Immunity, Vol. 7, 813–820, December, 1997, Copyright ©1997 by Cell Press

The Novel Receptor TRAIL-R4 Induces NF-κB and Protects against TRAIL-Mediated Apoptosis, yet Retains an Incomplete Death Domain

Mariapia A. Degli-Esposti,* William C. Dougall, Pamela J. Smolak, Jennifer Y. Waugh, Craig A. Smith, and Raymond G. Goodwin
Departments of Biochemistry and Molecular Biology
Immunex Corporation
51 University Street
Seattle, Washington 98101

Summary

A fourth member of the emerging TRAIL receptor family, TRAIL-R4, has been cloned and characterized. TRAIL-R4 encodes a 386–amino acid protein with an extracellular domain showing 58%–70% identity to those of TRAIL-R1, TRAIL-R2, and TRAIL-R3. The signaling capacity of TRAIL-R4 is similar to that of TRAIL-R1 and TRAIL-R2 with respect to NF-κB activation, but differs in its inability to induce apoptosis. Yet TRAIL-R4 retains a C-terminal element containing one third of a consensus death domain motif. Transient overexpression of TRAIL-R4 in cells normally sensitive to TRAIL-mediated killing confers complete protection, suggesting that one function of TRAIL-R4 may be inhibition of TRAIL cytotoxicity. Like TRAIL-R1 and TRAIL-R2, this receptor shows widespread tissue expression. The human TRAIL-R4 gene has been mapped to chromosome 8p22-21, clustered with three other TRAIL receptors.

Introduction

The process of programmed cell death, or apoptosis, is fundamental in developmental and homeostatic maintenance of complex biological systems. Of particular interest is the role of apoptosis in the processes of immune regulation. The tumor necrosis factor (TNF) family of cytokines and receptors has been the center of much attention, since several members of this family have been shown to be capable of regulating the apoptotic signals that shape the immune system (Smith et al., 1994; Nagata and Golstein, 1995; van Parijs and Abbas, 1996). TRAIL, the newest member of the TNF family of ligands (Wiley et al., 1995), can induce apoptosis in a wide variety of transformed cell lines of diverse lineage, but does not appear to kill normal cells (Wiley et al., 1995; Pitti et al., 1996). This finding has been puzzling since TRAIL message is expressed at significant levels in most normal tissues (Wiley et al., 1995), suggesting that regulation of TRAIL function takes place at the level of receptor expression.

The recent cloning of three TRAIL receptors implies a more complex scenario, one in which TRAIL may transduce different signals via different receptors. Two receptors for TRAIL, TRAIL receptor 1 (TRAIL-R1)/DR4 (Pan et al., 1997b) and TRAIL-R2/DR5 (Pan et al., 1997a; Sheridan et al., 1997; Walczak et al., 1997), are capable of mediating apoptosis. In contrast, the third TRAIL receptor, TRAIL-R3/TRID/DcR1 (Degli-Esposti et al., 1997; Pan et al., 1997a; Sheridan et al., 1997) is not able to transduce an apoptotic signal directly because it lacks a cytoplasmic domain. Indeed, some investigators have proposed that TRAIL-R3 acts as a decoy receptor to inhibit TRAIL signaling (Pan et al., 1997a; Sheridan et al., 1997). To date there is no description of signaling TRAIL receptors that inhibit apoptosis.

In this report we describe the identification of a fourth TRAIL receptor, one that does not signal apoptosis, can signal NF-κB activation, and is capable of protecting against TRAIL-mediated apoptosis.

Results

Isolation of TRAIL-R4 cDNA

To determine whether additional members of the TRAIL receptor family exist, we screened two cDNA libraries known to encode the previously characterized TRAIL-R1 (Pan et al., 1997b), TRAIL-R2 (Pan et al., 1997a; Sheridan et al., 1997; Walczak et al., 1997), and TRAIL-R3 (Degli-Esposti et al., 1997; Pan et al., 1997a; Sheridan et al., 1997) with a probe encompassing the extracellular ligand-binding domain of TRAIL-R3. Multiple cDNA clones were obtained encoding a type 1 transmembrane protein with homology to the previously identified TRAIL receptors. The new cDNA, referred to as TRAIL-R4, encodes a 386–amino acid (aa) protein with a predicted signal sequence cleavage site after serine 55, a 125 aa cysteine-rich extracellular domain, a 31 aa extracellular linker sequence, a 21 aa hydrophobic transmembrane region, and a 154 aa C-terminal cytoplasmic domain (Figure 1A). The extracellular domain of TRAIL-R4 contains one partial and two complete cysteine-rich pseudorepeats and shows the highest identity to the extracellular domains of TRAIL-R3 (70%), TRAIL-R2 (57%), and TRAIL-R1 (58%) (Figure 1B). Significant homology was also observed with other members of the TNF receptor (TNFR) family, especially TNFR1 and cytopathic avian leukosis–sarcoma virus receptor-1 (CAR1) (Figure 1B). Three potential N-glycosylation sites were identified (Figure 1A).

Two allelic variants of TRAIL-R4 were identified, differing at residues 35 (proline/serine) and 310 (serine/leucine) (Figure 1A). These variations, located in the putative signal peptide sequence and the cytoplasmic domain, respectively, have been shown to have no effect on receptor expression or ligand binding (data not shown).

The C-terminal cytoplasmic domain of TRAIL-R4 displays 58% identity to the cytoplasmic domains of both TRAIL-R1 and TRAIL-R2. However, TRAIL-R4 carries only a portion of the consensus death domain motif, having lost 52 of the 76 amino acids that encode the predicted TRAIL-R1 and TRAIL-R2 death domain (Walczak et al., 1997) (Figures 1A and 1C).

