNA was kept constant.

26. Supported by the Medical Scientist Training Program (A.M.C.) and NIH grant GM-07863 (A.M.C.). The initial EST clones used in this study were discovered as part of a joint collaboration between scientists at The Institute for Genomic Research and at Human Genome Sciences. We thank D. Goeddel for the TRAF2 and RIP constructs, I. Jones for manuscript preparation, and H. Duan and C. Vincenz for helpful discussions.

9 September 1996; accepted 7 October 1996