zero, then the total energy equation is

$$\rho w\left(E + \Phi + \frac{1}{2}\mathbf{u}^2\right) + pw - \kappa\frac{\partial(T + \delta T)}{\partial z} - \frac{\nu}{\rho}\frac{1}{2}\frac{\partial \mathbf{u}^2}{\partial z} = 0$$

where $\upsilon = (u, v, w)$ is the perturbation velocity, E, Φ, and $\mathbf{u}^2/2$ are the internal, potential, and kinetic energies, p is the total pressure (mean plus perturbation), and κ is the thermal conductivity [Gill (*8*), eq. 4.7.3 and 4.7.4; French and Gierasch, (*3*)]. The first term represents the advection of energy, the second term is the mechanical-energy work term, the third term is the transport of energy by thermal conduction, and the fourth term represents the (reversible) diffusion of kinetic energy, and is generally small. We drop terms that are higher than second order in perturbation variables, and take the average over a period (denoted by $\langle\ \rangle$), eliminating $\rho w\Phi$ and $\rho w(1/2)\mathbf{u}^2$. Finally, we write ρE as $c_v p/R$. Since $1 + c_v/R = \gamma/(\gamma - 1)$, this becomes

$$\kappa\frac{\partial T}{\partial z} = \frac{\gamma}{\gamma - 1}\langle(\delta p)w\rangle$$

where δp is the perturbation pressure. Evaluation of $\langle(\delta p)w\rangle$ with the WKB wave solution leads to (δ).

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The Receptor for the Cytotoxic Ligand TRAIL

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TRAIL (also known as Apo-2L) is a member of the tumor necrosis factor (TNF) ligand family that rapidly induces apoptosis in a variety of transformed cell lines. The human receptor for TRAIL was found to be an undescribed member of the TNF-receptor family (designated death receptor–4, DR4) that contains a cytoplasmic "death domain" capable of engaging the cell suicide apparatus but not the nuclear factor kappa B pathway in the system studied. Unlike Fas, TNFR-1, and DR3, DR4 could not use FADD to transmit the death signal, suggesting the use of distinct proximal signaling machinery. Thus, the DR4-TRAIL axis defines another receptor-ligand pair involved in regulating cell suicide and tissue homeostasis.

Apoptosis, or programmed cell death, is a process fundamental to the normal development and homeostasis of multicellular organisms. Deregulation of programmed cell death leads to a number of human diseases, including cancer, neurodegenerative disorders, and acquired immunodeficiency syndrome (*1*, *2*). The cell death machinery comprises effectors, activators, and negative regulators (*3*). Certain cytokines of the TNF ligand family and their cognate receptors, including TNFR-1 and Fas (also known as Apo-1 or CD95), are classic triggers of the suicide response (*1*, *3*). TNF and Fas ligand (also known as Apo-1L or CD-95L) induce apoptosis by binding to their respective death domain–containing receptors, TNFR-1 and Fas. The death domain is a protein-protein interaction motif that orchestrates the assembly of a signaling complex leading to the recruitment of pro-apoptotic proteases (caspases) related to the product of the *Caenorhabditis elegans* death gene *ced-3* (*3*–*7*).

Another member of the TNF ligand family, named TRAIL (Apo-2L), has been identified (*8*), and like Fas ligand (FasL), it induces rapid apoptosis in transformed cell lines of diverse origin (*8*). Unlike FasL, whose transcripts are predominantly restricted to stimulated T cells and sites of immune privilege (for example, corneal epithelium), TRAIL expression is detected in many normal human tissues, suggesting that TRAIL must not be cytotoxic to most tissues in vivo (*8*, *9*). Given that TRAIL is a member of the TNF ligand family and has marked pro-apoptotic potential for transformed cells, it was reasonable to assume that it bound a death domain–containing member of the TNF-receptor family. However, the inability of TRAIL to bind TNFR-1, Fas, or the recently identified death domain–containing receptor DR3 (also called Wsl-1, Apo-3, and TRAMP) (*8*, *10*) suggested that TRAIL may interact with a yet unknown member of the TNF-receptor family.

