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TRAIL-R4-β: A new splice variant of TRAIL-receptor 4 lacking the cysteine rich domain 1

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Abstract

Transcriptional modification by alternative splicing is known to be involved in the regulation of programmed cell death. Recently, alternative splice variants of the TNF-related apoptosis inducing ligand (TRAIL/APO2L) and of the death receptor TRAIL-R2/DR5 have been identified. In this study, we report the identification of a novel alternative splice variant of the decoy receptor with a truncated death domain TRAIL-R4 lacking exon 3, which we designated TRAIL-R4-β. As revealed by BLAST search we identified the genomic organisation of the TRAIL-R4 gene which consists of 9 exons. Loss of exon 3 resulted in the truncation of the first complete cysteine rich domain 1 which is known to be involved in ligand–receptor-complex. In conclusion, alternative splicing might be involved in functional fine-tuning of TRAIL-induced programmed cell death.

Keywords: Alternative splicing; TRAIL-R4; Apoptosis; Cysteine rich domain

TNF-related apoptosis inducing ligand (TRAIL/APO2L/TRAIL-α) is a type II membrane protein which in contrast to other members of the TNF family has been detected in a wide variety of non-neoplastic and neoplastic celltypes [1,2] and was found to induce programmed cell death in cancer cells but not in most normal cells [3–6].

Induction of apoptosis by TRAIL seems to be more complex than apoptosis mediated by other members of the TNF family, because five different TRAIL-binding receptors have been identified so far [7,8]. TRAIL-R1/DR4 and TRAIL-R2/DR5/TRICK2 exhibit an intracellular death domain and induce programmed cell death due to clustering of the death domains [3,9,10]. Recent studies demonstrated initiation of TRAIL-R1 and -R2 triggered apoptosis by formation of the death inducing signaling complex (DISC) after recruitment of FADD which leads to activation of caspase-8 [11–15]. While induction of apoptosis is the main pathway for the TRAIL death receptors, they also activate signaling via NF-κB and c-Jun [16–20]. In contrast, ectopic overexpression of TRAIL-R3/DcR1/TRID, which lacks a cytoplasmic region and is GPI-tethered to the membrane, rather protects cells from TRAIL-induced apoptosis [21]. TRAIL-R4/DcR2/TRUNDD which exhibits only a truncated intracellular death domain, lacking 52 of the 76 amino acids that encode the typical death domain, does not induce programmed cell death but can activate NF-κB upon overexpression [22]. However, TRAIL-receptor 2 has only two complete cysteine rich extracellular domains (CRD 2 + 3) and unlike CD95/Fas and TNFR1 only one partial CRD 1 domain with only one cysteine bond. The crystal structure of a complex between TRAIL and TRAIL-R2/DR5 revealed that the contact region is located at the interface between TRAIL monomers and the receptor CRD 2 and 3 [23,24]. Thereby the CRD 3 of soluble DR5 has been reported to interact much more extensively with TRAIL.

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Transcriptional modification by alternative splicing is known to be involved in the regulation of programmed cell death [25]. Thus, alternative splicing has also been identified in genes of the TRAIL-system. Beside the two isoforms of TRAIL-R2/DR5, i.e., TRICK2A and TRICK2B which differ in the presence of a 23 amino acid extension between the transmembrane region and the cysteine rich domain [26], we recently identified two alternative splice variants of TRAIL [27]. TRAIL-β and TRAIL-γ exhibit extensive truncation of the extracellular domain, loss of proapoptotic potential and different subcellular localisation. In this report, we describe the identification of a novel alternative splice variant of TRAIL-R4/DcR2/TRUNDD, designated TRAIL-R4-β. The lack of exon 3 of the TRAIL-R4 gene results in truncation of the first complete cysteine rich domain 1 which seems to be essential for TRAIL-binding.

