A Membrane-bound Fas Decoy Receptor Expressed by Human Thymocytes*

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Morgan Jenkins‡, Mary Keir‡, and Joseph M. McCune‡§

From the §Gladstone Institute of Virology and Immunology, University of California, San Francisco, California 94141-9100 and ‡Departments of Medicine and Microbiology and Immunology, University of California, San Francisco, California 94143

Human thymocytes at several stages of maturation express Fas, yet resist apoptosis induction through its ligation. A proximal step in apoptotic signaling through Fas is implicated in this resistance, as these cells undergo normal levels of apoptosis induction after exposure to tumor necrosis factor-α. We studied the Fas receptors expressed in human thymocytes to search for mechanisms of receptor-mediated inhibition of Fas signaling in these cells. We describe here a unique, membrane-bound form of Fas receptor that contained a complete extracellular domain of Fas but that lacked a death domain due to alternative splicing of exon 7. This Fas decoy receptor (FDR) was shown to have nearly wild-type ability to bind native human Fas ligand and was expressed predominantly at the plasma membrane. Unlike soluble forms of Fas receptor, FDR dominantly inhibited apoptosis induction by Fas ligand in transfected human embryonic kidney cells. Titration of FDR in Fas-expressing cells suggests that FDR may operate through the formation of mixed receptor complexes. FDR also dominantly inhibited Fas-induced apoptosis in Jurkat T cells. In mixing experiments with wild-type Fas, FDR was capable of inhibiting death signaling at molar ratios less than 0.5, and this relative level of FDR: wild type message was observed in at least some thymocytes tested. The data suggest that Fas signal pathways in primary human cells may be regulated by expression of a membrane-bound decoy receptor, analogous to the regulation of tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis by decoy receptors.

Although many mechanisms may regulate the sensitivity of Fas-expressing cells to Fas-induced apoptosis (12), alterations in the Fas receptor itself have received the greatest attention. In particular, alternative splicing of Fas mRNA in both mouse (13) and human (14–17) cells has been reported to result in an array of receptor proteins with various deletions or truncations. These variants include soluble forms of the Fas receptor that do not require proteolytic cleavage from the cell surface and that are also potential inhibitors of Fas ligand. However, these soluble Fas receptors appear to lack the ability to inhibit Fas signaling in the tissues in which they are found, including mouse thymocytes and human peripheral blood mononuclear cells (PBMCs) (13, 14, 16). Thus, their contribution to Fas resistance in vivo remains unproven.

We recently reported that most human thymocytes express Fas but are resistant to Fas-induced apoptosis (18). Such resistance was shown to be species-specific, specific for Fas and not for tumor necrosis factor α, and not abrogated by cycloheximide treatment. We hypothesized that the mechanism of resistance was receptor-intrinsic and therefore characterized the Fas receptors expressed on human thymocytes. In addition to previously described truncated receptor proteins, we found a unique form of Fas generated by alternative splicing of exon 7, resulting in a protein lacking a death domain but with an intact transmembrane domain. This Fas decoy receptor (FDR) is expressed on the plasma membrane, has wild-type ability to bind Fas ligand, but is incapable of transmitting a death signal, as assayed with several readouts including annexin V binding, hypodiploid analysis, and the TdT-mediated dUTP nick end labeling (TUNEL) technique. FDR interferes with Fas apoptosis signaling in a dominant negative fashion in both 293T cells and in Jurkat cells. FDR message was quantified by exonspecific RT-PCR in fetal thymocytes and PBMC. A ratio of FDR-to-wild-type message, which was sufficient to account for Fas resistance, was observed in some but not all fetal thymocyte samples studied but was not seen either in unstimulated PBMC or in phytohemagglutinin-stimulated blasts or Jurkat cells. These data suggest that Fas-induced apoptosis may be regulated in part by the tissue-specific expression of a membrane-bound decoy receptor, in a manner similar to that described for TRAIL (19, 20).

EXPERIMENTAL PROCEDURES

Human Tissue—Human fetal thymus specimens were derived from elective abortions, and PBMC were obtained from healthy adult volun-

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† An Elizabeth Glaser Scientist, supported by the Elizabeth Glaser Pediatric AIDS Foundation. To whom correspondence should be addressed: Gladstone Institute of Virology and Immunology, P. O. Box 419100, San Francisco, CA 94141-9100. Tel.: 415-695-3828; Fax: 415-826-8449; E-mail: mmmcune@gladstone.ucsf.edu.

