Temperature-sensitive Differential Affinity of TRAIL for Its Receptors

DR5 IS THE HIGHEST AFFINITY RECEPTOR*

TRAIL is a member of the tumor necrosis factor (TNF) family of cytokines which induces apoptotic cell death in a variety of tumor cell lines. It mediates its apoptotic effects through one of two receptors, DR4 and DR5, which are members of the TNF receptor family, and whose cytoplasmic regions contain death domains. In addition, TRAIL also binds to 3 “decoy” receptors, DcR2, a receptor with a truncated death domain, DcR1, a glycosylphosphatidylinositol-anchored receptor, and OPG a secreted protein which is also known to bind to another member of the TNF family, RANKL. However, although apoptosis depends on the expression of one or both of the death domain containing receptors DR4 and/or DR5, resistance to TRAIL-induced apoptosis does not correlate with the expression of the “decoy” receptors. Previously, TRAIL has been described to bind to all its receptors with equivalent high affinities. In the present work, we show, by isothermal titration calorimetry and competitive enzyme-linked immunosorbent assay, that the rank order of affinities of TRAIL for the recombinant soluble forms of its receptors is strongly temperature dependent. Although DR4, DR5, DcR1, and OPG show similar affinities for TRAIL at 4 °C, their rank-ordered affinities are substantially different at 37 °C, with DR5 having the highest affinity (KD = 2 nM) and OPG having the weakest (KD = 400 nM). Preferentially enhanced binding of TRAIL to DR5 was also observed at the cell surface. These results reveal that the rank ordering of affinities for protein-protein interactions in general can be a strong function of temperature, and indicate that sizeable, but hitherto unobserved, TRAIL affinity differences exist at physiological temperature, and should be taken into account in order to understand the complex physiological and/or pathological roles of TRAIL.

TRAIL (1) (systematic name, TNFSF10; formally Apo-2L (2) or TL2(3)), a type II membrane protein, is a recently described member of the TNF family of cytokines which induces apoptotic cell death in a variety of tumorigenic or transformed cell lines but not in normal cells (1). To date, five receptors, all members of the TNFR family, have been shown to bind to TRAIL. Of these, DR4 (4), DR5 (5–10), and DcR2 (11, 12) are membrane-anchored proteins with transmembrane and cytoplasmic domains, where DR4 and DR5 have functional death domains, while DcR2 contains a truncated, “non-functional” death domain. A fourth receptor, DcR1 (5, 6, 8, 13), is glycosylphosphatidylinositol-anchored, which is unique among the TNFR family, and contains no transmembrane or cytoplasmic regions, while the fifth receptor, OPG, is a secreted protein with no known membrane anchor.

Four of the TRAIL receptors, DR4, DR5, DcR1, and DcR2, have all been mapped to a cluster within 8p22-p21 (10, 11, 14). OPG, the least related of the TRAIL receptor genes, by its sequence homology, structure, and ligand recognition patterns, including its high affinity for another ligand, RANKL, has been mapped to the other end of the same chromosome, to 8q24 (3). TRAIL itself has been mapped to 3q26 (1).

TRAIL-induced apoptosis appears to require expression of one or both of its death domain containing receptors DR4 or DR5 (15), and is mediated via a Fas-associated death domain-dependent pathway (7). However, the expression patterns of DR4 and DR5 mRNA had suggested that DR4 and/or DR5 expression is necessary but not sufficient for TRAIL-induced apoptosis (15). The explanation for this latter observation was thought to be due to the expression of the “decoy” TRAIL receptors DcR1 and/or DcR2. Although ectopic expression, through transfection, of either of these two receptors into reporter cell lines (Ref. 5 and 12 for DcR1 and DcR2, respectively) has been shown to be protective against TRAIL-induced cell death, mRNA or protein (16) expression patterns of these two receptors did not support the hypothesis on their anti-apoptotic roles with respect to transformed cell lines, since both sensitive and resistant cell lines have been shown to express DcR1 and/or DcR2 mRNA.