Materials and methods

Cell lines and cultures. All cell lines ($n = 10$) used in this study were derived from typical representatives of the clear cell, chromophilic/papillary and chromophobe types of renal cell carcinoma (RCC), established in our laboratory as previously described [28–30]. The cell lines were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco, Karlsruhe, Germany) supplemented with 10% foetal calf serum (FCS), penicillin, and streptomycin and cultured at 37 °C in an atmosphere with 5% CO_2.

RNA extraction. Total RNA was isolated from cultured RCC cell lines using the RNeasy kit (Qiagen, Hilden, Germany). RNA concentration was measured by photometry at 260 nm and the integrity of RNA was controlled by electrophoresis after staining with ethidium bromide.

Reverse transcription (RT) and PCR amplification. Synthesis of cDNA was performed in a final volume of 30 μl containing 2 μg of total RNA, 25 μM of each dNTP (Stratagene, Heidelberg, Germany), 100 pmol random hexamer primer (Stratagene), 20 U of recombinant RNAse inhibitor Rnasin (Promega, Heidelberg, Germany) as well as 5 U of AMV reverse transcriptase (Promega) with the corresponding buffer. The reverse transcription reactions were incubated at 55 °C for 1 h.

For PCR amplification of human TRAIL-R2, TRAIL-R4, and GAPDH 1.5 μl first strand cDNA solution was diluted in a final volume of 50 μl containing 2.5 U of *Taq* polymerase (Sigma, Seelze, Germany), 1× PCR buffer, 12.5 μM of each dNTP (Stratagene) and 25 pmol of each TRAIL-R2 specific primers (forward primer, GAT TGT ACA CCC TGG AGT GAC ATC G and reverse primer, CCA CAG TAA AGA CTT GCA AAC AAA CAC) (GenBank Accession No. AF018658), TRAIL-R4 specific primers (forward primer, GAC CCC AAG ATC CTT AAG TTC G and reverse primer, TGT TCT ACA CGT CCG GCA CAT C) (GenBank Accession No. AF029761) and GAPDH specific primers (forward primer, ACG GAT TTG GTC GTA TTG GGC G and reverse primer, CTC CTG GAA GAT GGT GAT GG) (GenBank Accession No. J04038). The initial denaturation step at 94 °C for 4 min was followed by 35 cycles (TRAIL-R2 and -R4) or 27 cycles (GAPDH) of denaturation for 1 min or 30 s, annealing for 1 min at 55 °C (TRAIL-R2 and -4) or 64 °C (GAPDH), extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min. PCR amplification of TRAIL was performed as recently described [27]. PCR products were separated by electrophoresis on 3% agarose gels containing ethidium bromide and visualised by UV transillumination.

Sequence analysis. The ethidium bromide stained and visualised bands of interest were excised from agarose gels and eluated using the QIAquick gel extraction kit (Qiagen), ligated into pGEM-T-cloning vector (Promega) and cloned in accordance to standard protocols. Plasmid DNA was isolated employing the Plasmid mini kit (Qiagen), sequenced using SP6 and T7 site specific primers and analysed with an ABI Prism 310 sequencing apparatus (Applied biosystems, Weiterstadt, Germany).

Computational analysis. The NetGene2 server service [32,33] was used for prediction of splice donor and acceptor sites at the exon–intron boundaries of the TRAIL-R4 gene.

Sites of protein modification were analysed by PROSITE scan [31]. Database analysis for the genomic organisation of the TRAIL-R4 gene was performed by running the advanced BLAST search.

Results

Identification of a novel TRAIL-R4 splice variant

As revealed by RT-PCR amplification, all investigated RCC cell lines irrespective of their histological types, expressed the ligands TRAIL-α, -β -γ, TRAIL-R2, and its alternative splice variants TRICK2A and TRICK2B