*To whom correspondence should be addressed (e-mail: mdeglisposti@immunex.com).

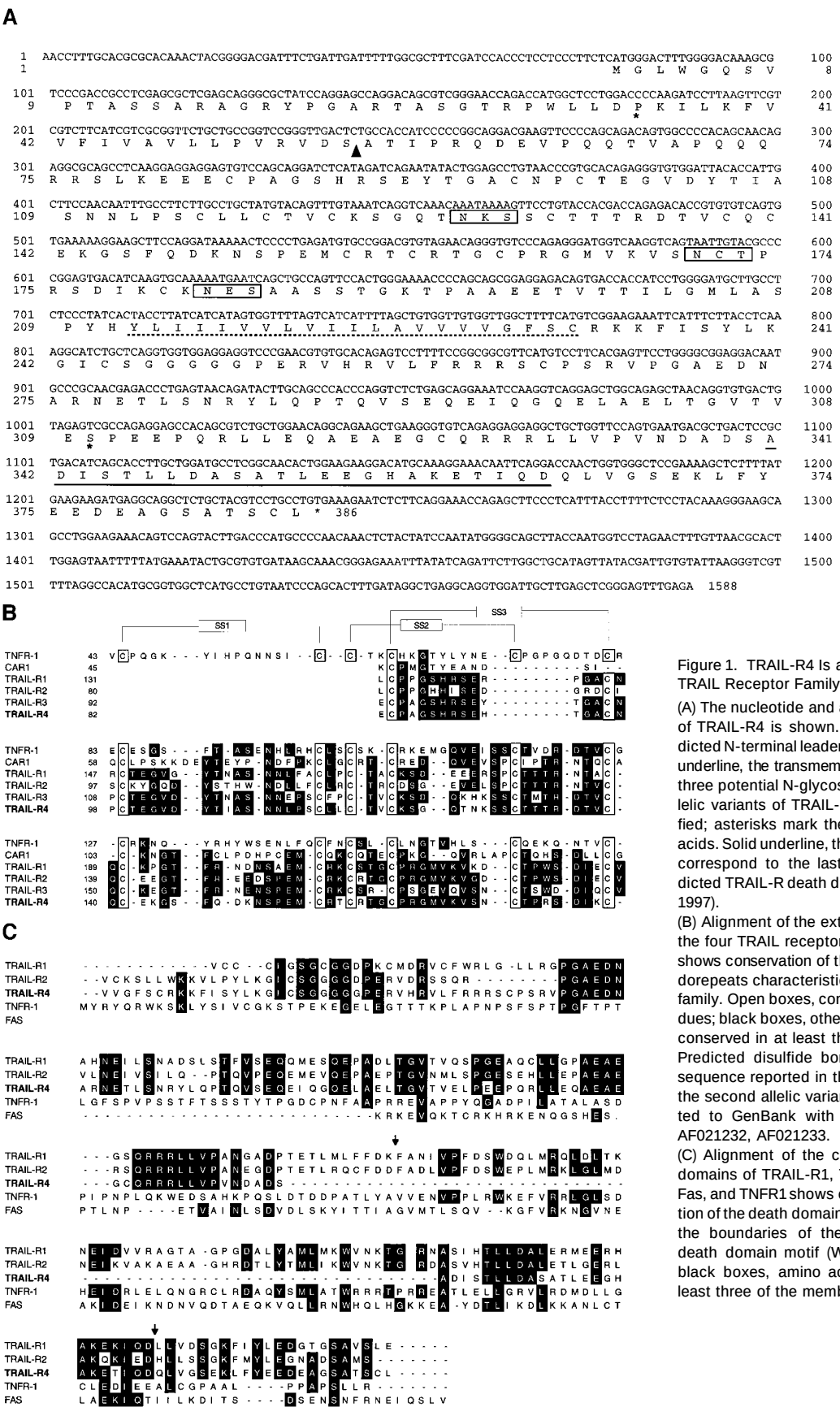

Figure 1. TRAIL-R4 Is a New Member of the TRAIL Receptor Family

(A) The nucleotide and amino acid sequence of TRAIL-R4 is shown. Arrowhead, the predicted N-terminal leader cleavage site; dotted underline, the transmembrane domain; boxes, three potential N-glycosylation sites. Two allelic variants of TRAIL-R4 have been identified; asterisks mark the polymorphic amino acids. Solid underline, the 24 amino acids that correspond to the last portion of the predicted TRAIL-R death domain (Walczak et al., 1997).

(B) Alignment of the extracellular domains of the four TRAIL receptors, TNFR1, and CAR1 shows conservation of the cysteine-rich pseudorepeats characteristic of the TNF receptor family. Open boxes, conserved cysteine residues; black boxes, other amino acids that are conserved in at least three of the members. Predicted disulfide bonds are shown. The sequence reported in this article and that of the second allelic variant have been submitted to GenBank with accession numbers: AF021232, AF021233.

(C) Alignment of the complete cytoplasmic domains of TRAIL-R1, TRAIL-R2, TRAIL-R4, Fas, and TNFR1 shows conservation of a portion of the death domain in TRAIL-R4. Arrows, the boundaries of the predicted TRAIL-R death domain motif (Walczak et al., 1997); black boxes, amino acids conserved in at least three of the members.