1 The abbreviations used are: PBMC, peripheral blood mononuclear cells; FDR, Fas decoy receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; wt, wild type; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; FADD, Fas-associated death domain protein; RT-PCR, reverse transcription polymerase chain reaction; rs, recombinant-soluble; wt, wild type.
ers. Protocols and consent procedures governing the collection and use of all human specimens were approved by the University of California, San Francisco Committee on Human Research.

**Cell Culture—**Jurkat E6 clone 1 cells were maintained in RPMI medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 10 µg/ml streptomycin. The human embryonic kidney cell line 293T was grown in Dulbecco’s modified Eagle’s medium supplemented in a similar manner. Ficol-separated PBMC were stimulated with phytohemagglutinin (5 µg/ml) (Sigma) and interleukin-2 (10 units/ml) (Roche Molecular Biochemicals) for 5 days before harvest.

**Cloning of Human Thymocyte Fas cDNAs—**Total RNA was prepared from nearly confluent human fetal thymocytes (15–18 weeks of gestation) or from 4 × 10^6 human thymocytes stimulated with tumor necrosis factor (TNF)-α and mitogen, and 10^6 human fetal thymocytes (95 °C for 60 s, 50 °C for 60 s, and 72 °C for 90 s, performed for 40 cycles). PCR products were digested with EcoRI and XbaI and ligated into pCDNA3 (Invitrogen, Carlsbad, CA). Individual transformed colonies were screened for insert size by restriction mapping or by PCR product size analysis using SP6 and T7 primers. 88 clones were isolated and sequenced plasmids served as vectors for the expression of wild-type Fas. Fas transcript cDNAs were amplified by Taq polymerase (Roche Molecular Biochemicals)-catalyzed PCR with primers (5’ Eco Fas and 3’ Xba Fas) (Table I), which correspond to the first or last 15 coding nucleotides and the 6 flanking noncoding nucleotides of the human Fas-coding sequence, as reported by Itoh et al. (21) (GenBank® accession number M67454). Thermal cycling parameters were 95 °C for 60 s, 50 °C for 60 s, and 72 °C for 90 s, performed for 40 cycles. PCR products were digested with EcoRI and XbaI and ligated into pCDNA3 (Invitrogen, Carlsbad, CA). Individual transformed colonies were screened for insert size by restriction mapping or by PCR product size analysis using SP6 and T7 primers. 88 clones were isolated and sequenced plasmids were then digested with SauI and PstI to generate fragments containing the cytomegalovirus promoter, Kozak and initiation sequences, and Fas-coding regions. These were ligated into XhoI-PstI-digested pEGFP-1 (CLONTECH, Palo Alto, CA) to create promoter-receptor-GFP fusion protein expression vectors. Surface expression of GFP-Fas fusion proteins was assessed by staining unfixed Jurkat cells with mouse anti-Flag monoclonal antibody to Fas (BD Biosciences, San Jose, CA) at 24 h after transfection and 2-color analysis on a FACScan (BD Biosciences).

**Transfection of Cell Lines—**Nearly confluent 293T cell cultures were split 1/20 into 6-well plates one day before calcium phosphate transfection. 0.5–1 µg of plasmid expression vector for Fas, Fas ligand, or variant Fas was used in a similar manner and the total amount of transfected DNA was kept constant at 2 µg/well with the addition of vector DNA. Transfection of Jurkat cells for transient expression was accomplished by washing and resuspending 10^6 cells in 400 µl of serum-free RPMI medium containing 60 µg of expression plasmid DNA in a 0.4-cm electroporation cuvette (Bio-Rad) followed by electroporation in a Bio-Rad Gene pulser at 250 mV, 950 microfarads.

**Intracellular Fas Ligand Trapping Assay—**An expression plasmid for receptor soluble human Fas ligand covalently linked to a Flag epitope (rs Fas ligand-Flag), similar to that described by Schneider et al. (22), was created from the extracellular portion of cloned Fas ligand fused C-terminal to a prolactin leader sequence and a Flag epitope, as described (18). 24 h after transfection, 293T cells were detached with trypsin, treated with Fix and Perm reagent (Caltag, Burlingame CA) according to the manufacturer’s instructions, and serially stained with biotinylated M2 anti-Flag monoclonal antibody (Sigma) or isotype and fluorescein isothiocyanate-conjugated avidin (BD Biosciences). Cyto- metric analysis was performed on a FACScan (BD Biosciences).

