Characterization of two receptors for TRAIL

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Abstract Two receptors for TRAIL, designated TRAIL-R2 and TRAIL-R3, have been identified. Both are members of the tumor necrosis factor receptor family. TRAIL-R2 is structurally similar to the death-domain-containing receptor TRAIL-R1 (DR-4), and is capable of inducing apoptosis. In contrast, TRAIL-R3 does not promote cell death. TRAIL-R3 is highly glycosylated and is membrane bound via a putative phosphatidylinositol anchor. The extended structure of TRAIL-R3 is due to the presence of multiple threonine-, alanine-, proline- and glutamine-rich repeats (TAPE repeats). TRAIL-R2 shows a broad tissue distribution, whereas the expression of TRAIL-R3 is restricted to peripheral blood lymphocytes (PBLs) and skeletal muscle. All three TRAIL receptors bind TRAIL with similar affinity, suggesting a complex regulation of TRAIL-mediated signals.

Key words: Apoptosis; Death receptor; TRAIL; Tumor necrosis factor

1. Introduction

Members of the tumor necrosis factor (TNF) receptor family are type I membrane molecules containing multiple cysteine-rich repeats in their extracellular domain. The cytoplasmic portions generally lack sequence homology with the exception of a motif called the 'death domain' (DD) which is found in four of the family members: Fas [1,2], TNF-R1 [3], TRAMP (wsl/Apo-3/DR-3) [4–7] and TRAIL-R (DR-4) [8]. These four receptors efficiently transmit death signals through DD-containing adaptor proteins. TRAIL receptor (TRAIL-R) is the most recently identified death receptor [8]. Its ligand TRAIL shows a broad tissue distribution [9] and, like FasL, induces rapid apoptosis of various transformed TRAIL-R-positive cell lines [9–11]. We and others have recently characterized a family of viral and mammalian proteins, called FLIPs, which interfere with death receptor signaling pathways by binding to FADD and FLICE [12–18]. FLIPs block TRAIL-R-mediated death signals even though it has been reported that TRAIL-R transmit signals independently of FADD [8,11]. We therefore considered the possibility that an as yet unknown alternative TRAIL receptor exists that signals through the FADD-FLICE pathway. Here we report on the identification and the structural and functional characterization of two novel receptors for TRAIL.

2. Materials and methods

2.1. Reagents and cell lines

A Flag-tagged version of the extracellular domain (residues 139–281) of human Fas:Fc and human TNFR1:Fc was produced in 293 HEK cells [19]. Flag-tagged recombinant soluble human TRAIL (residues Thr^{95}–Gly^{281}) [13] and Flag-tagged human TNFα (residues Ser^{85}–Leu^{233}) were produced in bacteria.

2.2. Northern blot analysis

Northern blot analysis was performed using Human Multiple Tissue Northern Blots I and II (Clontech #7760-1 and #7759-1). The membranes were incubated in ExpressHyb hybridization solution (Clontech #8015-1) for at least 1 h at 62°C. The random-primed cDNA probes (Boehringer Mannheim) were synthesized using the extracellular domains of TRAIL-R1 and TRAIL-R3 and the intracellular domain of TRAIL-R2 as template. The heat-denatured cDNA probe was added at 1.5×10^6 cpm/ml in fresh ExpressHyb. The membrane was hybridized for 12–24 h at 62°C, washed three times in 2×SSC containing 0.05% SDS and exposed at −70°C.

2.3. Cloning of TRAIL receptor cDNA

Several EST clones that potentially encoded novel TRAIL receptors were identified in the dbEST data base at the National Center for Biotechnology Information by performing a data base search using the generalized profile method [20] based on the sequence homology to the N-terminal domain of TRAIL-R1. Two clones from a cDNA library of the Ntera-2 neuroepithelial cell line encoded TRAIL-R2 (GenBank accession numbers: AA223440 and AA223122). Two clones from a cDNA library of human pregnant uterus (GenBank accession numbers: AA031883 and AA150849) and one clone from a cDNA library of human fetal liver and spleen (GenBank accession number: T71406) encoded TRAIL-R3. The inserts were sequenced by conventional methods, and the resulting sequences were aligned.