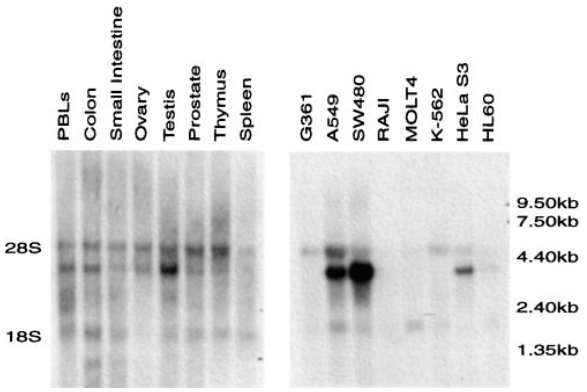

Figure 2. TRAIL-R4 Shows Widespread Tissue Expression

Northern analysis showing the distribution of TRAIL-R4 mRNA in whole human tissues and tumor cell lines. The source of the mRNA is shown above each lane. The position of the 28S and 18S ribosomal RNA species, as determined by methylene-blue staining, is marked on the left; the position of RNA size markers is shown on the right. A TRAIL-R4 transcript of 4.0 kb is detected in most tissues and in some tumor cell lines.

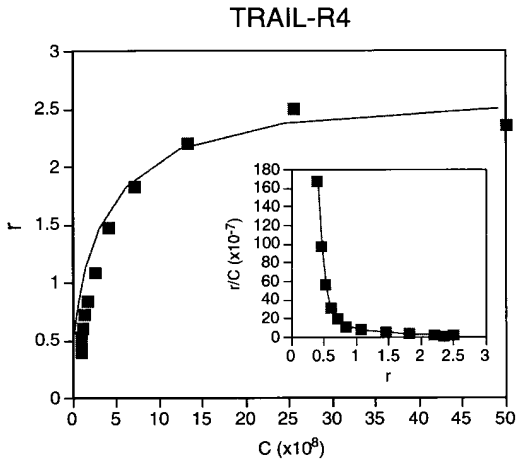

Figure 3. Equilibrium Binding Isotherms of TRAIL-R4

TRAIL-R4-Fc bound to goat-anti-human-Fc immobilized to plastic was used in equilibrium binding assays with LZ-TRAIL plus ^{125}I-labeled M15 anti-LZ antibody, as described (see Experimental Procedures). The binding data plotted in the Scatchard coordinate system are shown.

The TRAIL-R4 Gene Is Part of a TRAIL-R Cluster Located on Human Chromosome 8p

Analysis of two independent radiation hybrid panels using a primer pair specific for human TRAIL-R4 has mapped the gene to human chromosome 8p22-21. By linkage analysis the TRAIL-R4 gene has been placed into a cluster, located approximately 49 cM from the telomere, that also contains the genes for TRAIL-R1, TRAIL-R2, and TRAIL-R3 (Degli-Esposti et al., 1997). Two similar TNFR family gene clusters, on chromosomes 1p and 12p, have been described previously. However, in this instance, all of the genes in the cluster are receptors for a common ligand and share a much higher degree of identity than other members of the TNFR family.

TRAIL-R4 Shows Widespread Tissue Distribution

The mRNA expression of TRAIL-R4 in human tissues and tumor cell lines was determined by Northern blot analysis. A TRAIL-R4 transcript of approximately 4.0 kb was clearly detected in all tissues (Figure 2). Hybridization was also observed at positions 18S and 28S of ribosomal RNA migration, which we presume is not specific for TRAIL-R4. The lowest detectable levels of hybridization to the 4.0 kb TRAIL-R4 transcript were observed in thymus and spleen (Figure 2). This is similar to the widespread tissue distribution observed for TRAIL-R1 (Pan et al., 1997b) and TRAIL-R2 (Pan et al., 1997a; Sheridan et al., 1997; Walczak et al., 1997) but contrasts with the more restricted distribution observed for TRAIL-R3 (Degli-Esposti et al., 1997; Pan et al., 1997a; Sheridan et al., 1997). In addition, TRAIL-R4 message was expressed at a high level in three tumor cell lines (A549, SW480, and HeLa S3) (Figure 2).

TRAIL-R4 Binds TRAIL

Given the identity that exists between the extracellular ligand-binding domain of TRAIL-R4 and those of the previously characterized TRAIL receptors, we predicted TRAIL to be the ligand for this new receptor. The physical interaction between TRAIL and TRAIL-R4 was confirmed by autoradiographic analysis using soluble leucine zipper (LZ)–TRAIL and TRAIL-R4 transiently expressed on the cell surface of CVI/EBNA cells, as described previously (Degli-Esposti et al., 1997) (data not shown).

Quantitative equilibrium binding isotherms between the TRAIL ligand and TRAIL-R4, determined using a TRAIL-R4-Fc chimera, demonstrated specific and saturable binding (Figure 3) via two classes of sites ($K_{d(high)}$ = 0.085 nM; $K_{d(low)}$ = 39.2 nM). Thus, this receptor binds the TRAIL ligand with affinities comparable to those of the three previously characterized TRAIL receptors (Degli-Esposti et al., 1997).

TRAIL-Induced Apoptosis Is Inhibited by Soluble TRAIL-R4-Fc

To determine whether the extracellular ligand-binding domain of TRAIL-R4 can successfully block TRAIL-mediated activities, we tested the ability of a soluble TRAIL-R4-Fc fusion protein to block TRAIL-mediated apoptosis of Jurkat T cells. Jurkat cells are highly sensitive to TRAIL, and when cultured in its presence they are effectively killed within 6–12 hr. As expected, TRAIL-induced apoptosis was efficiently and specifically inhibited by coculturing with concentrated supernatants from CVI/EBNA cells transiently expressing soluble TRAIL-R4-Fc (Figure 4). Furthermore, TRAIL-R4-Fc was unable to block Fas-L–mediated killing (data not shown), and Fas-Fc was unable to block TRAIL-mediated killing (Figure 4).