**Radioimmunoprecipitation—**Calcium phosphate-transfected 293T cells were grown in 10-cm^2 dishes for 24 h and then labeled for 2.5 h in methionine-free Dulbecco’s modified Eagle’s medium with 1 µCi of [35S]methionine. 1 ml of reserved supernatant or of cells lysed in radioimmune precipitation buffer with protease inhibitors (Complete Mini, Roche Molecular Biochemicals) were precloned with Protein A/G Plus beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight and then immunoprecipitated with 1 µg of biotinylated anti-Flag antibody (M2, Sigma) and Protein A/G beads (Santa Cruz Biotechnology), according to the manufacturer’s instructions. Half of the recovered protein was analyzed on a 10% polyacrylamide SDS minigel before autoradiographic exposure for 24 h at room temperature.

**RT-PCR for Fas Splice Variants—**Poly(A) RNA was reverse-transcribed with murine leukemia virus reverse transcriptase and random hexamer primers and subjected to 45 cycles of PCR with primers to the fifth and eighth exons of Fas (Table I). PCR products were separated on an 8% polyacrylamide gel and stained with ethidium bromide before photography and band densitometry analysis. PCR products from plasmid templates for wild-type, Δ exon 6, and Δ exon 7 Fas were used as migration standards to estimate the relative abundance of message encoding each of these splice variants in mRNA from samples of fetal thymocyte cells, PBMCs, and phytohemagglutinin-stimulated blasts.

**Apoptosis Assays—**293T cells were assayed for apoptosis induction 24 h after transfection by three methods that gave similar results. First, flow cytometric quantitation of hypodiploid DNA was carried out using a modification of a published protocol (23). After a brief fixation in 1% paraformaldehyde, cells were stained with 40 µg/ml propidium iodide in 0.1% Nonidet P-40 and 0.1% sodium citrate for 30 min at room temperature and analyzed on a FACScan (Beckton Dickinson). The pulse width–peak area plot was used to exclude doublets, and forward and side scatter gates were drawn to exclude debris. The percent of remaining cells with subdiploid DNA content was measured on DNA histograms. Second, the TUNEL technique (24) was used with reagents from Roche Molecular Biochemicals following the manufacturer’s protocols. In other experiments, phycoerythrin-conjugated annexin V (Pharmingen, San Diego CA) was used to enumerate apoptotic cells in parallel with other readouts.

**RESULTS**

The resistance of human thymocytes to apoptosis induced by Fas ligation was the subject of a prior study (18). Since apoptotic signaling in steps distal to FADD recruitment were normal in human thymocytes stimulated with tumor necrosis factor α, the mechanism of Fas resistance appeared to be proximal to the FADD signaling step. Moreover, we observed that protein synthesis with cycloheximide did not alter the Fas resist-

### Table I: Oligonucleotide primers

| Name     | Location | Sequence                               |
|----------|----------|-----------------------------------------|
| 5’ Eco Fas | Sense    | gcc gaa ttc aca acc atg ctg ggc acc tgg  |
| 3’ Xba Fas | Antisense | cct tca agt ttc aca gtc cag caa gct tgg  |
| wt Δ stop | Antisense | gct cta gac tgc agg acc aag ctt tgg att tca tt |
| FDR Δ stop | Antisense | gct cta gac tgc agg ata aat tta ttg cca ctg tt |
| wt exon 7 | Antisense | gct cta gac tgc agt ttc tgt act tcc ttt ctc tt |
| Exon 5 Fas | Sense    | acc aag tgc aat gga gag gga               |
| Exon 8 Fas | Antisense | aga taa att tat tgt cag               |

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Fas transcripts from human thymus encode a variety of soluble and membrane bound receptors. Exon organization deduced from sequences of human fetal thymus Fas cDNAs are aligned with reference to wt human receptor. Sequences are numbered according to Itoh et al. (21) (GenBank® accession number M 67454) and exon-intron divisions according to Cheng et al. (35). Nucleotide numbers refer to the first base of each exon. Functional protein domains are symbolically mapped as follows: CRD, cysteine-rich domain; TM, transmembrane domain; DD, death domain. Angular brackets denote coding regions removed by alternative splicing. Dotted lines denote frame-shifted sequences, and × indicates premature stop codons.

