Identification and Molecular Cloning of Two Novel Receptors for the Cytotoxic Ligand TRAIL*

(Received for publication, July 28, 1997)

Marion MacFarlane‡, Manzoor Ahmad‡, Srinivasa M. Srinivasula, Teresa Fernandes-Alnemri, Gerald M. Cohen‡, and Emad S. Alnemri

From the Center for Apoptosis Research and the Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the MRC Toxicology Unit, Hodgkin Building, University of Leicester, Leicester, LE1 9HN United Kingdom

A human receptor for the cytotoxic ligand TRAIL (TRAIL receptor-1, designated DR4) was identified recently as a member of the tumor necrosis factor receptor family. In this report we describe the identification of two additional human TRAIL receptors, TRAIL receptor-2 and TRAIL receptor-3, that belong to the tumor necrosis factor receptor family. Interestingly, TRAIL receptor-2 but not TRAIL receptor-3 contains a cytoplasmic "death domain" necessary for induction of apoptosis and is hence designated death receptor-5 (DR5). Like DR4, DR5 engages the apoptotic pathway independent of the adaptor molecule FADD/MORT1. Because of its lack of a death domain, TRAIL receptor-3 is not capable of inducing apoptosis. However, by competing for TRAIL, it is capable of inhibiting TRAIL-induced apoptosis. Thus, TRAIL receptor-3 may function as an antagonistic decoy receptor to attenuate the cytotoxic effects of TRAIL in most tissues that are TRAIL**D, DR4**, and DR5**.

Apoptosis is an intrinsic and fundamental biological process that plays a critical role in the normal development of multicellular organisms and in maintaining tissue homeostasis (1). Consequently, deregulation of apoptosis may contribute to diseases such as cancer and neurodegenerative disorders (2). Some of the well known regulators of apoptosis are cytokines of the tumor necrosis factor (TNF)1 ligand family, such as Fas ligand (Fas L) and TNF, which induce apoptosis by activation of their corresponding receptors, Fas and TNFR-1 (3). These two receptors belong to a rapidly expanding family (collectively known as the TNF receptor family) containing at least 11 known members (3, 4). Members of this family contain an extracellular ligand-binding domain, of 2–6 cysteine-rich repeats, which is about 25% conserved between different family members. The cytoplasmic region is less conserved between various members except for a stretch of about 80 amino acids present in Fas, TNFR-1, DR3/Wsl-1/Apo-3/TRAMP, CAR-1, and DR4 (Refs. 3 and 4 and references therein). This intracellular region, which has been designated the cytoplasmic "death domain," is responsible for transducing the death signal.

Activation of Fas results in recruitment of the Fas-associated death domain-containing molecule FADD/MORT1 to the receptor complex (5–7). The resulting signaling complex then triggers activation of the caspase apoptotic pathway through interaction of the N-terminal death effector domain of FADD with the corresponding motifs in the prodomain of caspase-8 (Mch5/MACH/FLICE) and probably caspase-10 (Mch4) (8–11). In contrast to Fas, activation of TNFR-1 or DR3 results in recruitment of another death domain-containing adaptor molecule known as TRADD (12, 13). TRADD can associate with a number of signaling molecules, including FADD, TRAF2, and RIP, and as a result can transduce an apoptotic signal as well as activate NF-κB (14, 15). Consequently, engagement of TNFR-1 or DR3 can signal an array of diverse biological activities.

Recently, a new member of the TNF family known as TRAIL or Apo-2 ligand was identified and shown to induce apoptosis in a variety of tumor cell lines (16–18). A human receptor for TRAIL was also recently identified and designated DR4 (4). DR4 contains a cytoplasmic death domain, and like Fas, it induces apoptosis but not NF-κB activation. However, unlike Fas, TNFR-1, and DR3, DR4 does not recruit FADD to the receptor complex and thus signals death independently of FADD (4).