More recent data using mAbs for detection of TRAIL receptors on the cell surface corroborate the earlier mRNA data and show that the expression of the decoy receptors does not correlate with sensitivity or resistance of tumor cell lines to TRAIL-induced cell death (16). Possible explanations which have been

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1 The abbreviations used are: TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; ITC, isothermal titration calorimetry; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DD, death domain.
invoked to account for the discrepancy between the expression patterns of the TRAIL death and decoy receptors and sensitivity to TRAIL-induced cell death include intracellular regulation of caspase activation or the ability of DcR2 to provide intracellular anti-apoptotic signals, possibly through transcriptional regulation of other anti-apoptotic genes (15, 17). However, despite these early indicators, other additional mechanisms are likely, and the protective mechanisms involved in the DR4 and DR5 apoptotic pathways, as well as the role of the decoy receptors in normal physiology or in pathological conditions remain to be elucidated.

One potential mechanism by which apoptotic responses to TRAIL may be regulated is by the expression levels of these receptors. In addition, the possibility that TRAIL may cross-link different receptors to form heteromeric complexes cannot be ruled out. In these cases, the affinity of TRAIL for the different receptors may be important to determine the cellular consequences of ligand binding. DR4, DR5, DcR1, and DcR2 have been shown to exhibit high affinity binding to TRAIL with comparable, subnanomolar KD (12, 13, 15, 18). Similarly high affinity binding for OPG has also been reported (19), making it difficult to account for the known sequence and inferred structural diversity of this set of receptors that are recognized by the same ligand. As has been common practice for these types of studies, all these experiments were conducted at subphysiological temperatures. We have re-examined the affinities of recombinantly expressed DR4, DR5, DcR1, and DcR2 for recombinant TRAIL, using isothermal titration microcalorimetry (ITC) at 37 °C, and by standard competition ELISA at 4 and 37 °C. Here, we report, substantial temperature-sensitive differences in the relative affinities of these receptors for TRAIL which likely have important physiological implications. These findings are discussed in the context of the sequence and structural similarities and differences of these receptors.

MATERIALS AND METHODS

Expression and Purification of Ig Fusion Proteins—The cloning, expression, and purification of the Ig-fusion proteins DR5-Ig, DcR1-Ig, OPG-Ig, and HVEM-Ig has previously been described (19–21). DR4-Ig (amino acids 95–281) (1) was constructed in the pCDN mammalian expression vector. The leader and extracellular domains of the receptors were amplified by sequence analysis, and subsequently subcloned into the pCR2.1TOPO (Invitrogen), confirmed by product was subcloned into pCR2.1TOPO (Invitrogen), confirmed by sequencing, and subsequently subcloned into the EcoRI and Asp718I sites of pFCizOA (Invitrogen). The resulting plasmid, pFCizOA–TRAIL, has sTRAIL expression under the control of the methanol-inducible AOX1 promoter (24) and secretion under the control of the Saccharomyces cerevisiae a-factor signal sequence. This plasmid was transformed with SacI, electroporated into P. pastoris strain KM71 (alpha/Δ-SARG4, his4, arg5, trp1), and transformed according to protocols provided by Invitrogen. Strain MM49 was selected as the highest expressing strain as estimated by immunoblot analysis with a monoclonal antibody specific for the hexa-histidine tag (CLONTECH, Palo Alto, CA). Production was carried out in shaken flasks with a yield of about 25 mg/liter. TRAIL was then purified by capturing the protein-A- and protein-G-fused medium on a Ni-NTA column (Qiagen) equilibrated in PBS, pH 7. The column was washed with 30 ml imidazole (Calbiochem) in PBS and eluted with 300 ml imidazole in PBS. 30 mg of His6 TRAIL was recovered.

ITC of TRAIL and Its Receptors—The solution affinities of TRAIL for various receptors were measured by ITC (26, 27), which detects the extent of binding from the intrinsic binding heat of the interaction. ITC is a quantitative method for measuring binding constants because it enables unmodified, native forms of proteins to be characterized in solution phase. ITC also measures binding at equilibrium and does not involve separation steps to quantitate bound and unbound species. Reactant concentrations were determined from molar extinction coefficients calculated from amino acid sequences (28). Titrations were conducted in 1 ml of PBS, pH 7.4, at 37 °C. Phosphate buffer was chosen by virtue of its small ionic strength and enthalpy change of approximately 1 kcal/mol (26). The observed TRAIL-receptor binding enthalpy enthalpy changes therefore closely approximate the molecular binding enthalpy changes, regardless of whether binding is coupled to changes in protonation. Circular dichroism thermal melting experiments demonstrated that TRAIL and the receptor constructs were stable against thermal unfolding up to at least 50 °C.