We therefore sought to characterize the Fas receptors expressed by human thymocytes. RNA was isolated from fetal human thymocytes and from PBMC blasts, and RT-PCR was performed to amplify cDNA of Fas transcripts. The cloned cDNAs were screened by restriction analysis or by PCR to identify transcripts that would likely represent alternatively spliced RNAs (16, 17) (i.e. those shorter in length than wt Fas cDNA). Although most thymocyte clones represented full-length Fas transcripts, 17 of 88 clones (in contrast to only 1 of 16 cDNAs from PBMC blasts) revealed internally truncated transcripts. Nucleotide sequence analysis revealed five forms of truncated Fas receptor cDNAs from human thymocytes, all of which were apparently produced by alternative splicing of mRNA with deletion of one to three exons of the full-length receptor (Fig. 1). Four of these forms have been previously identified: Δ3,4,6 Fas (15, 16), Δ4,6 Fas (16), Δ4,7 Fas (15), and Δ6 Fas (14). The first three of these encode Fas molecules that have partial extracellular domains but that lack transmembrane anchor regions and death domains. The fourth, Δ6 Fas, contains a coding sequence for a death domain but, like the first three forms, lacks a transmembrane domain.

In addition to these forms, we found a cDNA encoding a previously undescribed Fas receptor bearing a deletion of exon 7 alone. The Δ7 Fas cDNA encodes a receptor protein of 181 amino acids (designated Fas decoy receptor or FDR), which contains a full-length extracellular ligand binding domain as well as a transmembrane anchor region. However, due to a frameshift and misreading of exon 8, FDR has a premature stop codon after coding only 8 amino acid residues of the intracellular tail and so lacks a death domain. These unique features of FDR made it a potential candidate as a functional membrane-bound decoy receptor for Fas ligand in primary cells. We therefore evaluated critical functional properties that might be associated with a dominant negative inhibitor of Fas signaling: expression on the plasma membrane, ability to bind Fas ligand, and inability to transmit a death signal.

Membrane localization was assessed by expressing fusion proteins of either wt Fas or FDR linked N-terminal to GFP in 293T cells. Cells transfected with either construct demonstrated surface Fas expression that was proportional to GFP expression, confirming that both receptors could be expressed on the plasma membrane (Fig. 2). Fluorescence microscopy also revealed that cells transfected with expression vectors for GFP-tagged wt Fas, FDR, or wt Fas truncated at exon 7 exhibited fluorescence predominantly at the plasma membrane, in contrast to the cytoplasmic localization of GFP in cells transfected with expression vectors for GFP alone (data not shown).

We next assessed the ability of the various Fas receptors to bind Fas ligand using an expression vector for a Flag epitope-tagged, soluble form of Fas ligand (rs Fas ligand-Flag). Transfection of this vector into 293T cells resulted in export of protein into the culture supernatant. If, however, this vector was co-transfected with a vector expressing wt Fas, Fas ligand was trapped intracellularly, as demonstrated by intracellular staining with anti-Flag monoclonal antibody and flow cytometric analysis (Fig. 3A). Radiolabeled rs Fas ligand-Flag was immunoprecipitated from the conditioned medium of cells transfected with the rs Fas ligand-Flag expression vector alone, but less material was recovered from conditioned medium of cells co-transfected with wt Fas (Fig. 3B), confirming that Fas ligand interaction with wt Fas resulted in intracellular trapping. In addition, anti-Flag immunoprecipitates of the cellular lysate demonstrated predominantly mature glycosylation forms of Fas ligand protein, but co-expression of wt Fas resulted in a predominance of lower molecular weight forms of Fas ligand.
We describe here a unique FDR that is expressed in human thymocytes and that acts as a dominant negative inhibitor of Fas signaling. Unlike most Fas receptors generated by alternative splicing, FDR is not secreted. It may thereby be able to act in a cell-specific manner. FDR is also distinguished from most soluble Fas receptors in that it appears to be fully capable of binding to Fas ligand. Perhaps most importantly, FDR is unique in its ability to substantially protect cells expressing Fas ligand, variant Fas receptors, and an equivalent amount of expression vector for wt Fas (Fig. 4B). Once again, FDR was unique among the variant Fas receptors in its ability to block apoptosis induction. These data demonstrate that FDR can inhibit Fas signaling of apoptosis in a dominant negative fashion.