Here, we report the identification of two additional human receptors for the cytotoxic ligand TRAIL (designated DR5 and TRAIL-R3). These represent as yet undescribed members of the TNF receptor family. Interestingly, DR5 but not TRAIL-R3 contains a cytoplasmic death domain necessary for induction of apoptosis, and like DR-4, DR5 engages the apoptotic pathway independent of the adaptor molecule FADD/MORT1. TRAIL-R3 on the other hand, can bind TRAIL but does not induce apoptosis and thus may function as an antagonistic receptor.

MATERIALS AND METHODS
cDNA Cloning—The full-length DR5 cDNA was cloned from a human Jurkat Uni-ZAP XR cDNA library (10) by PCR using specific primers derived from the nucleotide sequences of human GenBank™ EST clones 650744 and 664685. Similarly, TRAIL-R3 was cloned by PCR using specific primers derived from the nucleotide sequences of human GenBank™ EST clones 470799, 129137, and 504745.

Mammalian Expression Vectors—T7-epitope tagging was done as described recently (19). To generate N-terminal Flag-tagged receptor and receptor mutants, PCR-generated cDNAs encoding Fas, DR4, DR4A (residues 86 to 351), DR5, and DR5A (residues 1 to 268) were inserted in a modified pcDNA-3 vector that allowed for in-frame fusion with a Flag epitope tag that is preceded by Fas signal peptide. To generate C-terminal Flag-tagged receptors, PCR generated cDNAs encoding Fas (residues 16 to 158), DR4 (residues 86 to 217, with N-terminal Fas signal peptide-Flag tag), DR5 (residues 51 to 133), and TRAIL-R3 (residues 63 to 217) extracellular domains were inserted into a mod-
ified pcDNA3 vector that allowed for in-frame fusion with the Fc portion of the mouse IgG. For apoptosis assays we used the mammalian double expression vector pRSC (20), which allows for expression of lacZ under the Rous sarcoma virus promoter, and the test cDNA (DR4, DR4, DR5, DR5, TRAIL-R3) under the CMV promoter. CrmA, FLAME-1, caspase-8-DN (C345A), or caspase-10-DN (C355A) (19) were expressed using pcDNA3 (Invitrogen).

**Transfection, Immunoprecipitation, and Immunoblot Analysis—** These were done as described recently (19).

**TRAIL Binding Assay—** Recombinant soluble TRAIL with N-terminal T7 and His, tags was tagged by nickel affinity purification from bacteria transformed with a pET28c-TRAIL (residues 95 to 281) vector. Receptor-Fc chimeras were obtained by harvesting conditioned media of 293 cells transfected with constructs encoding Fas-, DR4-, DR5-, or TRAIL-R3-Fc fusion proteins as described (4). Binding of TRAIL to the receptor-Fc chimeras was performed as described (4).

**RESULTS AND DISCUSSION**

**Identification and Cloning of DR5 and TRAIL-R3—** To identify additional members of the TNF receptor family, we searched the GenBank™ EST database for sequences that are homologous to the TRAIL receptor-1, DR4. Several EST clones were identified, and their 3' and 5' sequences were compiled. Based on the compiled sequences, PCR primers were generated and used to clone two cDNAs that encode two new DR4-related sequences (Fig. 1, A and B). The first cDNA encodes a protein of 411 amino acids with an overall −59% identity to DR4 (Fig. 1A). Its predicted domain structure is highly related to DR4 and the other members of the TNF receptor family. It contains a putative N-terminal signal peptide (amino acids −51 to −1) followed by an extracellular domain containing two cysteine-rich pseudorepeats. Following the extracellular domain is a transmembrane domain (amino acids 132 to 152) and a cytoplasmic domain. Within the cytoplasmic domain there is a stretch of 67 amino acids (amino acids 273 to 339) comprising a death domain homology region (Fig. 1C). Based on these criteria and its apoptotic activity (see below) the new protein was designated death receptor-5 (DR5).