ELISA Binding of TRAIL to the Soluble Receptors—ELISAs were performed according to standard techniques. Nunc MaxiSorp Flat-bottomed plates were coated overnight at 4 °C with either DR5-Ig or OPG-Ig (20 ng/well, 100 μl/well) in 0.05 mM carbonate/bicarbonate buffer, pH 9.5–9.6. After washing with PBS-Tween (0.05% Tween 20) four times and blocking with 1% bovine serum albumin in phosphate-buffered saline for 2 h, serial dilutions of TRAIL-Ig were added to the plates. The ELISA plates were washed five times and incubated with alkaline phosphatase-conjugated streptavidin (1:1000, Vector Laboratories, Inc.) for 1 h at 37 or 4 °C. The plates were washed four times with PBS-Tween buffer and incubated with alkaline phosphatase-conjugated streptavidin (1:1000, Vector Laboratories, Inc.) for 1 h at room temperature. The ELISA plates were washed five times and p-nitrophenyl phosphate disodium salt (pNPP Tablets, Pierce, Rockford, IL) (100 μl/well) was added according to the manufacturer’s instructions for 15 min. The absorbance was measured at 405 nm on a microtiter plate reader (MRX, Dynatech Laboratories).

Cell Surface Expression of TRAIL Receptors—The full-length cDNA clones of DR4, DR5, without their death domains (DR5ΔDD residues 1 to 103) were ligated to the signal sequence of tissue plasminogen activator signal sequence upstream of the FLAG-718I sites of pPICZ A (Invitrogen). The resulting plasmid, pPICZ ΔAsp718I (Invitrogen). The resulting plasmid, pPICZ ΔAsp718I, was digested with EcoRI and cloned into modified pcDNA3 vector that allowed in-frame fusion with a Flag epitope tag (8). DcR1 (residues 10 or 30 liters of cell media on a 2.5 1000 cells/well. After blocking the plates were washed three times with PBS and incubated with flow cytometry using biotin-labeled affinity purified goat antibodies to the TRAIL receptor (R&D Systems Inc., Minneapolis, MN) with streptavidin (1:1000, eBioscience) as the secondary staining reagent. Both 0.1 and 1 μg/ml antibodies produced similar results and so only data with 1 μg/ml is reported in this study. The data was analyzed as relative mean fluorescence intensity representing the receptor density of the positive cells (% positive × mean fluorescence intensity of positive cells) as described previously (31).

TRAIL-Biotin Binding to Cell Surface Receptors by Europium Method—Cells were harvested, washed three times with PBS, and plated in presaturated (with PBS) 96-well U-bottom polystyrene plates (Costar, Corning Inc.) at 4 × 105 cells/well. After blocking with PBS + 1% bovine serum albumin, the cells were incubated with 0, 1, and 10 μg/ml TRAIL-biotin in PBS at 37 °C for 1 h. After incubation, the plates were washed three times with PBS and incubated with europium-labeled streptavidin (100 dilution) (DENTLIA, Wallac Oy, Finland).
land) for 30 min at room temperature. The cells were washed three
times with PBS and incubated with 100 μl of enhancement solution
(DELFIA, Wallac Oy, Finland) according to the manufacturer’s instruc-
tion. A parallel experiment with alkaline phosphatase-labeled strepta-
vidin, instead of europium, produced similar results.

RESULTS AND DISCUSSION

Biochemical Characterization of Recombinant TRAIL Receptor-Ig Fusion Proteins—
In the present investigations, we used
the CHO expression system to produce the soluble TRAIL receptors DR5, DR4, DeR1, and OPG as Ig-fusion proteins (19, 20). The purified proteins were characterized by SDS-PAGE (Fig. 1), analytical size exclusion chromatography, matrix-assisted laser desorption ionization-mass spectrometry, and N-
terminal sequence analysis. The key properties are summa-
rized in Table I. The heterogeneous appearance of SDS-PAGE bands and the disparity between the observed molecular weights and those calculated from the amino acid sequences (Table I) are indicative of glycoforms of the expressed proteins.

