An Antagonist Decoy Receptor and a Death Domain–Containing Receptor for TRAIL

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TRAIL, also called Apo2L, is a cytotoxic protein that induces apoptosis of many transformed cell lines but not of normal tissues, even though its death domain–containing receptor, DR4, is expressed on both cell types. An antagonist decoy receptor (designated as TRID for TRAIL receptor without an intracellular domain) that may explain the resistant phenotype of normal tissues was identified. TRID is a distinct gene product with an extracellular TRAIL-binding domain and a transmembrane domain but no intracellular signaling domain. TRID transcripts were detected in many normal human tissues but not in most cancer cell lines examined. Ectopic expression of TRID protected mammalian cells from TRAIL-induced apoptosis, which is consistent with a protective role. Another death domain–containing receptor for TRAIL (designated as death receptor–5), which preferentially engaged a FLICE (caspase-8)–related death protease, was also identified.

Apoptosis, or programmed cell death, is a regulated process that is central to metazoan development and tissue homeostasis (*1*). One mechanism of immune-mediated killing is the engagement of death receptors (*2*). Two ligand–death receptor pairs, FasL-Fas (CD95L-CD95) and tumor necrosis factor–α (TNF-α)–TNFR1, are well-characterized inducers of the cell suicide program. These receptors, including DR3 (Wsl, Apo3, TRAMP, or LARD) and DR4, all contain a stretch of 60 to 80 amino acids within their cytoplasmic domains, which are termed the death domain (*3*, *4*). When activated, the receptor death domains bind directly to the adaptor molecule FADD (Fas-associated death domain–containing molecule, also called MORT1) or indirectly through TRADD (TNFR1-associated death domain protein), an intervening bridging molecule (*3*, *5*). FADD functions as a central conduit for the flow of death signals: dominant negative versions that retain the death domain but lack the NH_2-terminal segment effectively block TNFR1-, CD95-, and DR3-induced cell death (*3*, *6*). Because the NH_2-terminal segment of FADD functions to engage downstream components of the death pathway, it has been termed the death effector domain (DED), which is also present in the prodomain of the death protease FLICE (FADD-like interleukin-1β converting enzyme, also called MACH or caspase-8) (*7*). The DED of FADD binds to the corresponding sequence within the FLICE prodomain and recruits this death protease to the receptor signaling complex. Like FasL-Fas, the TRAIL-DR4 ligand-receptor complex engages the caspase cascade but does so in a FADD-independent manner.

In contrast to FasL, which is predominantly expressed on activated T lymphocytes, on natural killer cells, and at sites of "immune privilege" (*2*), TRAIL and its receptor DR4 are expressed in many human tissues, including the spleen, lung, and prostate (*4*, *8*). However, normal tissues are resistant to TRAIL-DR4, whereas most transformed cells are sensitive to it (*8*). A potential mechanism for the resistance of normal tissues to DR4-TRAIL could be the existence of a naturally occurring antagonistic receptor that binds and sequesters TRAIL but is incapable of transducing an intracellular signal.

To identify such a receptor, we searched an expressed sequence tag (EST) database using the extracellular, cysteine-rich, ligand-binding domain of DR4 (Fig. 1C). A number of human EST clones were initially identified. Analysis of these clones revealed two related but distinct cDNAs. One encoded a protein of 411 amino acids that had features of a cell surface receptor. Database searches, protein sequence alignment, and comparative analyses indicated that this molecule (DR5) was a member of the TNF receptor family and contained a