The second cDNA encodes a protein of 299 amino acids with overall −40 and 36% identity to DR4 and DR5, respectively (Fig. 1B). This protein contains a putative N-terminal signal peptide (amino acids −63 to −1) followed by an extracellular domain containing two cysteine-rich pseudorepeats and five nearly identical PAAEETMN/T/TSFGTPA repeats. Following the extracellular domain is a C-terminal transmembrane domain (amino acids 217 to 236). Unlike DR4 and DR5, this molecule does not contain a cytoplasmic domain. Based on these criteria and its ability to bind TRAIL (see below), this protein was designated TRAIL-R3.

**Expression of DR5 and TRAIL-R3 in Normal and Tumor Cells—** Northern blot analysis of equivalent amounts of mRNA samples from normal human tissues and tumor cell lines with a DR5 riboprobe detected a ~4-kilobase transcript in all the samples (Fig. 1D, upper panels). Interestingly, the amount of DR5 transcript was at least 100-fold more in most tumor cell lines than in normal tissues. Autoradiography for less than 2 h was sufficient to detect the DR5 message in tumor cell lines, compared with 48 h in the case of the normal tissues. Other normal tissues such as testes, ovary, colon, small intestine, and lymphoid tissues had detectable but low expression of DR5 transcript (not shown), similar to that observed in the normal tissues shown in Fig. 1D. The TRAIL-R3 riboprobe detected a 5-kilobase message in both normal human tissues and tumor cell lines (Fig. 1D, lower panels). A significantly elevated expression of TRAIL-R3 mRNA in normal compared with tumor cells was observed. Given the activities of these two receptors (see below), this could explain the high sensitivity of tumor cell lines to TRAIL compared with normal cells (16–18).

**DR5 and TRAIL-R3 Are Receptors for the Cytotoxic Ligand**

**FIG. 1. Sequence analysis and tissue distribution of DR5 and TRAIL-R3.** Predicted amino acid sequences of human DR5 (A) and TRAIL-R3 (B) are shown. The mature DR5 and TRAIL-R3 are predicted to start at Glu and Tyr (indicated by black diamonds), respectively. The putative signal peptide and transmembrane domains are single- and double-lined, respectively. The five identical repeats in the extracellular domain of TRAIL-R3 (B) are marked by black triangles. The intracellular cytoplasmic death domain of DR5 (A) is boxed. C, colinear alignment of the death domains of members of the TNF receptor family. Identical residues in at least three of six sequences are shaded. The death domain of DR5 is 64, 30, 30, 20, and 31% identical to the corresponding domains in DR4, DR3, TNFR-1, Fas, and CAR1, respectively. D, Northern blot analysis of the expression of DR5 (upper panels) and TRAIL-R3 (lower panels) mRNAs in normal tissues and tumor cell lines. X-ray film exposure time in the two lower panels and the upper left panel is 48 h, whereas in the upper right panel it is 2 h. The cell lines are: HL-60, promyelocytic leukemia; HeLa cell S3, K-562, chronic myelogenous leukemia; MOLT-4, lymphoblastic leukemia; Raji, Burkitt’s lymphoma; SW480, colorectal adenocarcinoma; A549, lung carcinoma; and G861, melanoma. The numbers on the left indicate kilobases.

**TRAIL**—Because of the high degree of sequence homology between the extracellular domains of DR4, DR5, and TRAIL-R3, we decided to test whether DR5 and TRAIL-R3 are capable of binding TRAIL. The extracellular ligand-binding domains of Fas, DR4, DR5, and TRAIL-R3 were expressed as fusion proteins with the Fc region of mouse IgG (Fig. 2A, lower panel). As shown in Fig. 2A (upper panel), DR4-Fc, DR5-Fc, and TRAIL-R3-Fc were all capable of binding TRAIL to the same extent (lanes DR5-Fc, DR4-Fc, and TR3-Fc). As expected Fas-Fc was unable to bind TRAIL (lane 4). Furthermore, DR4-Fc, DR5-Fc,
Cloning of Two TRAIL Receptors