The 4 kbp long insert of clone AA223440 had a 106 bp deletion (bases 146–251, the A of the ATG being base number 1) when compared to clone AA223122. This deletion resulted in both a frame shift and a loss of homology with TRAIL-R1 and was obviously not the result of alternative splicing. As we were unable to obtain the expected sequence from clone AA223122 supplied by two different companies, we generated a complete cDNA coding for TRAIL-R2 using a double PCR approach with primers designed on the sequence of clone AA223122. In a first round of the PCR, two products were produced with clone AA223440 as template using (a) the forward oligonucleotide 5′-AAGCTTGCCACCATGGAACAACGGGGACAGAACGCCCCG-3′ and primer 5′-GGCCCCACAACAAAAGAGGTCCAGCCCCTCAGAGGGATTGTGTCCACCTGGACACCATATCTC-3′, and (b) the reverse oligonucleotide 5′-TTAGGACATGGCAGAGTCTGCATTACC-3′ and primer 5′-CTTTTGTTGTGGGGCCACTCTCTGCTGGGGAGCTAGGTCTTGTTGGGTGATCAGAGCAGACTCAGCTGAGACCAACAGCAGGACCG-3′. These purified PCR products, containing the 5′ and 3′ portions of TRAIL-R2, respectively, were then mixed and allowed to undergo three cycles of PCR before amplification with the forward and reverse oligonucleotides described above.

2.4. TRAIL receptor expression vectors

Full length TRAIL-R1 (aa 1–465), TRAIL-R2 (aa 1–439) and

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The accession numbers (BankIt) for the two TRAIL receptor cDNA sequences reported in this paper are 130586 (TRAIL-R2) and 130595 (TRAIL-R3).

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TRAIL-R3 (aa 1–259) and their respective extracellular domains (TRAIL-R1, aa 1–239; TRAIL-R2, aa 1–211; and TRAIL-R3, aa 1–240) were amplified by PCR with primers containing suitable restriction sites and 5′ Kozak consensus sequence [21]. An EST clone (GenBank accession number: AA100865) was used as a template for TRAIL-R1 amplification. PCR products were cloned into pCR0-blunt (InVitroGen), then subcloned into the pCRIII mammalian expression vector (InVitroGen) as *Hin*dIII/*Xho*I fragments for full length receptors or, for extracellular domains, as *Hin*dIII/*Sal*I fragments in frame with a *Sal*I/*Not*I cDNA cassette encoding the hinge, CH2 and CH3 domains (aa residues 231–447) of human IgG1 [22]. TRAIL-R3 (aa 25–240) was amplified by PCR and cloned into the *Pst*I and *Eco*RI sites of the pCRIII-derived vector pHAFlag [19], in frame with the signal peptide of hemagglutinin and a Flag epitope. The resulting plasmid, pHAFlag-TRAIL-R3, was digested with *Sma*I and religated to yield pHAFlag-TRAIL-R3Δ4 which lacks four out of the five threonine-, alanine-, proline- and glutamine-rich (TAPE) repeats.

2.5. *Identification of N- and O-linked glycosylation using DIG-labeled lectins*

Lectin binding to TRAIL-R3 was performed using the DIG Glycan Differentiation Kit (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, purified TRAIL-R3 (about 1 μg protein for peanut agglutinin and 0.1 μg for other lectins) was subjected to SDS-PAGE and Western blotting, together with 1 μg of the proper control glycoprotein. After blocking, the lectins were allowed to bind for 1 h at room temperature in 10 mM Tris-HCl pH 7.5, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. After incubation with anti-DIG alkaline phosphatase conjugate, bound lectins were revealed using 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium chloride.

2.6. *Enzymatic treatments of TRAIL-R3*

Peptide *N*-glycanase F: Flag-TRAIL-R3 was heated in 20 μl of 0.5% SDS, 1% 2-mercaptoethanol for 3 min at 95°C. Samples were cooled and supplemented with 10% Nonidet P-40 (2 μl) and 0.5 M sodium phosphate pH 7.5 (2 μl). Peptide *N*-glycanase F (125 U/μl, 1 μl) was added (or omitted in controls), and samples were incubated for 3 h at 37°C prior to analysis by Western blotting.