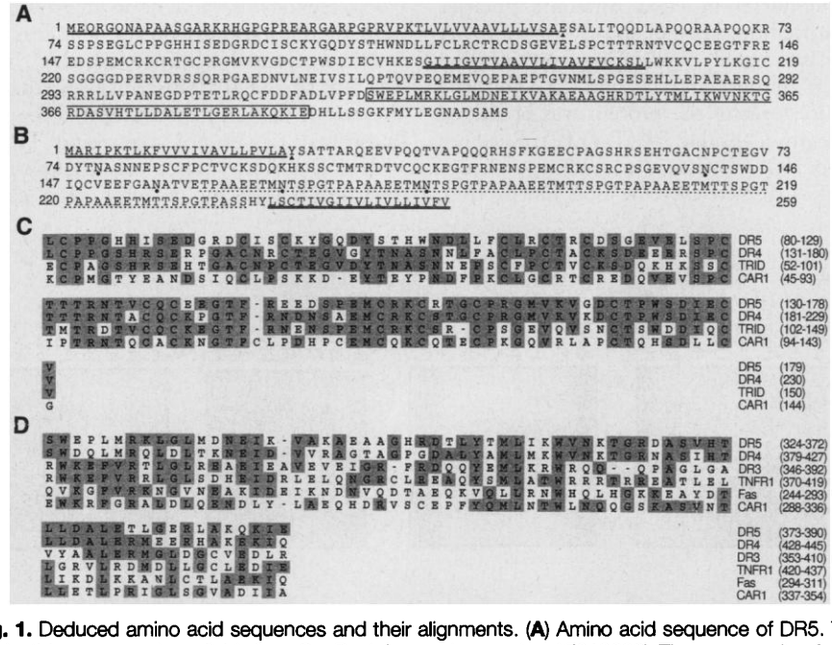

Fig. 1. Deduced amino acid sequences and their alignments. (**A**) Amino acid sequence of DR5. The nucleotide sequence is available from GenBank (accession number AF012628). The open reading frame for DR5 defines a type I transmembrane protein of 411 amino acids. The mature protein is predicted to start at amino acid 52 (Glu, black triangle). The putative signal peptide and transmembrane domain are singly and doubly underlined, respectively. The intracellular death domain is boxed. Abbreviations for the amino acids 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. (**B**) Amino acid sequence of TRID. The nucleotide sequence is available from GenBank (accession number AF012629). The open reading frame for TRID defines a cell surface protein of 259 amino acids. The mature protein is predicted to start at amino acid 24 (Tyr, black triangle). The putative signal peptide and transmembrane sequence are singly and doubly underlined, respectively. Five potential N-glycosylation sites are indicated by black dots. The dotted line indicates the five tandem sequence repeats. (**C**) Sequence alignments of cysteine-rich domains. (**D**) Sequence alignments of death domains. Alignments were done with Megalign (DNASTAR) software. Shading represents identical amino acids.

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cytoplasmic death domain (Fig. 1D). A putative signal peptide was present (amino acids 1 to 51), with the mature receptor predicted to start at amino acid 52 (Glu) (9). Like DR4, residues 84 to 179 consisted of a cysteine-rich domain with two cysteine-rich motifs. A transmembrane domain (amino acids 184 to 206) was followed by an intracellular region containing a stretch of 70 COOH-terminal amino acids with significant similarity to the death domains of known TNF receptor family members, including TNFR1, Fas, DR3, CAR1, and DR4 (Fig. 1D). DR5 was 66 and 64% identical to DR4 within the cysteine-rich and death domains, respectively. These sequence identities were higher than those observed among other related TNF receptor family members (20 to 30% and 25% for the cysteine-rich and death domains, respectively) (*10*). Given the presence of a death domain and its ability to trigger apoptosis (see below), this receptor was designated as death receptor–5, or DR5.