TRAIL-R4 Does Not Signal Apoptosis

Two previously characterized members of the TRAIL-R family have been shown to transmit a death signal (Pan et al., 1997a, 1997b; Sheridan et al., 1997; Walczak et al., 1997). Like other members of the TNFR family capable of transducing such a response, TRAIL-R1 and TRAIL-R2 share an approximately 80 aa region of homology in

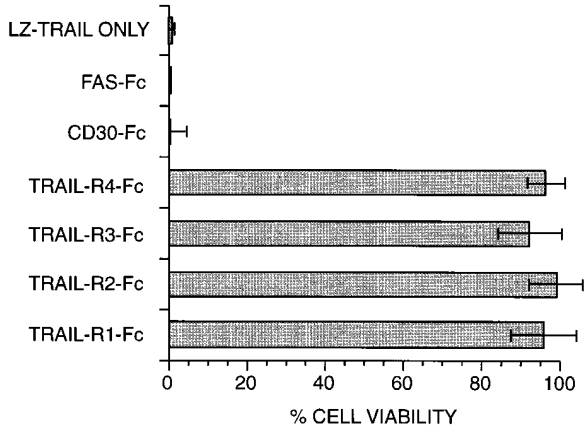

Figure 4. TRAIL-R4-Fc Inhibits TRAIL-Mediated Killing

Soluble TRAIL-R4-Fc, TRAIL-R3-Fc, TRAIL-R2-Fc, TRAIL-R1-Fc, or Fas-Fc or CD30-Fc proteins were added to cultures of Jurkat cells in the presence of soluble LZ-TRAIL (150 ng/ml). The MTT readings obtained in the absence and presence of LZ-TRAIL correspond to the maximum and minimum viability values, respectively. All TRAIL-R-Fc proteins blocked TRAIL-mediated apoptosis of Jurkat cells.

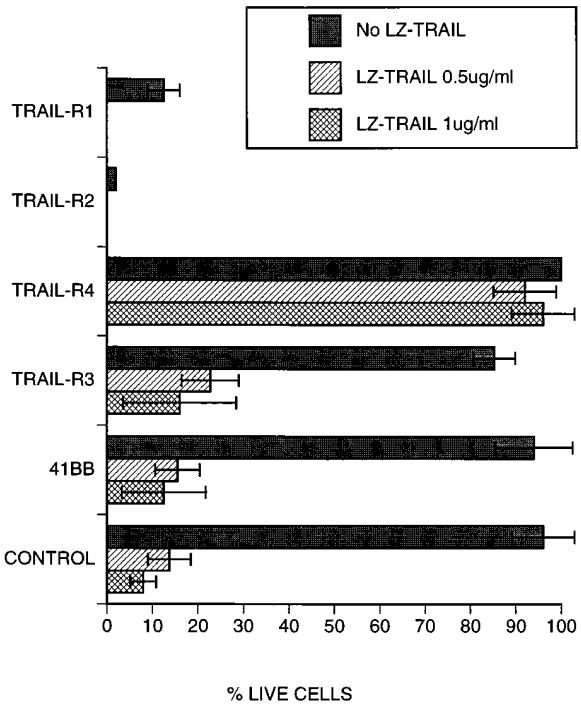

Figure 5. TRAIL-R4 Impedes TRAIL-Mediated Killing of CVI/EBNA Cells

CVI/EBNA cells were transiently cotransfected with an expression plasmid encoding the Escherichia coli *lacZ* gene plus an expression plasmid for TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, 41BB, or an unrelated type 1 transmembrane protein (control). Forty-eight hours after transfection the cells were incubated with or without soluble LZ-TRAIL (0.5 or 1.0 μg/ml) and assayed 16 hr later for apoptosis. Cells were checked for the morphological changes characteristic of apoptosis and stained for β-galactosidase activity. The percentage of live cells was quantitated by scoring the percentage of staining cells. The values plotted represent the mean and standard deviation of three separate experiments.

the cytoplasmic region that corresponds to the death domain, the region critical for the induction of apoptotic signals (Itoh and Nagata, 1993; Tartaglia et al., 1993). In contrast, TRAIL-R4 lacks 52 of the 76 amino acids that encode the predicted TRAIL-R1 and TRAIL-R2 death domain (Walczak et al., 1997), suggesting that this receptor may not be capable of transducing an apoptotic signal.

Self-oligomerization of cytoplasmic signaling sequences, as induced by overexpression, mimics ligand-mediated juxtaposition of the cytoplasmic regions required for productive signaling. Using such a system, both TRAIL-R1 and TRAIL-R2 were shown capable of signaling apoptosis (Pan et al., 1997a, 1997b; Sheridan et al., 1997; Walczak et al., 1997) (Figure 5). In contrast, transient overexpression of TRAIL-R4 in CVI/EBNA cells did not lead to cell death (Figure 5).

TRAIL-R4 Overexpression Confers Resistance to TRAIL-Mediated Apoptosis

Given the inability of TRAIL-R4 to trigger an apoptotic signal due to lack of the complete death domain sequence, we investigated the possibility that this receptor may actively modulate TRAIL signaling. To do so we tested the effect of overexpression of TRAIL-R4 in cells that are normally sensitive to TRAIL-mediated apoptosis. CVI-EBNA cells can be killed by incubation with soluble LZ-TRAIL (Figure 5). These cells were transiently transfected with plasmids encoding the full-length sequence of TRAIL-R4, TRAIL-R3, 41BB, and a control type 1 transmembrane protein, and their sensitivity to soluble LZ-TRAIL was assessed 48 hr after transfection. Overexpression of TRAIL-R4 conferred complete resistance to TRAIL-mediated killing (Figure 5). In contrast, TRAIL-R3, 41BB, and the control protein were unable to diminish the sensitivity of CVI/EBNA cells to TRAIL. A minor effect of TRAIL-R3 (<30% inhibition) was observed 6 hr after the addition of LZ-TRAIL (data not shown); however, this inhibition was no longer observed at 16 hr.