N-terminal sequencing gave single sequences for DR5-Ig, DR4-Ig, DeR1-Ig, and OPG-Ig, thus confirming the identity and high purity of the recombinant proteins. The N termini for DR5, DR4, and DeR1 aligned well with each other but less well with OPG. In conjunction with the overall identity for these proteins, where DR5 shows 59, 58, and 52% identity within the first 126 amino acids of the extracellular domains of DR5, DeR1, and DeR2, respectively, this data provides further evidence for the closer evolutionary relationship of these 4 TRAIL receptors. In contrast, OPG, which has a different N terminus and shows only 26% identity to DR5 within this region (see Table II), appears to have a more distant relationship to the other TRAIL receptors.

Biochemical Characterization of Recombinant TRAIL from
P. pastoris—N-terminal sequencing of purified TRAIL con-
firmed the predominant presence of His6-tagged TRAIL, al-
though two minor species (about 15%) with truncations within the His6 tag were also present in the purified material. The purity of TRAIL expressed in Pichia was superior to that pro-
duced using the CHO expression system (19), as determined by
SDS-PAGE and N-terminal sequence analyses. The purified
TRAIL from P. pastoris was found to be a stable trimer, down
to at least micromolar concentrations, by analytical ultracen-
trifugation, as was also shown by crystallography of Esche-
richia coli produced TRAIL (32, 33).

DR5 Shows Highest Reactivity for TRAIL in Competition
ELISAs—Biotinylated TRAIL binds, in a concentration de-
pendent manner, to DR5-Ig immobilized on microtiter plates
(Fig. 2A). Binding was more efficient at 37 °C than at 4 or 25 °C
(Fig. 2B). Immobilized OPG-Ig also bound to biotinylated
TRAIL but, under the same conditions, OPG-Ig was less effi-
cient than DR5-Ig.

DR5-Ig in solution was very efficient at competing with
TRAIL binding to immobilized DR5-Ig or OPG-Ig in competi-
tive ELISAs, both at 4 and 37 °C (Fig. 3). In contrast, OPG-Ig
was unable to compete with TRAIL binding to immobilized
DR5-Ig or OPG-Ig at 37 °C, but was able to compete at 4 °C,
although less efficiently than DR5-Ig (Fig. 3). This indicates
that at 37 °C, OPG-Ig has a weaker affinity for TRAIL than
does DR5-Ig. DeR1-Ig and DR4-Ig partially blocked TRAIL
binding to immobilized DR5-Ig, and required higher solution
concentrations when compared with inhibition by DR5-Ig (Fig.
4). However, they were almost equivalent to DR5-Ig at blocking
TRAIL binding to immobilized OPG-Ig, suggesting that
DeR1-Ig and DR4-Ig have intermediate affinities for TRAIL
when compared with the high affinity of DR5-Ig and low affin-
ity of OPG-Ig.

DR5 Is a High Affinity Receptor for TRAIL—The TRAIL
affinities of the receptor-Ig constructs in solution were meas-
ured directly at 37 °C by isothermal titration calorimetry (26).
Fig. 5 shows an example of titration calorimetry data for the
titration of TRAIL with DR4-Ig. The equilibrium $K_D$ of 70 nM
measured from the data in Fig. 5 is much weaker than values
measured previously at low temperatures (12, 13, 15, 18, 19).
However, the data in Fig. 5 also reveal that the binding enthalpy change for DR4-Ig is very large in comparison to typical
protein-protein interactions (34). Consequently, the binding affinity is a strong function of temperature, according to the
Gibbs-Helmholz relationship which is described elsewhere (35).

TRAIL binding experiments were also conducted with DR5-Ig,
DeR1-Ig, and OPG-Ig (Fig. 6) from which the $K_D$ and binding
enthalpy change values were determined (Table II). Of the four
DR5 Highest Affinity TRAIL Receptor

Summary of the biochemical characterization of CHO-expressed TRAIL receptors

| Receptor | TNF nomenclature | Aliases | N terminus | MW of Ig-fusion protein by MALDI-MS | Predicted MW of unglycosylated dimer |
|----------|------------------|---------|------------|------------------------------------|-------------------------------------|
| DR5      | TNFRSF10B        | KILLER, TRICK2A, TRAIL-R2, TRICKB | ALITQQDLAP | 111                                | 83,452                              |
| DR4      | TNFRSF10A        | Apo2, TRAIL-R1 | ATIKLHDSI | 91                                 | 82,334                              |
| DcR1     | TNFRSF10C        | TRAIL-R3, LIT, TRID | ATTARQEVEVP | ND*                               | 99,126                              |
| DcR2     | TNFRSF10D        | TRUNDD, TRAIL-R4 | ND        | ND                                 | NA                                  |
| OPG      | TNFRSF11B        | OCIP, TR1  | ETPFPKLHY | 161                                | 141,042                             |

* ND, not done, or for DcR1, no signal detected by MALDI-MS.