The other DR4-related cDNA encoded a protein of 259 amino acids with homology to the extracellular domains of TNF receptor family members. Sequencing of six clones from eight different libraries identified the same open reading frame. A putative signal peptide was present between amino acids 1 and 23, and the mature form of the protein was predicted to begin at residue 24 (Tyr) (9). Residues 52 to 150 constituted a cysteine-rich domain with two cysteine-rich motifs (Fig. 1C). Following the cysteine-rich domain were five tandem repeats of 13 amino acids. Similar repeat sequences are also found in other proteins, including Son of sevenless protein and endothelial leukocyte adhesion molecule 1 (*11*). However, there was no intracellular domain following the putative hydrophobic transmembrane domain in keeping with the possibility that this receptor did not signal after ligand binding. Sequence alignment and comparison revealed its extracellular cysteine-rich domain to be similar to the corresponding domains of both DR4 and DR5 with 69 and 52% amino acid identity, respectively. Because of the absence of an intracellular domain, this receptor was termed TRID (for TRAIL receptor without an intracellular domain). In addition, like DR4 and DR5, TRID had substantial homology to the cysteine-rich domain in CAR1, a chicken TNF receptor family member, with amino acid identities ranging from 42 to 48% (*12*) (Fig. 1D). A potential protective role for TRID was suggested by the finding that its transcripts were detectable in some normal human tissues, including heart, placenta, lung, liver, kidney, spleen, peripheral blood leukocytes, and bone marrow but were at substantially lower amounts in most transformed cell lines (Fig. 2A). The different size TRID transcripts were due to alterations in the 3′–untranslated region. In normal tissues not expressing TRID, such as the brain and colon, it is possible that another decoy receptor or a completely different mechanism is responsible for the resistance to TRAIL. In contrast, the transcript for DR5 was detected in both normal human tissues and cancer cell lines (Fig. 2B).

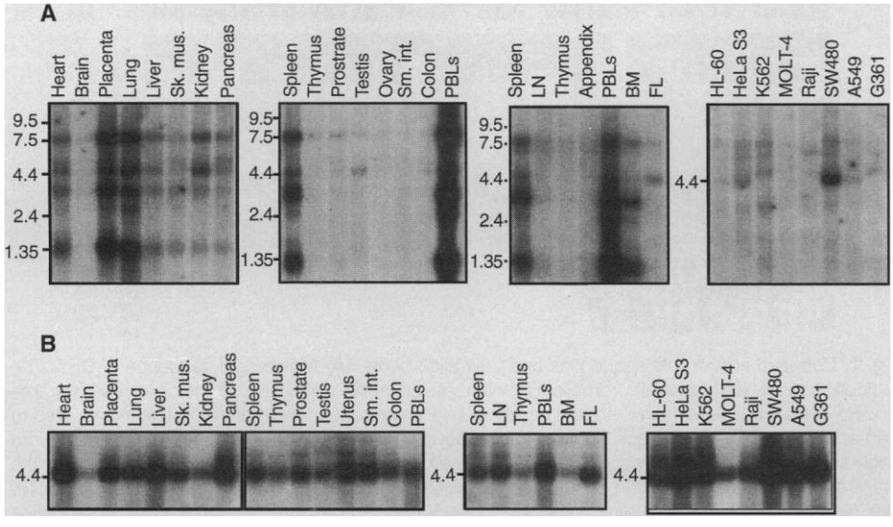

Fig. 2. Tissue distribution of TRID (**A**) and DR5 (**B**) transcripts. Human adult tissue, immune tissue, and cancer cell line Northern (RNA) blots (Clontech) were probed with an internal fragment of either DR5 or TRID cDNA (*15*). The blots were rehybridized with β-actin cDNA. Sk. mus., skeletal muscle; Sm. int., small intestine; LN, lymph node; BM, bone marrow; FL, fetal liver; PBLs, peripheral blood leukocytes; HL-60, promyelocytic leukemia; HeLa S3, HeLa cell line; K562, chronic myelogenous leukemia; MOLT-4, lymphoblastic leukemia; Raji, Burkitt's lymphoma; SW480, colorectal adenocarcinoma; A549, lung carcinoma; G361, melanoma.