To determine whether the observed resistance of human thymocytes to Fas-induced apoptosis was related to the expression of FDR, levels of wild-type Fas, ΔFas, and FDR mRNA were quantitated in human fetal thymocytes, PBMC, and PBMC blasts. As shown in Fig. 5, the level of FDR mRNA in Fas-susceptible PBMC and PBMC blasts was low, not exceeding 4% that of the level of wt Fas message in 8 samples tested. The ratio of FDR to wt Fas message was also less than 9% in Jurkat T cells, which were also sensitive to apoptosis through Fas signaling (data not shown). Fas-resistant human thymocytes were more variable in FDR expression levels, with three of six samples showing FDR:wt Fas ratios of 30–53% (Fig. 5 and data not shown). Taken together, these data suggest that FDR is preferentially expressed in human fetal thymocytes and that the level of expression is sufficiently high to account for resistance to Fas signaling in at least some human thymus.

**DISCUSSION**

The addition of wt Fas did not augment cell death to levels above those seen with Fas ligand alone (Fig. 4A, compare panels 2 and 3). Co-expression of equivalent amounts of variant Fas receptors which lacked transmembrane domains had no effect on the observed levels of apoptosis induction (Fig. 4A, panels 4–7). However, co-expression of FDR effectively abrogated Fas ligand-induced apoptosis to the levels of nontransfected controls (Fig. 4A, panel 8). To guarantee that the native level of Fas expression on 293T cells was not limiting for apoptosis induction, 293T cells were also transfected with Fas ligand, variant Fas receptors, and an equivalent amount of expression vector for wt Fas (Fig. 4B). Once again, FDR was unique among the variant Fas receptors in its ability to block apoptosis induction. These data demonstrate that FDR can inhibit Fas signaling of apoptosis in a dominant negative fashion.

We next assessed the ability of FDR to inhibit apoptotic signaling through the native Fas receptor on the Jurkat T cell line. The Fas receptor expressed on Jurkat cells is competent to transmit a death signal upon ligation with either anti-Fas monoclonal antibody or with cross-linked soluble Fas ligand. Expression of Fas-GFP fusion proteins in Jurkat cells allowed analysis of Fas ligand-induced apoptosis levels among successfully transfected cells. In three experiments, Jurkat cells expressing either FDR or truncated wild-type Fas were substantially protected from apoptosis compared with nontransfected cells in the same culture (Fig. 4D).

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plasma membrane, preventing the multimerization of receptor tails that is thought to be critical for FADD recruitment to the death signaling complex (25, 26).

Other mutations of the intracellular death domain of Fas have been reported in T cell lines selected for resistance to Fas (27) and from patients with the penetrant form of Canale-Smith syndrome (28, 29). These mutations do not prevent binding of Fas ligand with wt Fas but impair signaling through the death domain instead. Recently, the mechanism of Fas signaling defects in patients expressing Fas ligand and wt Fas or controls, as indicated, 24 h before quantitation of apoptosis by propidium iodide staining. C, 293T cells were transfected with various amounts of FDR expression plasmid in addition to Fas ligand and Fas. Induced apoptosis was assayed 24 h later using the TUNEL assay and is expressed as the percentage of the amount induced by Fas ligand and Fas alone. Data shown represent means ± S.E. of one experiment, representative of three performed. D, FDR expression inhibits apoptosis signaling by Fas on Jurkat T cells. Jurkat cells were electroporated with expression plasmids for GFP fusion proteins of wild-type Fas, FDR, or truncated Fas. Apoptosis was induced 24 h later with cross-linked soluble Fas ligand, and apoptotic morphology was assessed flow cytometrically after an additional 24 h. Apoptosis induction among cells expressing GFP fusion proteins was calculated with reference to GFP-negative cells in the same culture. Data shown represent means ± S.E. of three independent experiments.