TRAIL-R4 Induces NF-κB Activation

Despite its lack of a complete death domain, TRAIL-R4 encodes what appears to be a functional cytoplasmic signaling region. In several other members of the TNFR family the cytoplasmic region also contains sequences involved in activation of the transcription factor NF-κB. Since resistance to apoptosis may involve activation of NF-κB, leading to the induction of protective genes (Beg and Baltimore, 1996; Van Antwerp et al., 1996), we tested whether TRAIL-R4 is able to activate NF-κB directly.

By using an electrophoretic mobility shift assay, we found that in transfected 293/EBNA cells, overexpression of TRAIL-R4 showed activation of NF-κB relative to cells transfected with vector alone (Figure 6). Transfection of 293/EBNA cells with TRAIL alone also caused significant NF-κB activation (Figure 6). The level of NF-κB activation induced by TRAIL and/or its receptors, however, is inferior to that induced by TNFR1 (data not shown) but similar to that induced by CD30 (Figure 6).

Discussion

In this report we describe the cloning and characterization of TRAIL-R4, a novel member of the TRAIL-R family

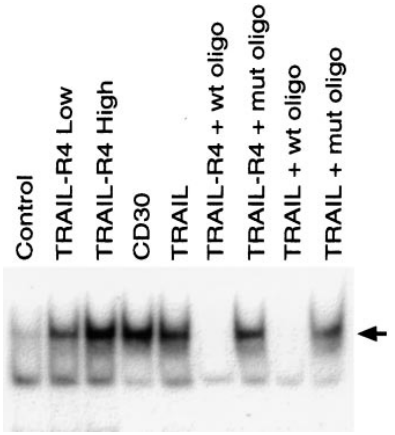

Figure 6. TRAIL-R4 Activates NF-κB

293/EBNA cells were transiently transfected with expression plasmids encoding soluble TRAIL (2.5 μg), TRAIL-R4 (1.0 μg = low; 5.0 μg = high), CD30 (5.0 μg), or empty pDC409 vector (1.0 μg = low; 5.0 μg = high). Nuclear extracts were incubated with a ^{32}P-labeled NF-κB-specific probe. NF-κB activation was assessed by gel shift analysis. Incubations with an oligonucleotide carrying a consensus NF-κB sequence (wt oligo) and an oligonucleotide carrying the same sequence but including a single point mutation (mut oligo), were included to determine the specificity of any band shifts.

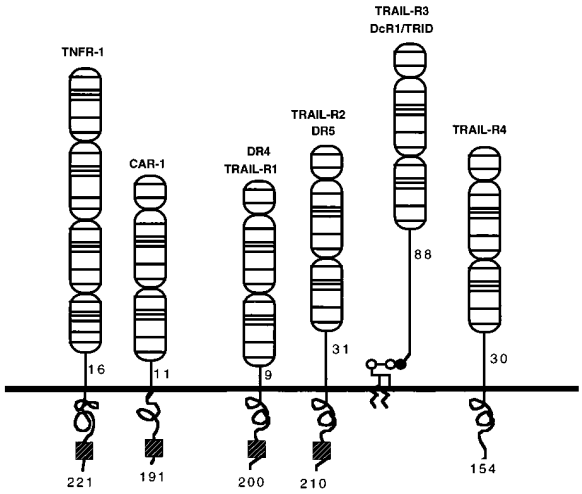

Figure 7. The TRAIL Receptor Family

A schematic representation of the four members of the TRAIL receptor family. Open boxes, homologous domains; horizontal lines, the conserved cysteine residues. The amino acid length of the extracellular linker and cytoplasmic regions are shown. Black boxes in the cytoplasmic regions represent death domains. The GPI anchor holding TRAIL-R3 onto the cell surface is shown. TNFR1 and CAR1, the closest relatives of TRAIL-Rs, are shown for comparison.

encoding a cell surface molecule capable of specific, high-affinity interaction with the cytotoxic TRAIL ligand. TRAIL-R4 shows a typical type 1 membrane protein architecture, with an extracellular ligand-binding region composed of three cysteine-rich pseudorepeats, the first of which is truncated.

TRAIL-R4 is the newest member of the TRAIL-R family, which also includes three previously described members (Figure 7). Two of the previously characterized receptors, TRAIL-R1/DR4 (Pan et al., 1997b) and TRAIL-R2/DR5 (Pan et al., 1997a; Sheridan et al., 1997; Walczak et al., 1997) induce apoptosis, whereas the third, TRAIL-R3/TRID/DcR1 (Degli-Esposti et al., 1997; Pan et al., 1997a; Sheridan et al., 1997), lacks a cytoplasmic domain and, as expected, does not signal apoptosis. Of particular significance is that TRAIL-R4 carries a cytoplasmic region lacking most of the death domain that characterizes TRAIL-R1 and TRAIL-R2 (Figures 1A and 1C) and that has been shown to be essential for the induction of apoptosis. To test whether TRAIL-R4 is indeed unable to signal apoptosis, we tested its ability to induce cell death in a transient overexpression system. This system relies on the fact that the self-aggregation of death domain motifs induced by overexpression simulates the ligand-mediated juxtaposition of signaling sequences essential for productive signaling (Boldin et al., 1995). Using this system, we have shown that TRAIL-R4 cannot induce apoptosis.