Transfected mammalian cells overexpressing any of the known death receptors, including TNFR1, Fas, DR3, and DR4, undergo apoptosis in a ligand-independent manner (*3*, *4*). Overexpression of DR5 also induced apoptosis in both MCF7 human breast carcinoma cells and human epitheloid carcinoma (HeLa) cells (Fig. 3A). Most of the transfected cells underwent morphological changes that are characteristic of apoptosis (*13*). As expected, the deletion of the death domain abolished killing ability. Like that of DR4, DR5-induced apoptosis was blocked by the caspase inhibitors CrmA and z-VAD-fmk, but dominant negative FADD was without effect (Fig. 3A), and DR5 did not interact with FADD or TRADD in vivo (Fig. 3B). Under conditions at which dominant negative FLICE effectively blocked TNFR1-induced apoptosis, it only partially inhibited DR4- or DR5-induced cell death. Instead, the dominant negative form of a related FLICE (designated as FLICE2 or caspase-10b) (*14*) blocked DR4- and DR5-induced apoptosis. Consistent with a more prominent role, FLICE2 was preferentially recruited to the DR4 and DR5 receptor signalling complexes (Fig. 3, C and D). Taken together, these data suggest that, like DR4, DR5 engages an apoptotic program that involves FLICE2 or a related molecule but that is independent of the adaptor molecule FADD. Because FLICE2 does not directly bind DR4 or DR5, a yet to be identified adaptor molecule is presumably responsible for this linkage.

Given the similarity of the extracellular, ligand-binding, cysteine-rich domains of DR5 and TRID to that of DR4 (Fig. 1C), it was anticipated that they would also bind TRAIL. To confirm this, we expressed the soluble extracellular ligand-binding domains of DR5 and TRID as fusions to the Fc portion of human immunoglobin G (IgG). DR5-Fc (like DR4-Fc) specifically bound TRAIL but not the related cytotoxic ligand TNF-α (Fig. 4A). Additionally, DR5-Fc blocked the ability of TRAIL to induce apoptosis but had no effect on TNF-α–induced cell death under conditions at which TNFR1-Fc completely abolished TNF-α killing (Fig. 4B). Similarly, TRID-Fc selectively bound TRAIL and blocked TRAIL-induced apoptosis. Given the absence of an intracellular signalling domain, it was likely that native TRID could itself similarly attenuate TRAIL-induced cell death. Overexpression of native TRID in TRAIL-sensitive