To identify such a receptor, we searched an expressed sequence tag (EST) database with the death domain of TNFR-1 (Fig. 1B). A human EST clone was initially found that encoded a previously unidentified death domain. Subsequent cDNA library screening and DNA sequence analysis led to the isolation of a clone encoding an open reading frame of 468 amino acids with features characteristic of a cell surface receptor (Fig. 1A) (*11*). Database searches, sequence alignment, and hydropathy analysis revealed the encoded protein (DR4) to be a member of the TNF-receptor family (Fig. 1). A putative signal peptide is present at the beginning of the molecule (amino acids –23 to –1), with the mature protein predicted to start at amino acid 24 (Ala). Residues 108 to 206 contain two cysteine-rich pseudorepeats that resemble corresponding regions in TNFR-1 (four repeats), DR3 (four repeats), Fas (three repeats), and CAR1 (two repeats) (*12*). Following the transmembrane domain (amino acids 227 to 245) is an intracellular region containing a 70–amino acid stretch with significant similarity to the death domains of TNFR-1, DR3, Fas, and CAR1 (Fig. 1B). The death domain of DR4 is 30, 29, and 28% identical to the corresponding domains in TNFR-1, CAR1, and DR3, respectively, but only 19% identical to the death domain of Fas. Four out of six residues in the death domain of TNFR-1 (Arg^{336}, Leu^{340}, Trp^{367}, and Ile^{397}) (*13*) that are essential for its signaling ability are conserved in the DR4 death domain (Arg^{362}, Leu^{366}, Trp^{392}, and Ile^{421}). Given the presence of a death domain and its ability to engage the death pathway (discussed below), we termed this receptor DR4 for death receptor–4.

Tissue distribution of the DR4 transcript was examined by Northern (RNA) blot analysis. Three major transcripts of 2.6, 4.6, and 7.2 kb were detected in most human tissues, including spleen, peripheral blood leukocytes, small intestine, and thymus (Fig. 1C). DR4 expression was also found in K562 erythroleukemia cells, MCF7 breast carcinoma cells, and activated T cells (*14*).

Upon overexpression, TNFR-1, Fas, and DR3 are activated in a ligand-independent manner and induce apoptosis (*7*, *10*). On the basis of this observation, we examined whether overexpression of DR4 could similarly trigger apoptosis. MCF7 human breast carcinoma cells and 293 human embryonic kidney cells were transiently transfected with a DR4-expressing construct. Most transfected cells underwent the morphological changes characteristic of apoptosis (Fig. 2, A to C). As anticipated, deletion of the death domain abolished the ability of DR4 to engage the death pathway. DR4-induced apoptosis was efficiently blocked by the caspase inhibitors z-VAD-fmk and CrmA, implicating the involvement of apical pro-apoptotic proteases (*7*) (Fig. 2D). Because apoptosis induced by TNFR-1, Fas, and DR3 is also attenuated by these same inhibitors (*7*, *10*), it is likely that the downstream death effector molecules are similar in nature.

FADD (also called MORT1) is the common intracellular adaptor molecule recruited by Fas, TNFR-1, and DR3 to engage the downstream death machinery (*6*, *10*, *15*). We tested if DR4 could also use FADD to transmit the death signal. We cotransfected 293

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cells with constructs expressing hemagglutinin (HA) epitope–tagged FADD (HA-FADD) and FLAG epitope–tagged DR4 (FLAG-DR4) or FLAG-Fas. Coimmunoprecipitation was done to determine whether in vivo associations occurred (Fig. 3A). Unlike Fas, DR4 did not bind FADD, and consistent with this, dominant-negative FADD had little effect on DR4- or TRAIL-induced apoptosis