The third member of the TRAIL-R family, TRAIL-R3/TRID/DcR1, is unable to transduce an apoptotic signal because it completely lacks a cytoplasmic domain. Unlike this receptor, TRAIL-R4 contains a large cytoplasmic domain that includes a portion of the canonical death domain described by Tartaglia and Itoh (Itoh and Nagata, 1993; Tartaglia et al., 1993). The TRAIL-R4 partial death domain includes 24 of the 76 amino acids that encode the predicted TRAIL-R1 and TRAIL-R2 death domain (Walczak et al., 1997) plus an additional 22 residues to the end of the coding sequence. The portion of the death domain that is missing in TRAIL-R4 corresponds to the region shown be essential for TNFR1 self-association–induced apoptosis (Boldin et al., 1995), but it may not be required for other signaling, including NF-κB activation. We therefore predicted that, unlike TRAIL-R3, the TRAIL-R4 receptor may be capable of inducing direct signals other than apoptosis. Indeed, we have shown that TRAIL-R4, like other members of the TNFR family, including TRAIL-R1 and TRAIL-R2, is capable of directly signaling NF-κB activation, confirming that in this system apoptosis and NF-κB activation represent two independent signaling pathways.

Since TRAIL-R4 is an actively signaling receptor we investigated the possibility that it may dynamically modulate the cytotoxic activity of TRAIL. Overexpression of TRAIL-R4 in cells normally killed by TRAIL conferred complete resistance to the cytotoxic effect of this cytokine, suggesting that expression of TRAIL-R4 may determine the sensitivity of different cells and tissues to TRAIL-mediated killing.

Other investigators have proposed that protection against TRAIL-mediated cytotoxicity is conferred by the presence of TRAIL-R3/TRID/DcR1 (Pan et al., 1997a; Sheridan et al., 1997), and indeed the presence or absence of TRID/DcR1 message has been taken as evidence to explain the differential sensitivity of normal tissues and tumor cell lines to the cytotoxic effects of TRAIL (Pan et al., 1997a; Sheridan et al., 1997). In light of the present findings, however, this appears to be an oversimplification of an increasingly complex biological system. First, the level of expression of TRAIL-R3/TRID/DcR1 in normal tissues is variable, and significant levels are detected only in peripheral blood lymphocytes,

Table 1. Correlation between TRAIL-R Expression and Sensitivity to TRAIL-Mediated Apoptosis

Cell Line	Lineage	Sensitivity to TRAIL-Mediated Apoptosis	TRAIL-R1	TRAIL-R2	TRAIL-R3	TRAIL-R4
CVI/EBNA	Fibroblast	++	+	−	−	−
293/EBNA	Epithelial	−	±	+	+	+
PS1	B cell	+++	+	+	−	+
RAJI	B cell	++	+	+	−	−
Jurkat	T cell	+++	±	+	−	+
U937	Monocytic	++	+	+	+	NT
HL60	Monocytic	+	+	+	+	+

Sensitivity to TRAIL-mediated apoptosis was quantitated in appropriate killing assays using soluble LZ-TRAIL. TRAIL-R expression was determined by PCR using locus-specific primer pairs. Northern analysis was also used for some of the illustrated lines, and in all cases it corroborated the PCR-derived results.
NT, not tested.

spleen (Degli-Esposti et al., 1997), and lung (Sheridan et al., 1997). Furthermore, extreme caution should be taken when cell surface expression of the TRAIL-R3 protein is predicted from message levels, especially because such extrapolations completely ignore the likely influence of secondary signaling mechanisms.

Second, the described decoy activity of TRAIL-R3 appears to be limited. As shown by others, expression of TRAIL-R3 reduces the killing potential of TRAIL. This effect, however, seems to be transient and at best partial. As described in the Results section, we observed an incomplete (~30%) inhibitory effect of TRAIL-R3 6 hr after addition of TRAIL. However, the inhibition was no longer observed at 16 hr. The extent of protection conferred by TRAIL-R3 noted in this study is equivalent to that previously reported by others (Pan et al., 1997a; Sheridan et al., 1997) and, together with the transient nature of the TRAIL-R3 protection, does not appear to justify a major role for TRAIL-R3 as a universal decoy receptor for TRAIL. Further complexity is generated by the discovery that TRAIL-R4 can bind TRAIL without eliciting an apoptotic signal and indeed, in the cell system described in this report, it can completely block the cytotoxic activities of TRAIL.

Third, TRAIL-R4 retains the ability to signal NF-κB activation. Since NF-κB activation has been shown to increase the anti-apoptotic threshold of cells and tissues exposed to cytotoxic cytokines such as TNFα (Beg and Baltimore, 1996; Van Antwerp et al., 1996), it is possible that TRAIL-R4 may similarly inhibit TRAIL-induced apoptosis via the active induction of genes whose products provide resistance to apoptosis. In this context, it is worthy to note that (1) the sensitivity of several cells to the cytotoxic effects of TRAIL is enhanced in the absence of protein synthesis (Sheridan et al., 1997; Griffiths and Kubin, personal communication), and (2) expression of the Flice inhibitory protein (FLIP) was shown to effectively modulate TRAIL-induced apoptosis (Irmler et al., 1997).

Fourth, the partial death domain in the cytoplasmic portion of TRAIL-R4 does not allow self-aggregation–induced apoptosis, but it retains a number of amino acids shown to play an essential role in TNFR1- and Fas-mediated apoptosis (Itoh and Nagata, 1993; Tartaglia et al., 1993). This effect may occur through the recruitment of secondary signaling factors. Thus, the incomplete death domain of TRAIL-R4 may actively prevent the cytotoxic effects of TRAIL by titration of adaptor proteins or by recruitment of such proteins to a heterocomplex that may result in the formation of an incomplete death-inducing signaling complex. In this regard, it will be relevant to determine whether signals generated via TRAIL-R4 are capable of blocking the apoptotic signals generated by other ligands in this family, such as TNF and Fas ligand.