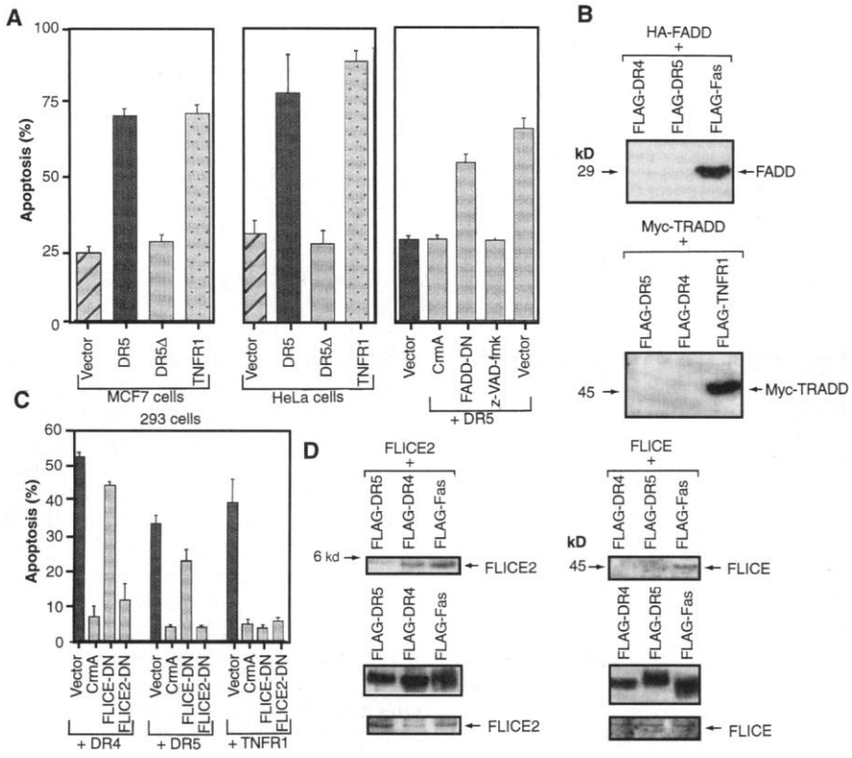

Fig. 3. DR5 induces apoptosis in mammalian cells. (**A**) Overexpression of DR5-induced apoptosis in both MCF7 (left) and HeLa (middle) cells. MCF7 and HeLa cells were cotransfected with a vector, DR5, DR5Δ (*16*), or TNFR1 together with a β-galactosidase (β-Gal) reporter construct. Twenty to 24 hours after transfection, cells were stained with 5-bromo-4-chloro-3-indoxyl-β-galactopyranoside and examined microscopically (*5, 17*). The data (mean ± SD) represent the percentage of round apoptotic cells as a function of total β-Gal–positive cells ($n = 3$). (Right) MCF7 cells were transfected with the DR5 expression construct in the presence of z-VAD-fmk (20 μM) or cotransfected with a threefold excess of CrmA, a dominant negative FADD (FADD-DN) expression construct, or a vector alone. (**B**) 293T cells were transfected (*4, 17*) with the indicated expression constructs and the presence of HA-tagged FADD (HA-FADD) or Myc-tagged TRADD (Myc-TRADD) detected by immunoblotting with polyclonal antibody to FADD or horseradish peroxidase (HRP)–conjugated antibody to Myc (Boehringer Mannheim) (*18*). (**C**) Dominant negative FLICE2 (FLICE2-DN) blocks both DR5- and DR4-induced apoptosis. 293 cells were cotransfected with DR5, DR4, or TNFR1 expression construct and a fourfold excess of CrmA, FLICE-DN, FLICE2-DN, or a vector alone in the presence of a β-Gal reporter construct as indicated. Cells were stained and examined 25 to 30 hours later. The data are represented as in (A). (**D**) FLICE2 is recruited to the DR5 and DR4 complex. In vivo interaction assays were as in (B). The lower panels confirm the expression of the cotransfected components as indicated. FLICE2 and FLICE were detected by immunoblotting with respective antibodies.

cells (MCF7) protected them from TRAIL-induced apoptosis as well as cells expressing the virally encoded caspase inhibitor CrmA (Fig. 4C). Overexpression of TRID by itself did not induce apoptosis.

Our findings are consistent with a potential guardian role for TRID that allows normal tissues to withstand the potentially deleterious effects of constitutively expressed TRAIL. Because not all normal tissues express TRID, there may be other decoy receptors that are as yet undetected. The identification of DR4, DR5, and an antagonist decoy receptor, TRID, adds further complexity to the biology of TRAIL-initiated signal transduction. Targeted gene disruption experiments will be required to reveal contributions made by individual receptors.

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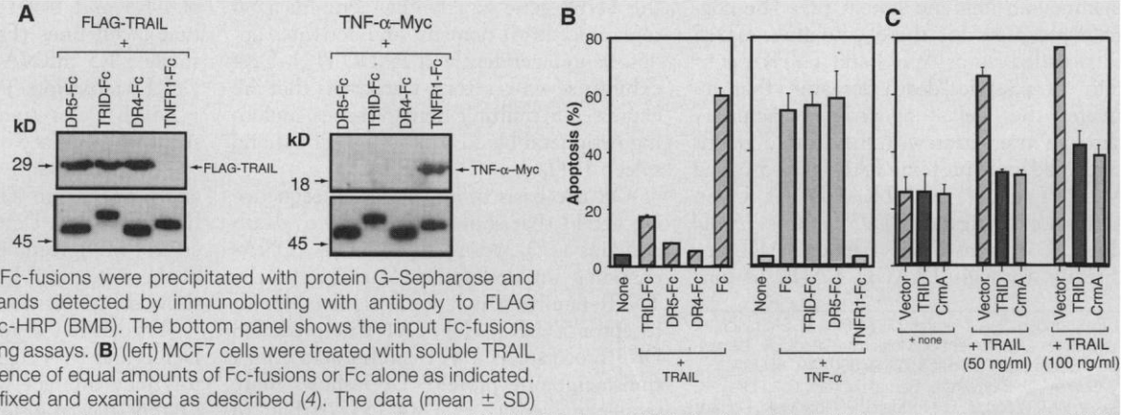