2.7. *In vitro translation*

Plasmids containing the cDNAs coding for TRAIL receptors were

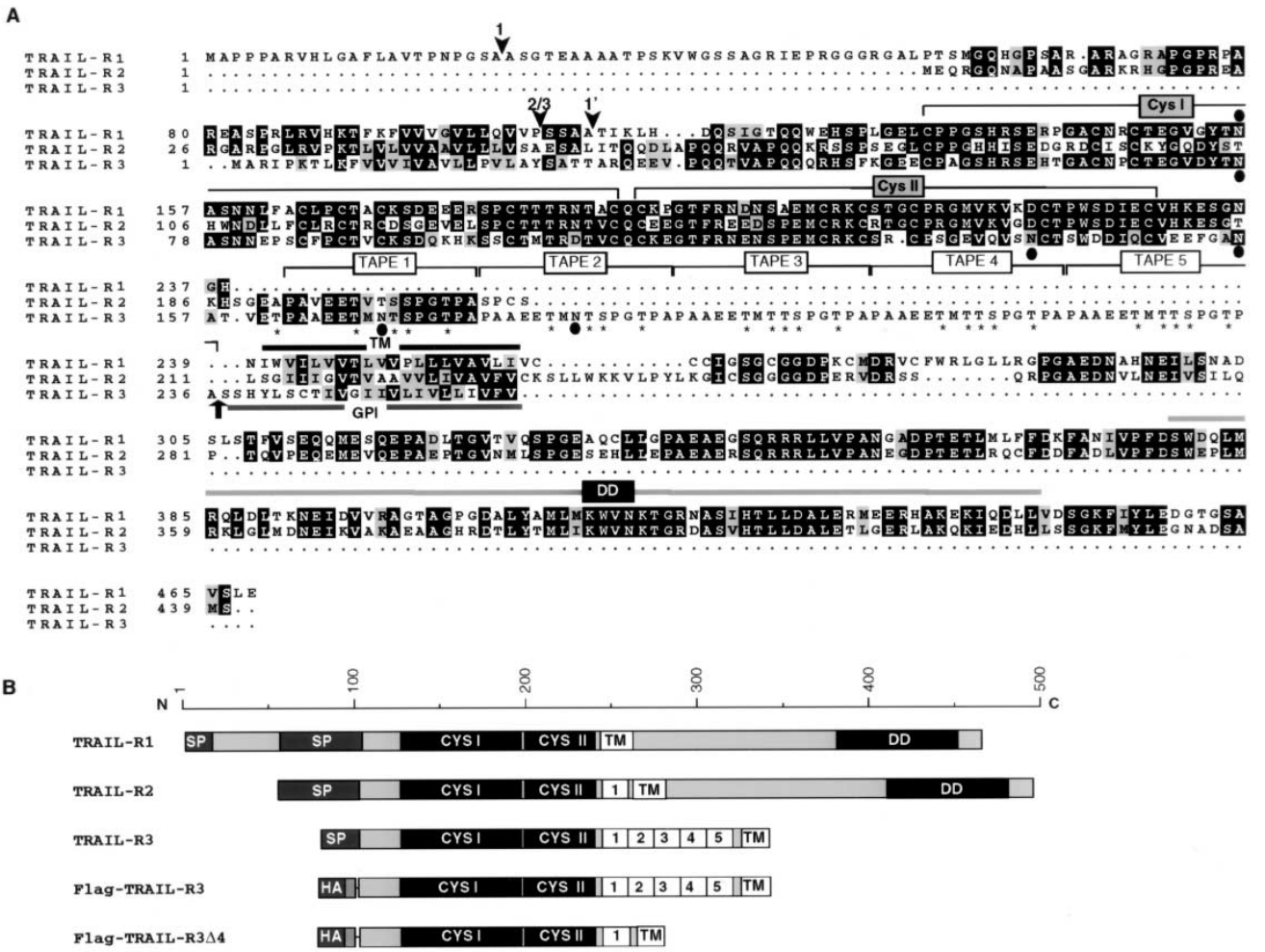

Fig. 1. Predicted amino acid sequence of TRAIL-R2 and TRAIL-R3 and their homology with TRAIL-R1 (DR-4). A: Amino acid sequence alignment of human TRAIL-R1 (DR-4), TRAIL-R2, and TRAIL-R3. Sequence numbering starts at the predicted signal peptide, the predicted start of the respective mature proteins is indicated by arrowheads (1 and 1′ for TRAIL-R1, 2/3 for TRAIL-R2 and -R3). The cysteine-rich domains (Cys I–II), the transmembrane segment (TM) and the death domain (DD) are indicated. The membrane proximal extracellular part of TRAIL-R2 and -R3 contains a highly conserved stretch of 15 aa (TAPE repeat) which is present once in TRAIL-R2 (TAPE 1) and five times in TRAIL-R3 (TAPE 1–5). The potential attachment site for a glycolipid anchor on TRAIL-R3 (Ala^{236}) is indicated with an arrow, and the putative carboxy-terminal signal sequence of the glycosyl phosphatidylinositol (GPI) addition is underlined. Potential *N*-glycosylation sites (one in TRAIL-R1, none in TRAIL-R2 and five in TRAIL-R3) and *O*-glycosylation sites present in the TAPE region of TRAIL-R3 are indicated by dots and asterisks, respectively. Identical and similar residues are represented in black and shaded boxes, respectively. B: Schematic representation of the TRAIL receptors and the Flag-tagged expression constructs of TRAIL-R3 (Flag-TRAIL-R3) and TRAIL-R3 lacking four TAPE repeats (Flag-TRAIL-R3Δ4). SP, predicted signal peptides; HA, hemagglutinin signal peptide.