FIG. 4. Inhibition of Fas ligand-induced apoptosis by FDR. A, apoptosis quantitated by analysis of hypodiploid DNA within 293T cells 24 h after transfection either with vector DNA (PC), Fas ligand alone (FL only), or Fas ligand and each of the alternatively spliced Fas receptors. Cellular DNA was stained with propidium iodide, and the number of apoptotic cells (y axis) with hypodiploid nuclei was analyzed by fluorescence intensity (x axis). B, expression plasmids for variant Fas receptors were co-transfected with Fas ligand and wt Fas or controls, as indicated, 24 h before quantitation of apoptosis by propidium iodide staining. C, 293T cells were transfected with various amounts of FDR expression plasmid in addition to Fas ligand and Fas. Induced apoptosis was assayed 24 h later using the TUNEL assay and is expressed as the percentage of the amount induced by Fas ligand and Fas alone. Data shown represent means ± S.E. of one experiment, representative of three performed. D, FDR expression inhibits apoptosis signaling by Fas on Jurkat T cells. Jurkat cells were electroporated with expression plasmids for GFP fusion proteins of wild-type Fas, FDR, or truncated Fas. Apoptosis was induced 24 h later with cross-linked soluble Fas ligand, and apoptotic morphology was assessed flow cytometrically after an additional 24 h. Apoptosis induction among cells expressing GFP fusion proteins was calculated with reference to GFP-negative cells in the same culture. Data shown represent means ± S.E. of three independent experiments.

FIG. 5. Relative levels of wt Fas, Δ6 Fas, and FDR mRNA in Fas-resistant and -sensitive tissues. Exon-specific RT-PCR was performed on RNA from two fetal thymi, two PBMC samples, and two PBMC blast cultures. PCR products from wt Fas, Δ6 Fas, and FDR are shown at left as size standards. Products were separated on 8% nondenaturing polyacrylamide gels, and densitometric analysis of products (adjusted for product size) was used to calculate relative abundance of FDR:wt Fas mRNA in the original sample.

plasma membrane, preventing the multimerization of receptor tails that is thought to be critical for FADD recruitment to the death signaling complex (25, 26).

Other mutations of the intracellular death domain of Fas have been reported in T cell lines selected for resistance to Fas (27) and from patients with the penetrant form of Canale-Smith syndrome (28, 29). These mutations do not prevent binding of Fas ligand with wt Fas but impair signaling through the death domain instead. Recently, the mechanism of Fas signaling defects in patients with lymphoproliferative syndromes and heterozygous CD95 mutations has been studied (30). The highly penetrant and dominantly inherited forms of this syndrome were seen only in those patients with mutations affecting the receptor death domain. The two critical features of these dominant negative inhibitors, wt ability to bind Fas ligand and loss of a functional death domain, are shared by the decoy receptor, which we describe here. The native expression in human thymocytes of this alternatively spliced receptor suggests that sensitivity to Fas-induced apoptosis may be developmentally modulated in part by the regulated expression of this dominant negative decoy receptor.

Whether FDR is solely or jointly responsible for the observed resistance of human thymocytes to Fas-induced apoptosis is still under investigation. The ability of T cells to undergo activation-induced cell death clearly depends not only upon the expression of Fas and Fas ligand but also upon the inactivation of cellular negative regulators of apoptosis (31–33). Redundant controls on cell death appear to have evolved as a protection against accidental cell death (12). In this light, the role of FDR in the human thymus may be to act as a Fas-specific negative regulator of apoptosis within cells that must pass through a stage of marked vulnerability to apoptosis induction (e.g. during negative selection).

The tissue-specific regulation of apoptotic signaling implied by these findings shares features with the TRAIL-TRAIL receptor system (34). As a transmembrane death receptor lacking a death domain, FDR resembles TRAIL-R4 or decoy receptor 2 (19, 20), a decoy receptor for TRAIL whose expression appears to correlate with sensitivity to TRAIL-induced apoptosis. Un-
like TRAIL-R4/decoy receptor 2, FDR arises from alternative splicing and so is itself regulated in a promoter-independent fashion.

Control of apoptotic signaling pathways is complicated by the convergence of multiple signal pathways at the stage of FADD recruitment and in distal steps. FDR provides a mechanism to exert stimulus-specific control of Fas-induced apoptosis in a cell-specific manner. Furthermore, by virtue of its severely abbreviated intracellular tail, it is unlikely that FDR is capable of transmitting non-apoptotic signals. These features make it attractive as a tool to dissect the role of Fas in human biology and as a potential therapeutic agent in autoimmune disease and in tissue transplantation.

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