Fig. 4. The extracellular domains of DR5 and TRID bind TRAIL and block TRAIL-induced apoptosis. (**A**) The Fc–extracellular domains of TRID, DR5, DR4, or TNFR1 and the corresponding ligands were prepared, and binding assays were performed as described elsewhere (*4*). The respective Fc-fusions were precipitated with protein G–Sepharose and coprecipitated soluble ligands detected by immunoblotting with antibody to FLAG (Babco) or antibody to Myc-HRP (BMB). The bottom panel shows the input Fc-fusions that are present in the binding assays. (**B**) (left) MCF7 cells were treated with soluble TRAIL (200 ng/ml) (*19*) in the presence of equal amounts of Fc-fusions or Fc alone as indicated. Six hours later, cells were fixed and examined as described (*4*). The data (mean ± SD) shown are the percentage of apoptotic nuclei among total nuclei counted ($n = 4$). (Right) DR5-Fc and TRID-Fc did not block TNF-α–induced apoptosis. MCF7 cells were treated with TNF-α (40 ng/ml, Genentech) in the presence of equal amounts of Fc-fusions or Fc alone as indicated. Nuclei were stained and examined 11 to 15 hours later. (**C**) TRID protects cells from TRAIL-induced apoptosis. MCF7 cells were transfected with TRID, CrmA expression construct, or a vector alone together with a β-Gal reporter construct. Twenty-four hours after transfection, TRAIL was added as indicated. Six hours later, cells were stained and examined as described in Fig. 3.

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15. Northern (RNA) blot analysis was performed with multiple tissue blots (Clontech, Palo Alto, CA) according to the manufacturer's instructions.
16. DR5, DR5Δ (amino acids 52 to 411), and TRID were cloned into pCMV1FLAG vector (IBI, Kodak). cDNAs encoding the extracellular domains of DR5 (amino acids 52 to 180) and TRID (amino acids 24 to 163) were obtained by polymerase chain reaction and cloned into a modified pCMV1FLAG vector that allowed for in-frame fusion with the Fc portion of human IgG. The constructs expressing DR4, soluble TRAIL, and TNF-α have been described (*4*).
17. Cell death assays were done as described (*5*). MCF7 and HeLa cells were transfected with the lipofectamine procedure (BRL, Grand Island, NY), according to the manufacturer's instructions. 293 cells and 293T cells were transfected with calcium phosphate precipitation.
18. In vivo interaction assays have been described elsewhere (*3*).
19. The preparation of receptor-Fc fusions and ligands and in vitro binding assays has been described (*4*).
20. We thank A. M. Chinnaiyan, D. Chaudhary, H. Duan, S. Hu, E. Humke, K. Luetke, J. McCarthy, M. Muzio, H.-N. Nguyen, K. O'Rourke, Q. Song, and C. Vincenz for useful discussions and reagents; Y. Kuang for technical help; I. Jones for preparing figures; and J. DeJohn for secretarial assistance. Supported by NIH grants ES08111 and DAMD17-96-1-6085.

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Control of TRAIL-Induced Apoptosis by a Family of Signaling and Decoy Receptors

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TRAIL (also called Apo2L) belongs to the tumor necrosis factor family, activates rapid apoptosis in tumor cells, and binds to the death-signaling receptor DR4. Two additional TRAIL receptors were identified. The receptor designated death receptor 5 (DR5) contained a cytoplasmic death domain and induced apoptosis much like DR4. The receptor designated decoy receptor 1 (DcR1) displayed properties of a glycophospholipid-anchored cell surface protein. DcR1 acted as a decoy receptor that inhibited TRAIL signaling. Thus, a cell surface mechanism exists for the regulation of cellular responsiveness to pro-apoptotic stimuli.