Fig. 1. The extracellular domains of DR5 and TRAIL-R3 bind TRAIL and can block TRAIL-induced apoptosis. A, conditioned media from cultures of 293 cells transfected for 72 h with empty vector (lane 1) or DR5 (lane 2), DR4 (lane 3), Fas (lane 4), or TRAIL-R3 (TR3-Fc) (lane 5) extracellular domain-Fc fusion proteins were incubated with purified soluble T7-His-6-TRAIL and then immunoprecipitated with anti-mouse IgG-agarose. After extensive washing the samples were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with a horseradish peroxidase-conjugated T7-antibody (upper panel). The corresponding receptor-Fc fusions in the conditioned media were also immunoblotted with anti-mouse Fc antibody (lower panel). B, aliquots of conditioned media containing receptor-Fc fusion proteins or no fusion protein (Vector) were incubated with equivalent amount of soluble TRAIL (250 ng/ml) and then added to MCF7 cells. Cells were stained 8 h later with propidium iodide, and the nuclei were examined by fluorescence microscopy. The graph shows the percentage of apoptotic nuclei (mean ± S.D.) as a function of total nuclei counted under each condition (n = 3).

and TRAIL-R3-Fc but not Fas-Fc were capable of blocking TRAIL-induced apoptosis in MCF7 cells (Fig. 2B). These data suggest that, like DR4, DR5 and TRAIL-R3 are receptors for TRAIL.

DR5 but Not TRAIL-R3 Induces Apoptosis in Human Cells—Ectopic expression of death domain-containing members of the TNF receptor family induces apoptosis in a ligand-independent manner. Consistent with this observation, we found that transient expression of DR5 in MCF7 or 293 cells triggers apoptosis (Fig. 3, A and B). The level of apoptosis induction was similar to that observed with DR4 (Fig. 3A). Induction of apoptosis was dependent on the presence of the cytoplasmic death domain, because deletion of this domain abolished the ability of DR4 and DR5 to induce apoptosis (Fig. 3, A and B). Accordingly, TRAIL-R3, which does not naturally contain a death domain, was also incapable of inducing apoptosis (Fig. 3, A and B). Interestingly, transient expression of TRAIL-R3 in MCF7 cells significantly blocked TRAIL-induced apoptosis (Fig. 3C), suggesting that it may function as an antagonistic decoy receptor.

Like DR4 and the other TNF receptor family members, DR5-induced apoptosis was efficiently blocked by the caspase inhibitors z-VAD-fmk and CrmA (Fig. 3D). DR4- and DR5-induced apoptosis was also significantly inhibited by the dominant negative inhibitors FLAME-1 (also known as Casper, FLIP, I-FLICE, and CASH) (Refs. 19 and 21 and references therein), caspase-8-DN, caspase-10-DN, and FLAME-1 (E). MCF7 cells were transfected with DR4 or DR5 expression constructs in the presence of z-VAD-fmk (20 μM) or co-transfected with a 4-fold excess of a CrmA, caspase-8-DN, caspase-10-DN, or FLAME-1 construct or an empty vector. The data are represented as in A and B.

Fig. 3. Expression of DR5 but not TRAIL-R3 induces apoptosis in human cells. MCF7 (A) and 293 (B) cells were transfected with the indicated pIRES-lacZ constructs. 30 h after transfection cells were stained with β-galactosidase and examined for morphological signs of apoptosis. The graphs show the percentage of round blue apoptotic cells (mean ± S.D.) as a function of total blue cells under each condition (n ≥ 3). C, ectopic expression of TRAIL-R3 attenuates TRAIL-induced apoptosis in MCF7 cells. MCF7 cells were transfected with TRAIL-R3 or vector alone for 36 h and then treated with soluble TRAIL (250 ng/ml) for 8 h. The data are represented as in A and B, after subtracting the background killing (12–15%) as a result of transfection. D and E, DR4- and DR5-induced apoptosis is inhibited by the caspase inhibitors, z-VAD-fmk and CrmA (D), and by the dominant negative inhibitors, caspase-8-DN, caspase-10-DN, and FLAME-1 (E). MCF7 cells were transfected with DR4 or DR5 expression constructs in the presence of z-VAD-fmk (20 μM) or co-transfected with a 4-fold excess of a CrmA, caspase-8-DN, caspase-10-DN, or FLAME-1 construct or an empty vector. The data are represented as in A and B.