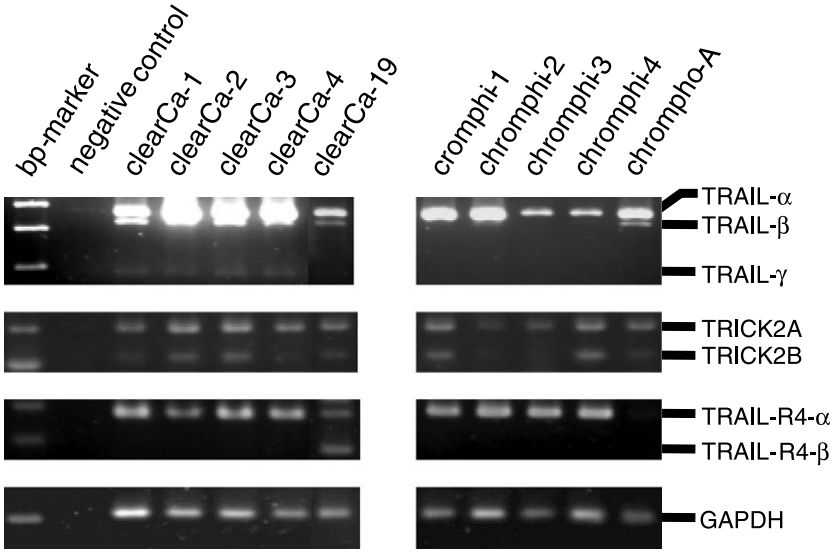

Fig. 1. Expression of different TRAIL-, TRAIL-R2- and TRAIL-R4-mRNA variants in human RCC cell lines.

as well as TRAIL-R4 (Fig. 1). Interestingly, besides the expected TRAIL-R4 amplification product of 380 bp, however, one additional band of 266 bp was coamplified in one of 10 RCC cell lines. These two amplification products of TRAIL-R4 were subsequently sequenced. The largest product was identified as regularly TRAIL-R4 and therefore designated TRAIL-R4-α, whereas the 266 bp band was identified as a variant of TRAIL-R4 which was characterised by a deletion of 114 nucleotides corresponding to nucleotides 258–372 of the TRAIL-R4-α cDNA (GenBank Accession No. AF029761). Because the genomic organisation and the exon–intron boundaries of the human TRAIL-R4 gene have not been published yet, we performed a database analysis using the advanced BLAST search, looking for a genomic clone containing the entire coding sequence of the human TRAIL-R4 gene. Surprisingly, we identified the Homo sapiens Chromosome 8 clone RP11-1149O23 (GenBank Accession No. AC100861.2) containing the entire coding sequence of the human TRAIL-R4 cDNA (Fig. 2). Subsequently, we determined the exon–intron boundaries of human TRAIL-R4 and found that the gene coding for human TRAIL-R4 is composed by nine exons. Of note, according to this genomic organisation, the 258–372 nucleotides deleted in TRAIL-R4 corresponding to exon 3 and was designated as TRAIL-R4-β.

Splice donor and acceptor sites at the intron–exon and exon–intron boundaries of exon 3

To investigate if the exon 3 is flanked by splice donor (SD) and splice acceptor sites (SA) matching to the consensus sequence of common SD sites ([C/A] AG GT [A/G] AGT) and SA sites ($[T/C]_{11}$ N [C/T] AG G), we performed computational analysis with the NetGene server service (Fig. 3A). As shown in Fig. 3B both the SA site at position 130,700–130,715 as well as the SD site at position 130,826–130,834 of exon 3 contain the AG bases of SA sites and the GT bases of SD sites that are necessary for posttranscriptional modification by splicing on intron–exon boundaries.