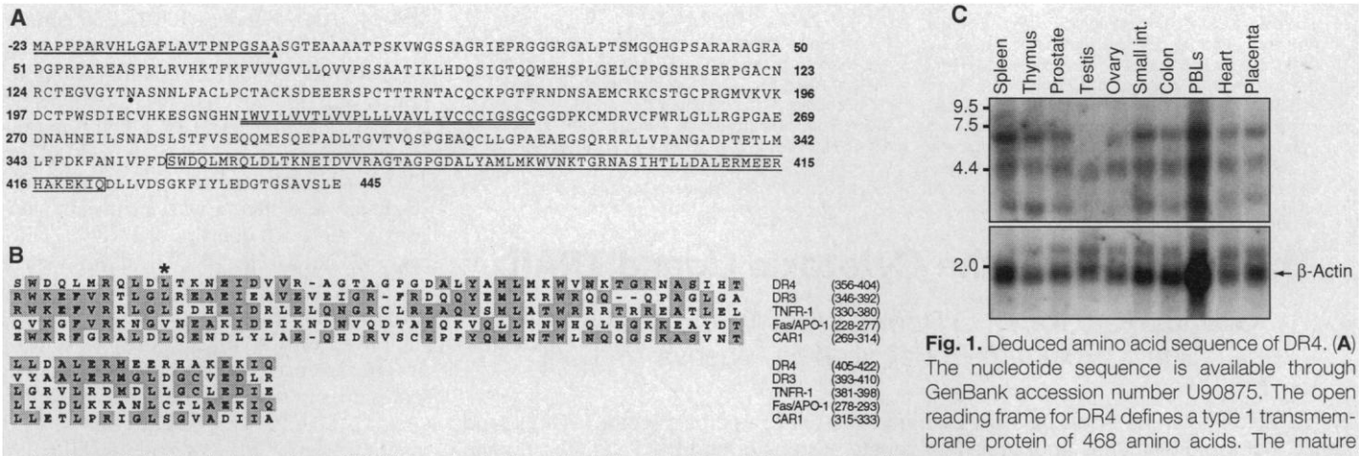

Fig. 1. Deduced amino acid sequence of DR4. (**A**) The nucleotide sequence is available through GenBank accession number U90875. The open reading frame for DR4 defines a type 1 transmembrane protein of 468 amino acids. The mature protein is predicted to start at amino acid 24 (Ala, indicated by a black triangle). The putative signal peptide and transmembrane domain are single and double underlined, respectively. A potential N-glycosylation site is indicated by a black dot. 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**) Sequence alignment of death domains. Alignment was done with Megalign (DNASTAR) software. Shading represents identical residues. The asterisk indicates a critical amino acid, mutation of which inactivates Fas (*18*). (**C**) Tissue distribution of the DR4 transcript. Human adult tissue Northern blots (Clontech) were probed with an internal fragment of DR4 cDNA according to the manufacturer's instructions. The blots were subsequently hybridized with β-actin cDNA. PBLs, peripheral blood leukocytes; Small int., small intestine.