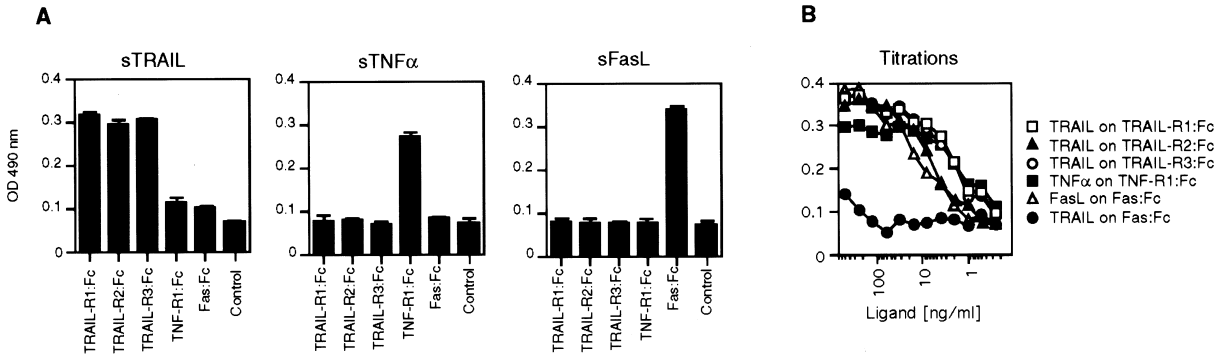

Fig. 2. TRAIL-R2 and TRAIL-R3 selectively bind TRAIL. A: Wells of microtiter ELISA plates were coated with fusion proteins of the indicated TNF receptor family members with human IgG Fc (rec:Fc), and binding of the Flag-tagged ligands detected with anti-Flag antibodies. In the control panel, no receptor was adsorbed onto the plate. B: As in A, but binding of the TNF ligand family members to the respective receptor:Fc constructs was tested at the ligand concentrations indicated in the figure.

in vitro translated using a coupled rabbit reticulocyte lysate system (TNT, Promega, USA) according to the manufacturer's instructions. Reaction volumes were 10 μl and contained 0.4 μg cDNA, 0.2 μl T7 TNT polymerase, 4 U RNAsin (Boehringer) and 5 μCi [^{35}S]methionine/cysteine. The reaction mixture was incubated at 30°C for 90 min and 10% of the reaction was subjected to SDS-PAGE under reducing conditions.

2.8. Ligand binding assay

Ligand binding assays were performed as previously described [19]. In brief, 96 well ELISA plates were coated with the receptor-Fc constructs (1 μg/ml in PBS, 100 μl, 2–16 h, 37°C). After saturation in blocking buffer (PBS containing 5% fetal calf serum, 1 h, 37°C) and three washes, Flag-tagged ligands were added (1 μg/ml in PBS containing 0.5% fetal calf serum, 100 μl, 1 h, 37°C, when not stated otherwise). Bound ligands were revealed with anti-Flag antibody (1 μg/ml in blocking buffer, 100 μl, 30 min, 37°C), rabbit anti-mouse IgG coupled to peroxidase (1/1000 dilution in blocking buffer, 100 μl, 30 min, 37°C) and *o*-phenylenediamine hydrochloride (0.3 mg/ml in 50 mM citric acid, 100 mM Na_2HPO_4, 0.01% H_2O_2). Absorbance was taken at 490 nm with an ELISA reader.