As mentioned above, it may be difficult to correlate the expression of message for the different TRAIL receptors with sensitivity to TRAIL-mediated cytotoxicity, and such a correlation may represent a nonphysiological oversimplification of the system. We have analyzed the sensitivity of several tumor cell lines in the context of the expression of different TRAIL receptors. As shown in Table 1, there is no clear correlation between the expression of the potential decoy receptors TRAIL-R3 and/or TRAIL-R4 and sensitivity to TRAIL. These data provide further evidence of the complexity of this system and warn us that a great deal of further analysis is required to elucidate the biological role of TRAIL and its receptors, and their potential as antitumor agents.

In conclusion, the identification of TRAIL-R4 adds further complexity to the biological relevance of the emerging TRAIL receptor family. The physiological function of each of these receptors remains to be elucidated. This task may prove to be more difficult than expected since the standard approach of targeted gene disruption may not be completely straightforward: the genes encoding each of the described TRAIL receptors are located in a chromosomal cluster and show a high degree of nucleotide identity. Continued evaluation of this system, however, is imperative since it may provide the first clues toward an understanding of the mechanisms that regulate the balance between cell survival and cell death.

Experimental Procedures

Isolation of TRAIL-R4 cDNA

A cDNA sequence with homology to TRAIL-R1 (Pan et al., 1997b), TRAIL-R2 (Pan et al., 1997a; Sheridan et al., 1997; Walczak et al., 1997), and TRAIL-R3 (Degli-Esposti et al., 1997; Pan et al., 1997a; Sheridan et al., 1997) was identified by screening peripheral blood lymphocyte and human foreskin fibroblast cDNA libraries. A [^{32}P]dCTP random-prime-labeled polymerase chain reaction (PCR) product encompassing the cysteine-rich extracellular domain of TRAIL-R3 was used as the probe for screening. The sequence of TRAIL-R4 was obtained using the ABI PRISM Dye Terminator Cycle

Sequencing Ready Reaction Kit, Protocol PN402078 (Perkin Elmer, Foster City, CA).

Chromosomal Mapping

Two independent radiation hybrid panels, the Stanford G3 Radiation Hybrid Panel RH01 and the Genebridge 4 Radiation Hybrid Panel RH02.02 (Research Genetics, Huntsville, AL) were used to determine the chromosomal location of TRAIL-R4. Each panel was screened with a set of oligonucleotide primers (5′-CACTACCTTATCATCATAG TGGTTTT-3′ and 5′-GAAGGACATGAACGCCGCCGGAAAAG-3′) that specifically amplify human TRAIL-R4 from genomic DNA. The results were electronically submitted to the appropriate servers (www.shgc.stanford.edu and www.genome.wi.mit.edu) for linkage analysis.

Northern Analysis

A human multiple tissue Northern blot and a tumor cell line Northern blot (Clontech, Palo Alto, CA) were probed with a ^{32}P-labeled riboprobe encompassing 248 nucleotides of the 3′ untranslated region of TRAIL-R4 immediately following the termination codon. The blots were hybridized overnight at 63°C in Stark's buffer (Wahl et al., 1979) and washed twice for 20 min in 0.1× SSC, 0.1% SDS at 68°C. Hybridizing bands were visualized by autoradiography.

Plasmid Construction

A full-length TRAIL-R4 transcript was cloned into the pDC409 mammalian expression vector by PCR. The TRAIL-R4-Fc fusion chimera was constructed as described (Smith et al., 1993) by fusing the extracellular domain, encoded between methionine 1 and proline 209, to the Fc portion of a mutein human IgG1 sequence.

Scatchard Analysis

The equilibrium binding isotherm between TRAIL and TRAIL-R4 was determined by Scatchard analysis using purified TRAIL-R4-Fc protein bound to plates previously coated with goat-anti-human-Fc. Serial dilutions (2×) of soluble human LZ-TRAIL (Walczak et al., 1997), starting at a concentration of 5 μg/ml in binding medium (3% bovine serum albumin, 20 mM HEPES [pH 7.4], 0.15 M NaCl, 0.04% NaN_3), were added to the plates for 30 min at room temperature. Unbound LZ-TRAIL was removed by washing with binding medium, and the plates incubated for 30 min at room temperature with ^{125}I-labeled M15 anti-LZ monoclonal antibody (125 ng/ml). Plates were again washed and specifically bound ligand released with 0.1 M glycine HCl [pH 3]. Nonspecific binding was calculated by inclusion of a 500-fold molar excess of unlabeled M15 anti-LZ antibody in duplicate reaction mixtures containing the first two dilutions of LZ-TRAIL. Specific binding values were calculated by subtracting linearly extrapolated nonspecific binding from each point. The RS1 software (BBN Software Products, Cambridge, MA) was used to plot the data in a Scatchard coordinate system with a nonlinear least squared fit.

Jurkat Killing Assay

Jurkat cells (1×10^4 cells/well) were incubated with soluble LZ-TRAIL (150 ng/ml) in the presence of concentrated supernatants (25×) containing equivalent amounts (75–125 μg/ml) of TRAIL-R1-Fc, TRAIL-R2-Fc, TRAIL-R3-Fc, TRAIL-R4-Fc, and CD30-Fc. Purified Fas-Fc was also included. Viability was measured after 16–20 hr by MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye conversion (Mosmann, 1983).