Apoptosis (programmed cell death) is crucial for the development and homeostasis of metazoans (*1*). The cell death program has three essential types of elements: activators, inhibitors, and effectors; in *Caenorhabditis elegans*, these components are encoded, respectively, by the *ced-4*, *ced-9*, and *ced-3* genes. The CD95 ligand (CD95L) and tumor necrosis factor (TNF) are important extracellular activators of apoptosis in the mammalian immune system (*2*). The cognate receptors for these cytokines, CD95 (also called Fas or Apo1) and TNFR1, contain cytoplasmic "death domains" that activate the cell's apoptotic machinery through interaction with the death domains of the adapter proteins FADD (also called MORT1) (*3*, *4*) and TRADD (*5*). Upon activation by ligand, CD95 recruits FADD directly, whereas TNFR1 binds FADD indirectly, through TRADD. FADD in turn activates the *ced-3*–related protease MACHα/FLICE (caspase 8), thereby initiating a series of caspase-dependent events that lead to cell death (*6*, *7*).

The cytokine TRAIL, also called Apo2L (*8*, *9*), is structurally related to CD95L and TNF; TRAIL activates rapid apoptosis in tumor cell lines, acting independently of CD95, TNFR1, or FADD (*9*, *10*). A receptor for TRAIL, designated DR4, belongs to the TNFR gene superfamily, contains a cytoplasmic death domain, and activates apoptosis independently of FADD (*11*). DR4 exhibits several mRNA transcripts that are expressed in multiple human tissues, including peripheral blood leukocytes (PBLs) and spleen (*11*).

On the basis of an expressed sequence tag (EST) that showed homology to death domains (*12*), we isolated human cDNAs encoding an undescribed member of the TNFR family, which we designated death receptor 5 (DR5) (Fig. 1A). The predicted DR5 precursor is a 411–amino acid type I transmembrane protein. DR5 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, namely, DR3 (also called Apo3, WSL-1, or TRAMP) (*13*–*16*) (29%), TNFR1 (19%), or CD95 (17%). DR5 and DR4 each contain two extracellular cysteine-rich domains (CRDs) (Fig. 1A), whereas other mammalian TNFR family members have three or more CRDs (*17*). DR5 contains a cytoplasmic death domain that shows substantially more identity to the death domain of DR4 (64%) than to the death domain of DR3 (29%), TNFR1 (30%), or CD95 (19%).

Using a signal sequence trap approach and extracellular domain (ECD) homology (*18*, *19*), we isolated an additional TNFR family member, which we named decoy receptor 1 (DcR1) (Fig. 1A). The DcR1 precursor is 259 amino acids long. DcR1 has a hydrophobic NH_2-terminal sequence, followed by two CRDs. Downstream of the CRDs are five nearly identical tandem repeats, each 15 amino acids long; these repeats are followed by a hydrophobic COOH-terminus without an apparent cytoplasmic tail (Fig. 1A). This latter feature, together with the presence of a pair of small amino acids (Ala^{223} and Ala^{224}) just upstream of the hydrophobic COOH-terminus, suggests that DcR1 may be processed into a glycosyl-phosphatidylinositol (GPI)–anchored cell surface protein (*20*). The ECD of DcR1 is most closely related to those of DR4 (60% identity) and DR5 (50% identity) and contains five potential N-linked glycosylation sites (Fig. 1A).

We investigated the mRNA expression of DR5 and DcR1 in human tissues and tumor cell lines (Fig. 1B). We detected a single DR5 mRNA transcript and several DcR1 transcripts in multiple tissues; the ~1.5-kb DcR1 transcript corresponded in size to the cloned DcR1 cDNA. DR5 expression was relatively high in fetal liver and lung, and in adult PBL, ovary, spleen, liver, and lung. DcR1 expression was highest in PBL, spleen, lung, and placenta. Most of the tumor cell lines expressed DR5, but showed little or no expression of DcR1.

The sequence similarities between DR5, DcR1, and DR4 suggested that these receptors may interact with a common ligand. Epitope-tagged fusion proteins based on the ECD of DR5 or DcR1 (*21*) each coprecipitated with soluble TRAIL (*22*) (Fig. 2A). Other cytotoxic TNF family members, namely, TNF, lymphotoxin-α, or CD95L,

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