3. Results

3.1. Identification, expression and chromosomal localization of TRAIL-R2 and TRAIL-R3

When the dbEST data base was searched using an improved profile search [20] based on an optimal sequence alignment of TRAIL-R1 with all TNF receptor family members, two sets of candidate cDNA clones were found showing high sequence homology with the published sequence of the TRAIL-R (DR-4, which we call TRAIL-R1 for the sake of clarity) [8]. One set of cDNA clones predicted a membrane receptor encoding a polypeptide of 440 aa with an overall structural organization similar to TRAIL-R1 (51% amino acid sequence identity). This receptor was named TRAIL-R2 (see below). TRAIL-R2 contains two cysteine-rich repeat units in its extracellular region, followed by a predicted hydrophobic transmembrane segment and a 209 aa long cytoplasmic tail containing a typical 'death domain' (Fig. 1). The second set of cDNA clones predicted a protein containing 259 aa. This short receptor (named TRAIL-R3) shows high sequence homology to both TRAIL-R1 and TRAIL-R2 in the extracellular domain (60% and 52% amino acid sequence identity, respectively), but ends immediately after a short predicted transmembrane domain (Fig. 1). Moreover, TRAIL-R3 contains a characteristic motif of 15 aa, rich in Thr, Ala, Pro and Glu residues, before the predicted transmembrane domain, which is repeated five times within the protein (TAPE repeats). The five TAPE repeats present in TRAIL-R3 are remarkably conserved diverging in only one out of the 15 aa positions. A single TAPE repeat is also present in TRAIL-R2, but not in TRAIL-R1, at a similar location (Fig. 1). Moreover, the cDNA sequence of TRAIL-R3 predicts a carboxy-terminal stretch of 15 hydrophobic amino acid residues which is reminiscent of a signal for the addition of a glycosyl-phosphatidylinositol (GPI) anchor [23]. In a GPI-linked protein, the COOH-terminal portion of the nascent protein is cleaved off and exchanged for a preformed GPI anchor by the action of a GPI transamidase. A functional signal for GPI addition fulfils several sequence requirements [23]: first, the GPI ac-

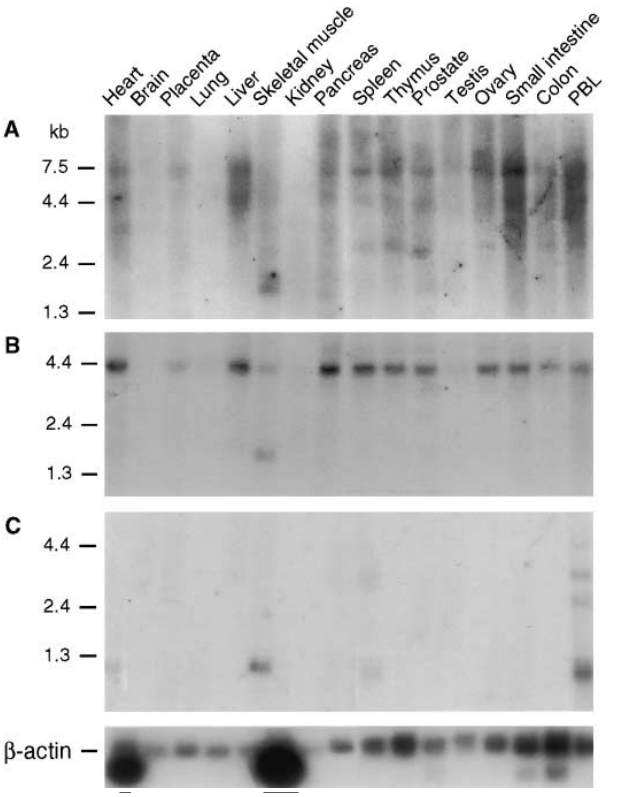

Fig. 3. Tissue distribution of TRAIL receptors. Northern blot analysis of poly(A$^+$) RNA (2 μg per lane) of various human tissues using probes for TRAIL-R1 (DR-4) (A), TRAIL-R2 (B) and TRAIL-R3 (C). The blots were subsequently reprobed with β-actin cDNA.

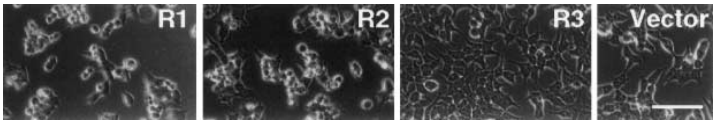

Fig. 4. Overexpression of TRAIL-R1 and TRAIL-R2, but not of TRAIL-R3 in 293T cells results in apoptosis. A: Morphology of 293T cells transiently overexpressing TRAIL-R1, -R2 or -R3. Cells were transfected with the indicated full length receptors and analyzed 32 h later by phase contrast microscopy. The control shows 293T cells transfected with the empty vector. The bar corresponds to 50 μm.

ceptor amino acid residue (at the ω site) and the amino acid residue at position ω+2 are small; second, the amino acid residue at position ω+1 is different from Trp or Pro; and third, the presence of a hinge region located between position ω+3 and approximately ω+8, followed by a stretch of approximately 8–20 highly hydrophobic amino acids, is observed. According to this rule, Ala^{236} would be the predicted attachment site for a GPI anchor in TRAIL-R3 (Fig. 1).