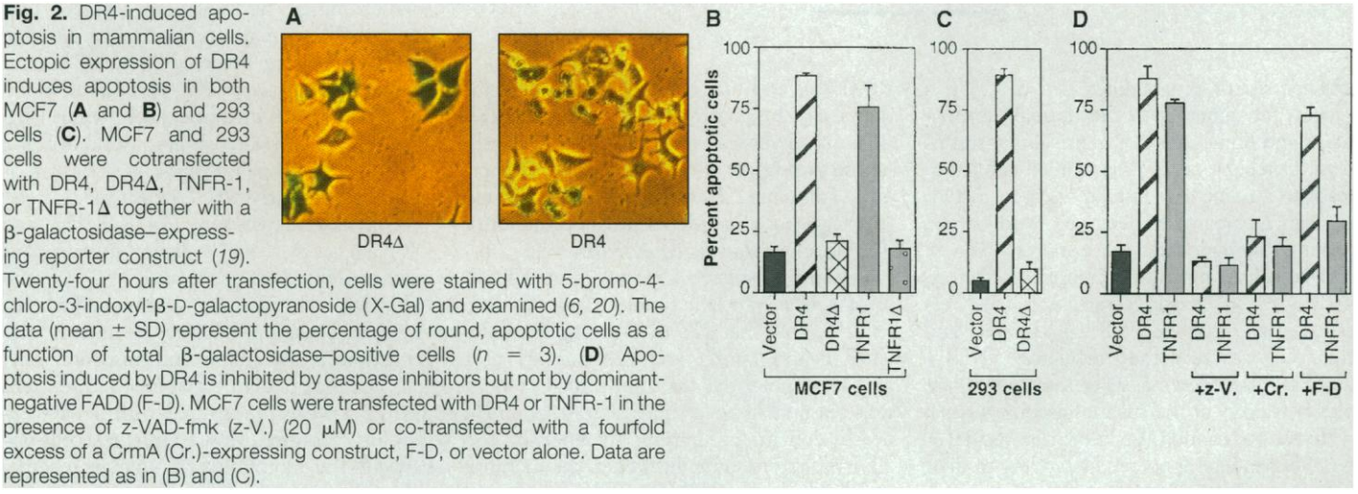

Fig. 2. DR4-induced apoptosis in mammalian cells. Ectopic expression of DR4 induces apoptosis in both MCF7 (**A** and **B**) and 293 cells (**C**). MCF7 and 293 cells were cotransfected with DR4, DR4Δ, TNFR-1, or TNFR-1Δ together with a β-galactosidase–expressing reporter construct (*19*). Twenty-four hours after transfection, cells were stained with 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) and examined (*6, 20*). The data (mean ± SD) represent the percentage of round, apoptotic cells as a function of total β-galactosidase–positive cells (n = 3). (**D**) Apoptosis induced by DR4 is inhibited by caspase inhibitors but not by dominant-negative FADD (F-D). MCF7 cells were transfected with DR4 or TNFR-1 in the presence of z-VAD-fmk (z-V.) (20 μM) or co-transfected with a fourfold excess of a CrmA (Cr.)-expressing construct, F-D, or vector alone. Data are represented as in (B) and (C).

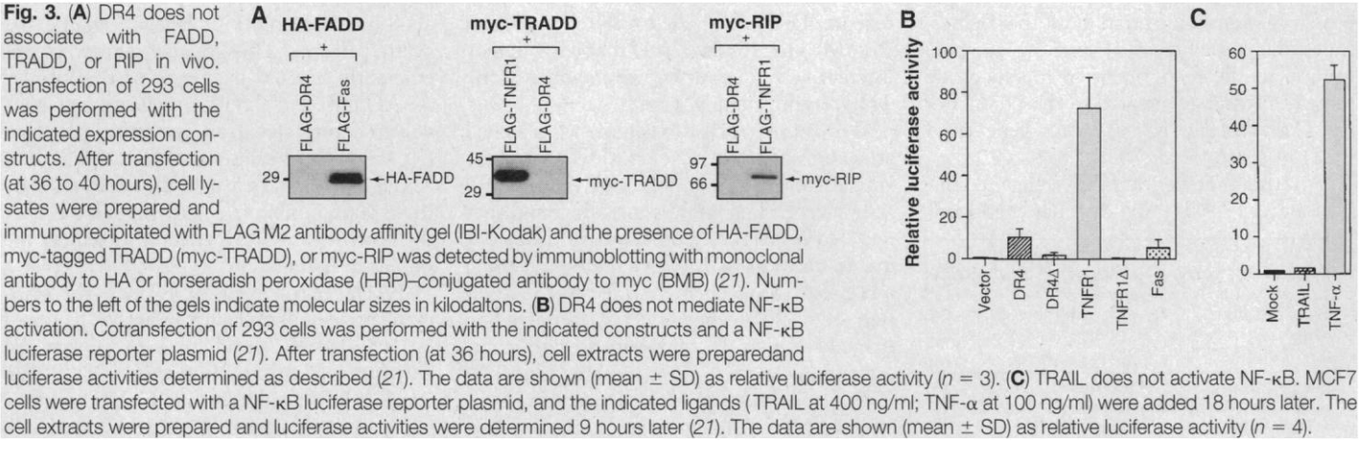

Fig. 3. (**A**) DR4 does not associate with FADD, TRADD, or RIP in vivo. Transfection of 293 cells was performed with the indicated expression constructs. After transfection (at 36 to 40 hours), cell lysates were prepared and immunoprecipitated with FLAG M2 antibody affinity gel (IBI-Kodak) and the presence of HA-FADD, myc-tagged TRADD (myc-TRADD), or myc-RIP was detected by immunoblotting with monoclonal antibody to HA or horseradish peroxidase (HRP)–conjugated antibody to myc (BMB) (*21*). Numbers to the left of the gels indicate molecular sizes in kilodaltons. (**B**) DR4 does not mediate NF-κB activation. Cotransfection of 293 cells was performed with the indicated constructs and a NF-κB luciferase reporter plasmid (*21*). After transfection (at 36 hours), cell extracts were preparedand luciferase activities determined as described (*21*). The data are shown (mean ± SD) as relative luciferase activity (n = 3). (**C**) TRAIL does not activate NF-κB. MCF7 cells were transfected with a NF-κB luciferase reporter plasmid, and the indicated ligands (TRAIL at 400 ng/ml; TNF-α at 100 ng/ml) were added 18 hours later. The cell extracts were prepared and luciferase activities were determined 9 hours later (*21*). The data are shown (mean ± SD) as relative luciferase activity (n = 4).