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115321 ctcccttctc ATGGGACTTT GGGGACAAAG CGTCCCGACC GCCTCGAGCG CTCGAGCAGG
115381 GCGCTATCCA GGAGCCAGGA CAGCGTCGGG AACCAGACCA TGGCTCCTGG ACCCCAAGAT
115441 CCTTAAGTTC GTCGTCTTCA TCGTCGCGGT TCTGCTGCCG gtgagtctcc ..........
124261 agcccatctc tgccttgtcc ccacagGTCC GGGTTGACTC TGCCACCATC CCCCGGCAGG
124321 ACGAAGTTCC CCAGCAGACA GTGGCCCCAC AGCAACAGAG GCGCAGCCTC AAGGAGGAGG
124381 AGTGTCCAGC AGgtgcactc ttatttttaa aaatcagttt atttttagtt ..........
130681 tgactcattc attaactttt ctctcccttc ccagGATCTC ATAGATCAGA ATATACTGGA
130741 GCCTGTAACC CGTGCACAGA GGGTGTGGAT TACACCATTG CTTCCAACAA TTTGCCTTCT
130801 TGCCTGCTAT GTACAGTTTG TAAATCAGgt acagaatgtg tggacccctt ..........
132181 atgctctgtt gtcagGTCAA ACAAATAAAA GTTCCTGTAC CACGACCAGA GACACCGTGT
132241 GTCAGTGTGA AAAAGGAAGC TTCCAGGATA AAAACTCCCC TGAGATGTGC CGGACGTGTA
132301 GAACAGGgta agacagcagc caggggctcc caacagcttt caggaacctg ..........
133321 ctgtctccct ctctgtgtgt acccagGTGT CCCAGAGGGA TGGTCAAGGT CAGTAATTGT
133381 ACGCCCCGGA GTGACATCAA GTGCAAAAAT GAATCAGCTG CCAGTTCCAC TGGGAAAACC
133441 CCAGCAGCGG AGGAGACAGT GACCACCATC CTGGGGATGC TTGCCTCTCC CTATCACTAC
133501 CTTATCATCA TAGTGGTTTT AGTCATCATT TTAGCTGTGG TTGTGGTTGG CTTTTCATGT
133561 CGGAAGAAAT TCATTTCTTA CCTCAAAGGC ATCTGCTCAG gtaggtgctg ..........
134161 agactgcttc ttttccagGT GGTGGAGGAG GTCCCGAACG TGTGCACAGA gtgagttggt
134221 ttctccagaa actgggggct tcgtgggttc aggaactgct tcccatccac ..........
134581 cctgaagctg agcccggggt gtggacttga gtgggctctt tgtcttccca agGTCCTTTT
134641 CCGGCGGCGT TCATGTCCTT CACGAGTTCC TGGGGCGGAG GACAATGCCC GCAACGAGAC
134701 CCTGAGTAAC AGATACTTGC AGCCCACCCA GGTCTCTGAG CAGGAAATCC AAGGTCAGGA
134761 GCTGGCAGAG CTAACAGGTG TGACTGTAGA GTCGCCAGAG GAGCCACAGC GTCTGCTGgt
134821 gagttgagga gggactgtgc cctgcctggc gtgcagcctg cacaggactt ..........
140941 actagactgc aggactcctg actctgacca gagcattccc cactgtgtgt tacagGAACA
141001 GGCAGAAGCT GAAGGGTGTC AGAGGAGGAG GCTGCTGGTT CCAGTGAATG ACGCTGACTC
141061 CGCTGACAgt aagtgttttg tgccttgaga cgcagcagga ggacagggaa ..........
141241 ctctcttctc agTCAGCACC TTGCTGGATG CCTCGGCAAC ACTGGAAGAA GGACATGCAA
141301 AGGAAACAAT TCAGGACCAA CTGGTGGGCT CCGAAAAGCT CTTTTATGAA GAAGATGAGG
141361 CAGGCTCTGC TACGTCCTGC CTGTGAaaga atctcttcag gaaaccagag cttccctcat
141421 ttacctttc tcctacaaag ggaagcagcc tggaagaaac agtccagtac ttgacccatg
141481 ccccaacaaa ctctactatc caatatgggg cagcttacca atggtcctag aactttgtta
141541 acgcacttgg agtaattttt atgaaatact gcgtgtgata agcaaacggg ..........
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Fig. 2. Genomic organisation of the human TRAIL-R4 gene. The human TRAIL-R4 cDNA is marked in bold letters within the Homo sapiens chromosome 8. SD and SA sites at the exon–intron boundaries are underlined, the stop codon is marked by an underlined grey-shaded square.