Affinities of TRAIL receptors determined by isothermal titration calorimetry

There was no detectable binding of HVEM-Ig, TNFRII-Ig, or LTβR-Ig to TRAIL in ELISAs and/or by surface plasmon resonance (BIAcore™) at up to 1 μM TRAIL (data not shown).

| Receptor | Known ligands | Identity of extracellular domains (CRDs) to DR5* | Apparent enthalpy change | Observed Kd for TRAIL in nM |
|----------|---------------|-----------------------------------------------|--------------------------|----------------------------|
| DR5      | TRAIL         | NA                                            | −35                      | ≤2b                       |
| DR4      | TRAIL         | 74/126 (59%)                                  | −88                      | 70                        |
| DcR1     | TRAIL         | 73/126 (58%)                                  | −25                      | 200                       |
| DcR2     | TRAIL         | 65/126 (52%)                                  | ND                       | ND                        |
| OPG      | RANK, TRAIL   | 33/126 (26%)                                  | −96                      | 400                       |

* Numbers represent identities between DR5 and the respective receptor over the 1st 126 N-terminal residues. CRDs stands for “cysteine-rich domains” of the TNFR motif.

b Value is at most 2 nM but may be tighter and cannot be determined with certainty by the ITC method for higher affinity interactions.

TRAIL-biotin Binding to Immobilized DR5-Ig

A

B

FIG. 2. Binding of biotinylated TRAIL to microtiter plate immobilized DR5-Ig. A, DR5-Ig was immobilized at different concentrations and TRAIL-biotin was titrated in for binding, as described under “Materials and Methods.” B, comparison of TRAIL binding to immobilized DR5-Ig (20 ng/well) at different temperatures.

TRAIL receptors evaluated, DR5-Ig very distinctly has the highest affinity at 37 °C, as can be judged by the steep transition of the binding data in Fig. 6. The TRAIL Kd of DR5-Ig is at least as tight as 2 nM at 37 °C, and is more than 30-, 100-, and 200-fold tighter than DR4-Ig, DcR1-Ig, and OPG-Ig, respectively. The observed Kd (2 nM) is very close to the instrumental lower limit and the true affinity could be tighter. Similar high affinity binding of DR5-Ig was also found for TRAIL expressed in CHO cells (Kd ≤ 1 nM), indicating that the high affinity binding to DR5-Ig is not unique to P. pastoris expressed TRAIL, and that TRAIL expressed in mammalian and yeast expression systems retains the appropriate folding for receptor recognition.

The temperature dependence of binding affinities for protein-protein interactions can be highly variable. The magnitude and direction of the change in affinity with temperature is governed by the binding enthalpy change (35). A key strength of isothermal titration calorimetry is that it measures directly the binding enthalpy change, along with the binding affinity, at a given temperature. In the course of measuring the TRAIL affinities of the various receptors we found that their binding enthalpy changes were considerably variable. Observed binding enthalpy changes ranged from −35 to −96 kcal/mol (Table II), reflecting 61 kilocalories difference in binding enthalpy. The affinities of some of these interactions are therefore much stronger functions of temperature than others. Consequently, the rank order of TRAIL affinities of the receptors at low temperatures (4 or 25 °C) is very different than the rank order at 37 °C. While all the receptors have similar TRAIL affinities at 25 °C, the affinities of DR4-Ig and OPG-Ig are strong functions of temperature, and they are greatly weakened at 37 °C. The affinity of DcR1-Ig is only moderately weaker at 37 °C, and the affinity of DR5-Ig exhibits the shallowest temperature dependence.