(Fig. 2D) (*8*). In a similar experiment, DR4 could not bind either TRADD or RIP (Fig. 3A), adaptor molecules involved in TNFR-1 and DR3 signaling (*8*, *16*), or RAIDD (*3*, *14*). Next, we determined if DR4 could activate nuclear factor kappa B (NF-κB), a characteristic of TNFR-1 and DR3 signaling. Like Fas, DR4 was incapable of NF-κB activation in this cell line (Fig. 3B). Similarly, TRAIL failed to activate NF-κB in MCF7 cells (Fig. 3C).

To determine if DR4 is capable of binding TRAIL, we expressed the extracellular ligand-binding domain of DR4 as a fusion to the Fc region of human immunoglobulin G (IgG) (DR4-Fc) (Fig. 4A). TRAIL selectively bound to DR4-Fc but not to the corresponding extracellular domains of TNFR-1 or Fas-Fc similarly expressed as Fc fusions. In addition, DR4-Fc did not bind either TNF-α or FasL under conditions where both of these ligands bound their cognate receptors (Fig. 4A). Further, the ability of TRAIL to induce apoptosis in MCF7 cells was specifically blocked by DR4-Fc but not by TNFR1-Fc, Fas-Fc, or Fc alone (Fig. 4, B and C). As expected, TNF-α–induced apoptosis was inhibited by TNFR-1–Fc but not by DR4-Fc, Fas-Fc, or Fc alone (Fig. 4D). Taken together, the data are consistent with DR4 being a receptor for TRAIL.

TNFR-1, Fas, and their respective ligands were the only death receptor–ligand pairs previously characterized. Here we identified DR4 as the receptor for the pro-apoptotic ligand TRAIL. Similar to TNFR-1, DR3, and Fas, DR4 contains a death domain capable of engaging the suicide machinery and the apoptotic proteases that compose it. Unlike the known death receptors, however, DR4 signaled cell death differently, independent of FADD. Functionally, the DR4-TRAIL complex is most related to the Fas-FasL complex, which can trigger apoptosis in a wide variety of cell lines and usually does not activate NF-κB. In vivo activation of the Fas pathway causes massive liver destruction in mice (*17*). It will be interesting to determine whether activation of the DR4 pathway will have similar toxicity in vivo. In addition, targeted disruption of the DR4 gene may provide insights into the physiological role of DR4.