The high sequence homology of the two novel receptors to TRAIL-R1 suggested that they probably interact with TRAIL. TRAIL-receptor:immunoglobulin Fc fusion proteins were constructed and their binding to soluble TRAIL (sTRAIL) was compared using an ELISA-based assay [7]. Each of the receptors interacted with TRAIL, whereas no binding was observed with TNFα and sFasL (Fig. 2A). Signal intensities were comparable suggesting that the three TRAIL receptors bind TRAIL with similar affinities (Fig. 2B).

3.2. Expression and chromosomal localization of TRAIL-R2 and TRAIL-R3

The expression pattern of TRAIL-R2 and -R3 in various tissues was examined by Northern blot analysis (Fig. 3). TRAIL-R1 [8] and TRAIL-R2 possessed a similar expression pattern and their respective mRNAs were detected in many tissues. While the TRAIL-R1 probe detected multiple transcripts (2.6 kbp, 4.6 kbp, and 7.2 kbp) [8], only one TRAIL-R2 transcript (4.4 kbp) was detected by Northern blotting (Fig. 3B). In contrast to TRAIL-R1 and -R2, the expression of TRAIL-R3 (mRNA size, 1.2 kbp) was restricted to skeletal muscle, peripheral blood lymphocytes, and the spleen.

For the determination of the chromosomal localization of the TRAIL-R, we took advantage of the known linkage of the EST clones to two sequence tagged sites (STS) [24]. The STS for TRAIL-R1 (STS: SHGC-9963) and TRAIL-R2 (STS:

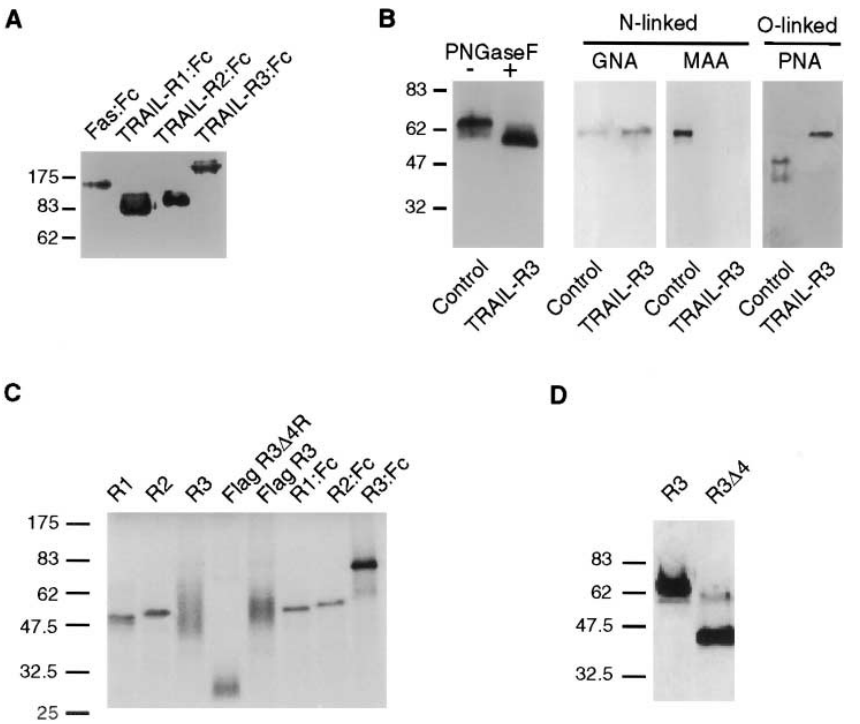

Fig. 5. TRAIL-R3: Glycosylation and the TAPE repeats. A: Western blot analysis under non-reducing conditions of the extracellular domains of Fas and TRAIL-R1, -R2 and -R3 in fusion with IgG Fc (Fas:Fc, R1:Fc, R2:Fc, R3:Fc). Protein A conjugated to peroxidase was used to reveal the blot. B: Western blot analysis of Flag-TRAIL-R3 expressed in 293T cells. The anti-Flag blot on the left hand side reveals a M_r of 65 kDa for the mature protein which decreases to 57 kDa upon the removal of *N*-linked carbohydrates by peptide *N*-glycanase F (PNGase F). Western blot showing Flag-TRAIL-R3 revealed with the lectins *Galanthus nivalis* agglutinin (GNA, detecting terminal α-Man residues), *Maackia amurensis* agglutinin (MAA, detecting sialylated oligosaccharides) and peanut agglutinin (PNA, detecting *O*-linked core disaccharide) are shown in the other panels. C: SDS-PAGE analysis under reducing conditions of in vitro translation products of the TRAIL receptors (R1, R2, R3), of the Flag-tagged TRAIL-R3 with (Flag R3) or without the last four TAPE repeats (Flag-R3Δ4), and of the extracellular domain of the receptors fused to IgG Fc (R1:Fc, R2:Fc, R3:Fc). The theoretically predicted loss of M_r between Flag-R3 and Flag-R3Δ4 is 5.8 kDa. D: Western blot analysis of cellular Flag-TRAIL-R3 (R3) and Flag-TRAIL-R3Δ4 (R3Δ4) produced in 293T cells. Samples were prepared in SDS-PAGE sample buffer containig reducing agent (DTT). The blot was probed with anti-Flag antibodies.

WI-11701) both mapped to human chromosome 8, 44 cM from the top of the linkage map 8p12–21, suggesting that the receptors are descendants of a common ancestral gene.

3.3. TRAIL-R1 and TRAIL-R2 signal apoptosis

The presence of a death domain in the cytoplasmic region of TRAIL-R2 suggested that activation of this receptor could result in the activation of a death signaling pathway. Experiments with other death receptors have revealed that cell death can be induced in the absence of the respective ligand through the overexpression of the receptors only [7]. 293T cells transfected with TRAIL-R1 or TRAIL-R2, examined 32 h after transfection, had rounded up and had undergone morphological changes typical of apoptosis (Fig. 4). In contrast, overexpression of TRAIL-R3 did not induce cytotoxic effects.

3.4. Structure of TRAIL-R3

While measuring the TRAIL-receptor:Fc-TRAIL interaction (see Fig. 2), we found that the TRAIL-R3 fusion protein had an unexpectedly high apparent molecular mass when overexpressed in 293T cells (Fig. 5A). A theoretical M_r value of approximately 100 kDa was expected. However, only TRAIL-R1 and -R2 showed this expected value, whereas the M_r of the TRAIL-R3:Fc was >180 kDa (Fig. 5A). Likewise, the expressed full length TRAIL-R3 exhibited an apparent M_r of 65 kDa under denaturing and reducing conditions instead of the theoretical M_r value of 24.5 kDa (Fig. 5C), suggesting the presence of extensive posttranslational modifications and/or unusual structural motifs.

This observation prompted us to analyze the carbohydrate modifications in TRAIL-R3. Upon incubation with peptide *N*-glycanase F, the M_r decreased by approximately 8 kDa (Fig. 5B), indicating that most of the five potential *N*-glycosylation sites (see Fig. 1) were glycosylated. These *N*-linked oligosaccharides are most likely of the high-mannose type since TRAIL-R3 reacted with *Galanthus nivalis* agglutinin which recognizes terminal mannose residues in α(1–3), α(1–6) or α(1–2) glycosidic linkages, and not with *Sambucus nigra* or *Maackia amurensis* agglutinins that recognize sialic acids in α(2–6) and α(2–3) glycosidic linkage to galactose (Fig. 5B, and data not shown). TRAIL-R3 also reacted with peanut agglutinin, a lectin that specifically binds to the unsialylated Galβ1–3GalNAc disaccharide core of *O*-linked carbohydrates (Fig. 5B), indicating the presence of *O*-linked carbohydrates. This is in concordance with the sequence of TRAIL-R3 which reveals more than 20 potential *O*-linked glycosylation sites [25] in the TAPE repeat region only (see Fig. 1).