and TRAIL-R3-Fc but not Fas-Fc were capable of blocking TRAIL-induced apoptosis in MCF7 cells (Fig. 2B). These data suggest that, like DR4, DR5 and TRAIL-R3 are receptors for TRAIL.

DR5 but Not TRAIL-R3 Induces Apoptosis in Human Cells—Ectopic expression of death domain-containing members of the TNF receptor family induces apoptosis in a ligand-independent manner. Consistent with this observation, we found that transient expression of DR5 in MCF7 or 293 cells triggers apoptosis (Fig. 3, A and B). The level of apoptosis induction was similar to that observed with DR4 (Fig. 3A). Induction of apoptosis was dependent on the presence of the cytoplasmic death domain, because deletion of this domain abolished the ability of DR4 and DR5 to induce apoptosis (Fig. 3, A and B). Accordingly, TRAIL-R3, which does not naturally contain a death domain, was also incapable of inducing apoptosis (Fig. 3, A and B). Interestingly, transient expression of TRAIL-R3 in MCF7 cells significantly blocked TRAIL-induced apoptosis (Fig. 3C), suggesting that it may function as an antagonistic decoy receptor.

Like DR4 and the other TNF receptor family members, DR5-induced apoptosis was efficiently blocked by the caspase inhibitors z-VAD-fmk and CrmA (Fig. 3D). DR4- and DR5-induced apoptosis was also significantly inhibited by the dominant negative inhibitors FLAME-1 (also known as Casper, FLIP, I-FLICE, and CASH) (Refs. 19 and 21 and references therein), caspase-8-DN, and caspase-10-DN (Fig. 3E). Among these, caspase-10-DN was the most effective in blocking DR4- and DR5-induced apoptosis. Inhibition of DR4- and DR5-induced apoptosis by FLAME-1 is consistent with recent observations that TRAIL-induced apoptosis is blocked by expression of FLIP (FLAME-1) (22). These data also suggest that the upstream caspases-8 and -10 are involved in both the DR4 and DR5 death signaling pathways.

DR4 and DR5 Recruit Caspase-8, Caspase-10, and FLAME-1 to the Death Signaling Pathway—Death domain containing adaptor molecules such as FADD/MORT1, CRADD/RAIDD, TRADD, and RIP are recruited by some members of the TNF receptor family to engage the upstream caspases (3, 19). Using co-immunoprecipitation experiments, we tested if DR5 could interact with these molecules to transmit the apoptotic signal. Unlike Fas, DR5 did not interact with FADD or CRADD (Fig. 4A) nor with RIP or TRADD (data not shown). A similar observation was reported with DR4 (4). Interestingly, full-length Fas, DR4, and DR5, but not death domain-deleted mutants, were all capable of forming complexes with caspase-8, caspase-10, and FLAME-1 (Fig. 4B). Because these proteins do not
interact directly, this suggests that formation of these complexes would require an adaptor molecule distinct from FADD.