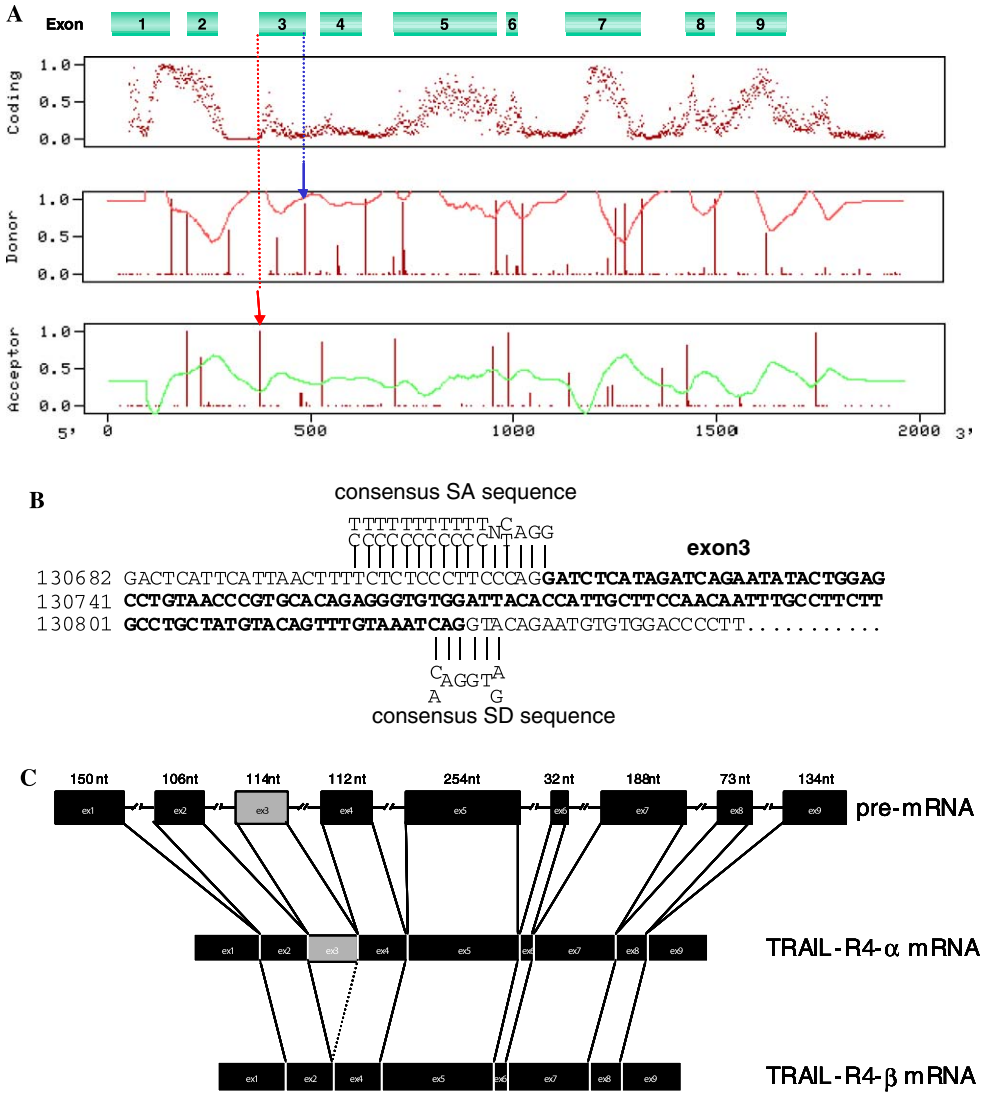

Fig. 3. SD and SA sites flanking exon 3. (A) Computational analysis with the NetGene2 [32,33] server identified the SD-site (red arrow) and the SA-site (blue arrow) at the exon–intron boundary of exon 3. (B) Alignment of the SD and SA sites matching to the consensus sequence of common splice sites and the resulting TRAIL-R4 mRNAs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

These data indicate that posttranscriptional processing of the TRAIL-R4-pre-mRNA generates two alternative splice variants of human TRAIL-R4, and therefore designated TRAIL-R4-α and TRAIL-R4-β (Fig. 3C).