Preferentially Enhanced Binding of TRAIL to Cell Surface DR5—To determine whether the high affinity binding of TRAIL seen with the recombinantly expressed soluble DR5 also translates to enhanced binding on the cell surface, full-length DR5, DR4, DcR1, or DcR2 were transfected for cell surface expression on HEK293 cells. In the case of the death receptors DR5 and DR4, to prevent triggering of downstream apoptotic events upon binding of TRAIL, death domain truncated versions of DR5 (DR5ΔDD) and DR4 (DR4ΔDD) were also transfected into these cells. OPG was not included in these experiments because it is a naturally secreted protein lacking a member anchor. In these experiments, Fas was used as a control for transfection and TRAIL binding. The level of surface expression of the receptors was evaluated by flow cytometry using antibodies specific for each receptor. The transfections resulted in very similar levels of expression of the various receptors. Roughly 50% (ranges from 47 to 51%) of all the transfectants were positive for the relevant transfected receptor with broad intensity of staining for each receptor ranging over 3 orders of magnitude on the intensity scale. The level of expression for each receptor (represented in Fig. 7A as relative mean intensity) was comparable for each transfected receptor. Similarly comparable expression levels were also observed by Western blot analysis.

TRAIL binding experiments were conducted at 37 °C, using biotinylated TRAIL as described under “Materials and Methods.” As is shown in Fig. 7B, TRAIL showed significantly
higher binding to the DR5 (DR5ΔDD) than to the DR4 (DR4ΔDD), DcR1, or DcR2 transfectants. Similar results were also obtained with the full-length (death domain untruncated) DR5 and DR4 (not shown). This assay has sufficient sensitivity to distinguish between the large (>70-fold) affinity differences observed in the ITC determinations between DR5 and the other TRAIL receptors (DR4 and DcR2), but not the smaller (2–5-fold) differences observed (or expected in the case of DcR2) among the lower affinity receptors. No binding of TRAIL was observed in the Fas-transfected cells. Thus, this data confirms that similar rank order of affinities govern the binding of TRAIL to its receptors on the cell surface.

Two groups have recently described the crystal structure of the TRAIL-DR5 complex (36, 37). As had been predicted from the known TNF-β-TNFR-I complex, the trimeric TRAIL binds three DR5 molecules, one each in the cleft formed at the interface of the TRAIL subunits. DR5 has one truncated, and two full-length pseudorepeats of cysteine-rich domains (CRD1, CRD2, and CRD3, respectively, with CRD3 as the membrane proximal domain). In its interaction with TRAIL, CRD2 and CRD3 straddle the TRAIL interface thus forming two major contact surfaces whereby patch A within CRD3 primarily contributes to the specificity of the interaction with TRAIL, while patch B within CRD2 contributes to more general hydrophobic
interactions (36). Alignment of the TRAIL receptors shows, with the exception of the less related OPG, there is a high degree of sequence conservation within the region represented by patch A, which drives the specificity of the interactions with TRAIL. Thus, these receptors have, for the most part, retained identical key contact residues or have conservative or semiconservative substitutions in this region. Although there are some differences within this region among the 4 closely related receptors, interestingly, the region represented by patch B within CRD2 displays the most divergence between DR5 on one side and DR4, DcR1, and DcR2 on the other. Within the putative TRAIL recognition regions, DR4 and DcR1 are more related to each other than either one is to DR5. Overall within the extracellular region of these receptors, DcR1 and DcR2 are very closely related within 93 identities within the first 126 residues, compared with 65/126 and 73/126 for DcR1 and DcR2, respectively, and DR5. Thus, based on sequence identity or similarity, DcR1, DcR2, and DR4 would be predicted to have closely related recognition elements in binding to TRAIL. Therefore, although precise affinity determination by the ITC method was not conducted for TRAIL binding to DcR2, based on the cell binding experiments, and the sequence identities and presumed structural similarities with the lower affinity receptors, DcR2 would be predicted to be closer to DcR1 and DR4 than to DR5.

In addition to affinity differences among the TRAIL receptors discussed in this report, other mechanisms may contribute to the functional differences of the receptors. These include their unique expression patterns in different tissues and cells types, receptor regulation in response to activation or cell differentiation, the relative receptor densities at the cell surface, ability to recognize other ligands (as is the case for OPG which binds to RANKL with high affinity, and which may also be the case for the other TRAIL receptors, with the possibility that there may be other unidentified ligands), and the important differences in their cytoplasmic domains which contribute to their ability or inability to couple to different signaling pathways. The relative contribution of these factors remains to be elucidated.

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