The maximum viability value corresponds to highest MTT reading, obtained in the absence of LZ-TRAIL.

Measurement of TRAIL-Mediated Apoptosis after TRAIL-R4 Transfection

CVI/EBNA cells (ATCC CRL 10478) (1.65×10^5 cells per slide) were transfected by the DEAE-dextran method (Sambrook et al., 1989) with 2.0 μg of DNA. Each DNA mixture contained 1.5 μg of test plasmid and 0.5 μg of pDC409-*E.coli lacZ*. Forty-eight hours post-transfection cells were incubated with soluble LZ-TRAIL (0.5–1.0 μg/ml) for 16 hr. The slides were then washed, fixed with glutaraldehyde, and stained with X-Gal (5-bromo-4-chloro-3-indoxyl-β-D-galacto-pyranoside) for β-galactosidase activity. Cell death correlates with a decrease in the number of cells expressing β-galactosidase (Hsu et al., 1995). Each experiment was repeated three times.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from 293/EBNA cells transfected with TRAIL, TRAIL-R4, CD30, or control vector 48 hr after transfection, as described (Yao et al., 1995). Oligonucleotides containing an NF-κB binding site were annealed, radiolabeled with [^{32}P]dATP, and combined with 10 μg of nuclear extracts for 20 min at room temperature. The specificity of the reaction was confirmed by competition with 50-fold molar excess of nonlabeled wild-type oligonucleotides or oligonucleotides containing a mutated NF-κB binding site. The protein–DNA complexes were resolved by 6% polyacrylamide gel electrophoresis in 0.25 × Tris-borate-EDTA buffer and visualized by autoradiography.

Acknowledgments

The authors are grateful to C-P. Huang for sequencing, C. Rauch for purification of LZ-TRAIL, K. Maggiora for transfections, and M. Petersen for purification of soluble Fc proteins.

Received September 12, 1997; revised October 22, 1997.

References

Beg, A.A., and Baltimore, D. (1996). An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science *274*, 782–784.

Boldin, M.P., Mett, I.L., Varfolomeev, E.E., Chumakov, I., Shemer-Avni, Y., Camonis, J.H., and Wallach, D. (1995). Self-association of the "death domains" of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. J. Biol. Chem. *270*, 387–391.

Degli-Esposti, M.A., Smolak, P.J., Walczak, H., Waugh, J., Huang, C.-P., DuBose, R.F., Goodwin, R.G., and Smith, C.A. (1997). Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. J. Exp. Med., *186*, 1165–1170.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. Cell *81*, 495–504.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.L., Schröter, M., Burns, K., Mattmann, C., et al. (1997). Inhibition of death receptor signals by cellular FLIP. Nature *388*, 190–195.

Itoh, N., and Nagata, S. (1993). A novel protein domain required for apoptosis: mutational analysis of human Fas antigen. J. Biol. Chem. *268*, 10932–10937.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. *65*, 55–63.

Nagata, S., and Golstein, P. (1995). The Fas death factor. Science *267*, 1449–1456.

Pan, G., Ni, J., Wei, Y.-F., Yu, G.-L., Gentz, R., and Dixit, V.M. (1997a). An antagonistic decoy receptor and a death domain-containing receptor for TRAIL. Science *277*, 815–818.

Pan, G., O'Rourke, K., Chinnaiyan, A.M., Gentz, R., Ebner, R., Ni, J., and Dixit, V.M. (1997b). The receptor for the cytotoxic ligand TRAIL. Science *276*, 111–113.

Pitti, R.M., Marsters, S.A., Ruppert, S., Donahue, C.J., Moore, A., and Ashkenazi, A. (1996). Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. *271*, 12687–12690.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Press).

Sheridan, J.P., Marsters, S.A., Pitti, R.M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C.L., Baker, K., Wood, W.I., et al. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science *277*, 818–821.

Smith, C.A., Gruss, H.J., Davis, T., Anderson, D., Farrah, T., Baker, E., Sutherland, G.R., Brannan, C., Copeland, N.G., Jenkins, N.A., et al. (1993). CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. Cell *73*, 1349–1360.

Smith, C.A., Farrah, T., and Goodwin, R.G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell *76*, 959–962.

Tartaglia, L.A., Ayres, T.M., Wong, G.H., and Goeddel, D.V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. Cell *74*, 845–853.

Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R., and Verma, I.M. (1996). Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science *274*, 787–789.

van Parijs, L., and Abbas, A.K. (1996). Role of Fas-mediated cell death in the regulation of immune responses. Curr. Opin. Immunol. *8*, 355–361.

Wahl, G.M., Stern, M., and Stark, G.R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA *76*, 3683–3687.

Walczak, H., Degli-Esposti, M.A., Johnson, R.S., Smolak, P.J., Waugh, J.Y., Boiani, N., Timour, M.S., Gerhart, M.J., Smith, C.A., Goodwin, R.G., et al. (1997). TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J. *16*, 5386–5397.

Wiley, S.R., Schooley, K., Smolak, P.J., Din, W.S., Huang, C.-P., Nicholl, J.K., Sutherland, G.R., Davis Smith, T., Rauch, C., Smith, C.A., and Goodwin, R.G. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity *3*, 673–682.

Yao, Z., Fanslow, W.C., Seldin, M.F., Rousseau, A.M., Painter, S.L., Comeau, M.R., Cohen, J.I., and Spriggs, M.K. (1995). Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity *3*, 811–821.

GenBank Accession Numbers

The nucleotide sequences of TRAIL-R4 have been deposited in GenBank under the accession numbers AF021232 and AF021233.