Taken together, we report the identification of DR5 and TRAIL-R3 as two new receptors for the cytotoxic ligand TRAIL. Consistent with the observation that TRAIL-induced apoptosis is independent of FADD (18), we show that DR5, like DR4 (19), does not bind FADD. However, both receptors recruit the upstream caspases-8 and -10 and the anti-apoptotic protein FLAME-1, suggesting that an as yet unidentified adaptor molecule is involved in the mechanism of apoptosis signaling by these receptors. Because TRAIL-R3 does not contain a cytoplasmic death domain and is capable of attenuating the cytotoxicity of TRAIL, it may function physiologically as an antagonist to DR4 and DR5. A correlation may exist between the high sensitivity of tumor cells to TRAIL and the elevated levels of DR5 in these cells. Further analysis of the regulation of DR4, DR5, and TRAIL-R3 expression in normal and tumor cells should lead to a better understanding of their normal physiological function.

Acknowledgments—We thank Drs. T. C. Tsang and W. J. LaRochelle for the pRSC vector and the Fc encoding plasmid MMThnu-HFc, respectively.

REFERENCES

1. Steller, H. (1995) Science 267, 1445–1448
2. Thompson, C. B. (1995) Science 267, 1456–1462
3. Nagata, S. (1997) Cell 88, 355–365
4. Pan, G., O’Rourke, K., Chinnaian, A. M., Gentz, R., Ehner, R., Ni, J., and Dixit, V. M. (1997) Science 276, 111–113
5. Boldin, M. P., Vargeloseev, E. E., Pancer, Z., Matt, I. L., Camonis, J. H., and Wallach, D. (1995) J. Biol. Chem. 270, 7795–7798
6. Chinnaian, A. M., O’Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
7. Kischkel, F. C., Hellbairdt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P., and Peter, M. E. (1995) EMBO J. 14, 5579–5588
8. Boldin, M. P., Goncharov, T. M., Golsteve, Y. V., and Wallach, D. (1996) Cell 85, 803–815
9. Muzio, M., Chinnaian, A. M., Kischkel, F. C., O’Rourke, K., Shevechenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827
10. Fernandes-Alnemri, T., Armstrong, R., Krebs, J., Srinivasu, S. M., Wang, L., Bullrich, F., Fritz, L., Trapani, J. A., Tomasselli, K. J., Litwack, G., and Alnemri, E. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7464–7469
11. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) Cell 87, 171
12. Chinnaian, A. M., O’Rourke, K., Yu, G.-L., Lyons, R. H., Garg, M., Duan, D. B., Xing, L., Gentz, R., Ni, J., and Dixit, V. M. (1996) Science 274, 990–991
13. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) Cell 81, 495–504
14. Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D. V. (1996) Cell 84, 299–308
15. Hsu, H., Huang, J., Shu, H.-B., Baichwal, V., and Goeddel, D. V. (1996) Immunity 4, 387–396
16. Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nieholl, J. K., Sutherland, G. R., Davis, T. D., Smith, C., Rauch, C., Smith, C. A., et al. (1995) Immunity 3, 673–682
17. Pitti, R. M., Marsters, S. A., Ruppert, S., Donahue, C. J., Moore, A., and Ashkenazi, A. (1996) J. Biol. Chem. 271, 12657–12660
18. Marsters, S. A., Pitti, R. M., Donahue, C. J., Ruppert, S., Bauer, K. D., and Ashkenazi, A. (1996) Curr. Biol. 6, 750–752
19. Srinivasu, S. M., Ahmad, M., Outtile, S., Bullrich, F., Banks, S., Wang, Y., Fernando-Alnemri, T., Croce, C. M., Litwack, G., Tomasselli, K. J., Armstrong, R. C., and Alnemri, E. S. (1997) J. Biol. Chem. 272, 18542–18545
20. Tsang, T. C., Harris, D. T., Akporiaye, E. T., Chu, R. S., Brailey, J., Liu, F., Vasanwala, F. H., Schluter, S. F., and Hersh, E. M. (1997) Bio/Technology 15, 98–101
21. Wallach, D. (1997) Nature 388, 123–125
22. Irmler, M., Thome, M., Hahne, M., Schneider, P. K., H., Steiner, V., Bodmer, J.-L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) Nature 388, 190–195