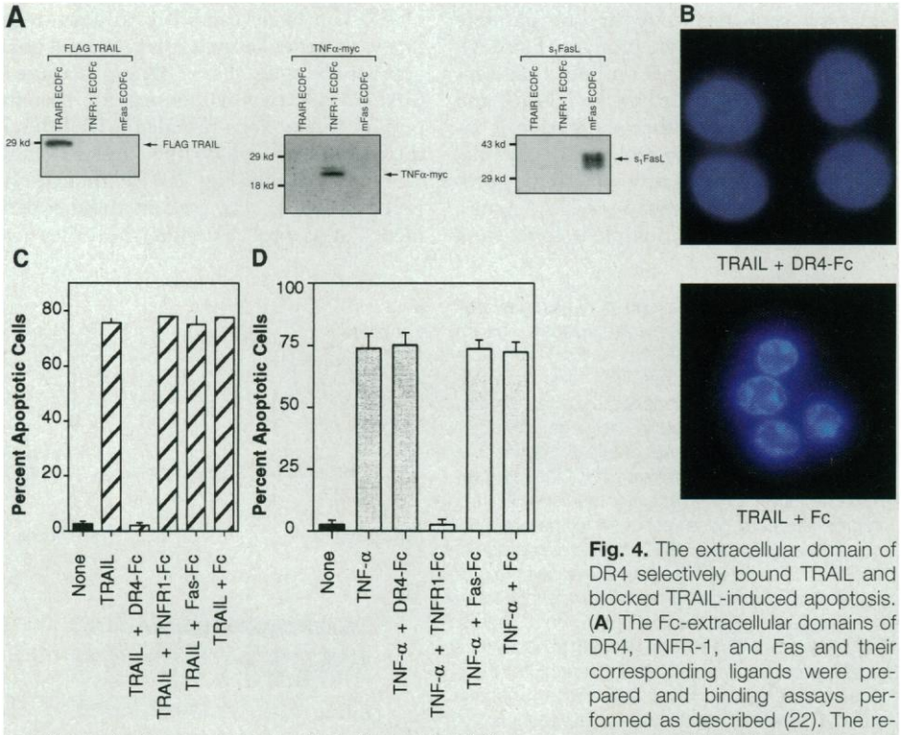

Fig. 4. The extracellular domain of DR4 selectively bound TRAIL and blocked TRAIL-induced apoptosis. (**A**) The Fc-extracellular domains of DR4, TNFR-1, and Fas and their corresponding ligands were prepared and binding assays performed as described (*22*). The respective Fc fusions were precipitated with protein G–Sepharose, and coprecipitated soluble ligands were identified by immunoblotting with antibodies to FLAG (Babco), to myc-HRP (BMB), or to FasL (Pharmingen). Numbers to the left of the gels indicate molecular sizes in kilodaltons. (**B** and **C**) MCF7 cells were treated with soluble TRAIL (400 ng/ml) (*23*) in the presence of equal amounts of Fc fusions or Fc alone as indicated. Five hours later, cells were fixed with formaldehyde, and the nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy with a fluorescein isothiocyanate range barrier filter cube (*10*). The data (mean ± SD) shown are the percentage of apoptotic nuclei among total nuclei counted (n = 3). (**D**) DR4-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. Between 15 and 18 hours later, nuclei were stained with DAPI.

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19. DR4 and DR4Δ (amino acids 1 to 351) were cloned into pCMV1FLAG (IBI Kodak). The constructs encoding TNFR-1 and TNFR-1Δ have been described (*15*). The cDNAs encoding the extracellular domains of DR4 (amino acids 87 to 217) and TNFR-1 (amino acids 37 to 205) were obtained by polymerase chain reaction (PCR) and cloned into a modified pCMV1FLAG vector that allowed for in-frame fusion with the Fc portion of human immunoglobulin G. cDNAs encoding soluble TRAIL (amino acids 95 to 281) and soluble TNF-α (amino acids 77 to 233) were obtained by PCR and subcloned into pCMV1FLAG and pSectagB (Invitrogen), respectively.
20. Cell death assays were performed as described (*6*). MCF7 cells were transfected by using the lipofectamine procedure (BRL) according to the manufacturer's instructions. The 293 cells were transfected by means of calcium phosphate precipitation.
21. In vivo interaction and NF-κB luciferase assays were done as described (*10*).
22. We transfected 293 cells with constructs encoding either receptor-Fc chimeras or ligands, and the conditioned media was harvested 72 to 80 hours later, clarified by centrifugation, divided, and stored at −80°C. For binding assays, equal amounts of receptor-Fc– and ligand-containing conditioned media were mixed in buffer containing 50 mM Hepes, pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.5 % NP-40, and a protease inhibitor mixture, and the sample was incubated at 4°C with continuous rotation for 4 hours. Receptor-Fc–ligand complexes were precipitated with protein G–Sepharose, extensively washed with the above buffer, boiled in SDS sample buffer, and resolved on a 12% SDS-polyacrylamide gel. Bound ligands were identified by immunoblotting.
23. A PCR fragment encoding soluble FLAG-TRAIL (amino acids 95 to 281) was cloned into the His-tag vector pET15b (Novagen). The His-FLAG-TRAIL was purified by nickel chelate affinity chromatography according to the manufacturer's instructions.

24. We thank S. Nagata for the Fas-Fc and soluble FasL expression constructs, J. Bretz and other members of the Dixit lab for helpful discussions, M. Garg for preparing His-FLAG-TRAIL protein, I. Jones for assistance in preparing the figures, and J. DeJohn for secretarial help. Supported by NIH grants ES08111 and DAMD17-96-1-6085.

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