However, the contribution of these posttranslational modifications on the M_r of TRAIL-R3 only partially explained its high apparent M_r. When the complete TRAIL receptors, or their extracellular domains of the TRAIL receptors fused to IgG Fc, were in vitro translated in the absence of microsomal membranes, the expected M_r for TRAIL-R3 was still considerably higher than the predicted value (Fig. 5C). In contrast, the apparent molecular mass of TRAIL-R1 and TRAIL-R2 roughly corresponded to the theoretical values. We therefore analyzed the effect of the TAPE repeats on the M_r of TRAIL-R3. A deletion mutant of Flag-TRAIL-R3 lacking four out of five TAPE repeats (Flag-TRAIL-R3Δ4) was constructed and Fig. 5C shows that this TAPE repeat-deficient receptor had an almost normal electrophoretic behavior by SDS-PAGE analysis. The large contribution of the TAPE repeats upon the M_r of TRAIL-R3 was also evident when comparing the M_r of TRAIL-R3 and TRAIL-R3Δ4 expressed in 293T cells (Fig. 5D).

4. Discussion

We have characterized two novel receptors for TRAIL which we have named TRAIL-R2 and TRAIL-R3, bringing the current total number of TRAIL receptors to three, and of death receptors to five. TRAIL-R2 exhibits high structural and functional homology to TRAIL-R1 (DR-4).

The genes for both TRAIL-R2 and TRAIL-R3 are clustered on the same 8p12–21 region of chromosome 8. No other genes of the TNF receptor family have been mapped to this region of chromosome 8. TRAMP, CD30-R, TNF-R2, and OX40-R have been mapped to chromosome 1. A second gene cluster has been found on chromosome 12 (CD27R, 12p13; LTβ-R, 12p13; TNFR1, 12p13.2) and the genes encoding Fas, CD40R, and NGFR are distributed on various other chromosomes.

The sequences of TRAIL-R2 and TRAIL-R3 contain one and five repeat units of 15 amino acids (TAPE repeats) respectively, which are located in the extracellular domain, close to the membrane interaction site. Both receptors are potent inducers of cell death. While this manuscript was in preparation, two other groups reported on the characterization of two TRAIL receptors, designated DR-5 and DcR1 (TRID) [26,27]. Whereas the sequence of DcR1 is identical to TRAIL-R3, DR-5 differs from TRAIL-R2 in that it lacks the TAPE repeat, suggesting that alternative splicing may occur. These TAPE repeat units are responsible for the anomalous migration of the receptors on SDS-PAGE gels (the TAPE repeats migrate approximately 4 times slower than predicted), a phenomenon which has already been observed in other polypeptides containing repetitive domains. For example, several protozoan parasite proteins such as the circumsporozoite protein of *Plasmodium falciparum* [28] or the procyclin of *Trypanosoma brucei* [29] migrate on SDS-PAGE gels with apparent molecular weights significantly greater than the molecular weights predicted from their sequence. The same is true for some mammalian proteins such as the C-kinase substrate (MARCKS) [30]. The Pro, Ala or Glu repeats of MARCKS are responsible for the rod-like structure of the protein (4.4×36 nm) [30], whereas a molecular model of the $(Asp\text{-}Pro)_2(Glu\text{-}Pro)_{29}$ sequence of procyclin indicates a structure of 18 nm in length for a diameter of 0.9 nm [31]. Therefore, it is likely that the repeat units of TRAIL-R3 also adopt an elongated, rigid structure. TRAIL-R3 is then predicted to expose its ligand binding site out of the plane containing the binding sites of TRAIL-R1 and R2.

The sequence of TRAIL-R3 also predicts several sites for posttranslational modifications including signal peptide cleavage, *N*- and *O*-glycosylation and GPI addition. The predicted signal peptide cleavage sites of TRAIL-R2 and TRAIL-R3 are found at corresponding positions, preceding the extracellular two cysteine-rich domains by approximately 25 aa. In TRAIL-R1 the proposed putative signal peptide cleavage site is more distal (additional 86 aa separate the start of the mature protein from the cysteine-rich repeats, see Fig. 1), although a second cleavage site is predicted in the TRAIL-R1 sequence at a position corresponding to the predicted site in TRAIL-R2 and TRAIL-R3. An antibody specifically react-

ing with a peptide spanning amino acid residues 1–24 following the first predicted signal peptidase cleavage site does not detect the TRAIL-R1:Fc fusion protein (data not shown), suggesting that the second cleavage site is used in vivo.

Both death-inducing TRAIL receptors show a broad tissue distribution. In contrast to mice treated with Fas ligand, mice survive the same dose of TRAIL (our unpublished observation) despite the high expression levels of TRAIL-R1 and -R2 in the liver. Targeted disruption of the TRAIL-R genes may therefore provide interesting insights into the physiological role of TRAIL-R.

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