Alterations of protein domains in the novel TRAIL-R4-β isoform

Computational translation of the novel TRAIL-R4 isoform revealed that the TRAIL-R4-β mRNA lacking exon 3 encoded an in-frame 348 amino acids long protein with loss of the first complete CRD 1 (Fig. 4A). Moreover, one potential protein kinase C phosphorylation site between amino acids 87–89 as well as two N-myristoylation sites between amino acids 86–91 and 102–107, which were identified by PROSITE scan in the TRAIL-R4-α-variant were lost in the TRAIL-R4-β-protein due to alternative splicing. Of note, no modifications of the three potential N-glycosylation sites identified in the TRAIL-R4-β protein were identified (Fig. 4B).

Discussion

In this study, we report the identification of a novel alternative splice variant of TRAIL-R4 which we designated as TRAIL-R4-β. As revealed by BLAST search we also identified the genomic organisation of the TRAIL-R4 gene, which consists of nine exons. According to the genomic organisation, the 114 nucleotides deleted in the TRAIL-R4-β amplification product corresponding to exon 3, resulted in the loss of the first complete cysteine rich domain CRD1.

Alternative splicing permits a high degree of protein diversity generating structurally and functionally distinct

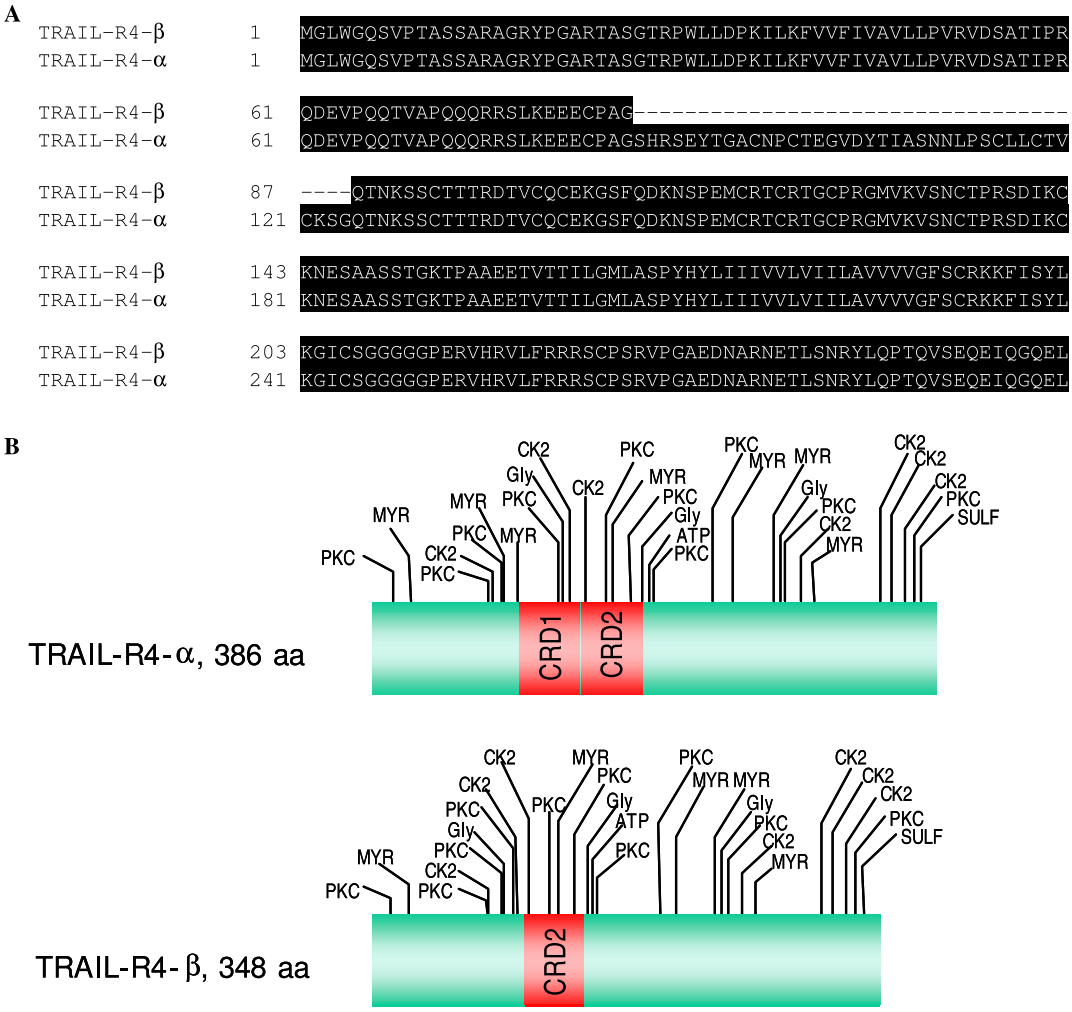

Fig. 4. Structural alterations of the TRAIL-R4 variants. (A) Multiple of TRAIL-R4-α and TRAIL-R4-β was performed by ClustalW [45] and enhanced with Boxshade. Dark shaded amino acids are highly conserved. (B) Protein sequences were analysed by Prosite scan [31]. Identified domains and potential modification sites are marked. SS, signal sequence, CRD, cysteine rich domain, TM, transmembrane region, PKC, protein kinase C phosphorylation site, CK2, casein kinase II phosphorylation site, MYR, N-myristoylation site, Gly, N-glycosylation site, ATP, ATP/GTP binding site motif A, SULF, Tyrosine sulfation site.

proteins that differ in their subcellular localisation, molecular targets, and functionality [25]. A large number of genes involved in programmed cell death have been found to be regulated and modified by alternative splicing. For example, the CD95 (Fas) receptor exists in several isoforms, whereas the soluble isoform mediates resistance towards CD95L induced apoptosis [34–36]. Moreover, different isoforms of caspases and members of the inhibitor of apoptosis protein family (IAP) have been identified [37–41]. Finally, alternative splicing is implicated in TRAIL-signaling as we recently described. Two novel isoforms of TRAIL/Apo2L, i.e. TRAIL-β and -γ, exhibiting an extensive loss of their extracellular binding domain and an absence of apoptotic potential [26].

The novel TRAIL-R4 isoform we identified exhibits a loss of the first complete CRD1 within the extracellular binding site. In contrast to other members of the TNF receptor family which are defined by the presence of a variable number of CRDs in their extracellular regions, TRAIL-R4-α consists of two complete CRDs. The CRDs of the TNF family play a critical role in receptor–ligand binding capacity and signal transduction [42,43]. Mutations in CRDs of the Fas receptor as well as aberrant splicing due to a insertion in the 5′ splice site affecting the CRDs are associated with specific Fas-mediated defects in lymphocytic programmed cell death of patients suffering from autoimmune lymphoproliferative disorder [44]. In this context, deletion of the CRDs in the CD95 receptor generated by in vitro mutagenesis implies a loss of binding capacity [45]. Consequently, it is reasonable to assume that the novel identified TRAIL-R4 isoform is unable for ligand binding and therefore fail to neutralise TRAIL. In consequence, TRAIL-R4-β may interfere with the functional fine tuning of TRAIL-R4-α signaling by two ways: The amplification of the deleted TRAIL-R4-β variant might be the first mechanism to quantitatively regulate gene expression and addi-

tionally receptor signalling, because the mRNAs of both TRAIL-R4 isoforms originate from the same pre-mRNA precursor pool [25]. On the other hand, TRAIL-R4 isoforms are able to form heterotrimeric complexes leading to a decreased or uneffective signalling complex which is supported by the observation that a weak ligand-dependent heterocomplex formation between TRAIL-R1 and TRAIL-R2 was obvious as revealed by co-immunoprecipitation [12].

Nevertheless, the identification of TRAIL-R4-β might therefore be helpful for the understandings of TRAIL-mediated programmed cell death under physiological conditions as well as tumorigenesis.

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