Crystal Structure of TRAIL-DR5 Complex Identifies a Critical Role of the Unique Frame Insertion in Conferring Recognition Specificity*

TRAIL is a cytokine that induces apoptosis in a wide variety of tumor cells but rarely in normal cells. It contains an extraordinarily elongated loop because of an unique insertion of 12–16 amino acids compared with the other members of tumor necrosis factor family. Biological implication of the frame insertion has not been clarified. We have determined the crystal structure of TRAIL in a complex with the extracellular domain of death receptor DR5 at 2.2 Å resolution. The structure reveals extensive contacts between the elongated loop and DR5 in an interaction mode that would not be allowed without the frame insertion. These interactions are missing in the structures of the complex determined by others recently. This observation, along with structure-inspired deletion analysis, identifies the critical role of the frame insertion as a molecular strategy conferring specificity upon the recognition of cognate receptors. The structure also suggests that a built-in flexibility of the tumor necrosis factor receptor family members is likely to play a general and important role in the binding and recognition of tumor necrosis factor family members.

The TNF family and the TNF receptor (TNFR) superfamilies play important roles in regulating many biological functions, especially as prominent mediators of immune regulation, inflammatory responses, bone development, and homeostasis (1–4). Ligands belonging to the TNF family are expected to function as a homotrimer as suggested by the crystal structures of TNFα, TNFβ, CD40L, and TRAIL. The monomeric unit of these proteins contains two β-pleated sheets, which form a β-sandwich conforming to the jellyroll topology. The TNFR family members are transmembrane proteins with several exceptions that contain an extracellular domain only. The extracellular domain of the receptors is characterized by the concatenated cysteine-rich domains (CRDs) (5) that are responsible for ligand binding. Although most of the intracellular domains of the receptor family members are not conserved, those of a subset of the receptors contain a common death domain. It is a protein-protein interaction motif that recruits cellular partners and ultimately activates a protease cascade leading to apoptosis. Despite the same structural scaffolds, namely the β-sandwich for the TNF family and the concatenated CRDs for the TNFR family, the recognition between cognate ligands and receptors is achieved in a highly specific manner with typical dissociation constants in nanomolar range.

TRAIL is a recently identified member of the TNF family (6, 7). It is a type II membrane protein that is processed proteolytically at the cell surface to form a soluble ligand (residues 114–281) (8). An unique feature of TRAIL distinguished from those of other TNF family members is an insertion of 12–16 amino acids (depending on the proteins compared with) near the N terminus (9), called AA loop, which is previously known to be important for the receptor binding of TNFs (10, 11). TRAIL interacts with at least four different receptors, which share a high sequence homology in the extracellular domains. Two of these are apoptosis-inducing receptors, DR4 (12) and DR5 (13–15), both of which contain an intracellular death domain. The other two are nonsignaling decoy receptors, DcR1 (13, 14, 16) and DcR2 (17, 18), which can bind to TRAIL but cannot trigger apoptosis. DcR1 lacks a cytoplasmic domain entirely (19), whereas DcR2 has a nonfunctional truncated death domain (14, 20). Another receptor, osteoprotegerin, binds to TRAIL with a dissociation constant of 400 nM, which is the lowest affinity of the known TRAIL receptors (21). In contrast to TNFs and FasL, TRAIL has been known to induce apoptosis in a variety of tumor cells and some virally infected cells but not in normal cells (6, 7). Consistently, administration of TRAIL to mice bearing human tumors actively suppressed tumor progression (22) and improved survival of the animal (23). Furthermore, repeated intravenous injections of TRAIL in nonhuman primates did not cause detectable toxicity to normal tissues and organs, including liver tissues (23). However, susceptibility of human normal hepatocytes to TRAIL was reported very recently (24). A working hypothesis for the selective antitumoral activity of TRAIL is that the decoy receptors are preferentially expressed in normal cells compared with tumor cells and interfere with the TRAIL action (13, 14, 17, 25).

The structure of TNFα in complex with the extracellular domain of TNF-R55 (sTNFR55) had been determined (10). The structure of TRAIL-receptor complex should provide valuable

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The Crystal Structure of Human TRAIL-DR5 Complex

insights into molecular recognition between the two families that are rapidly expanding. Recently, two crystal structures of TRAIL in complex with the extracellular domain of DR5 (sDR5) were reported (26, 27) and provided detailed views of the ligand-receptor interactions. However, neither of them sheds light on biological implication of the frame-insertion in the AA' loop. In this study, we independently determined the 2.2 Å crystal structure of TRAIL-sDR5 complex. It reveals extensive interactions of the AA' loop with the receptor, whereas previous structures show virtually no interaction between the two. The newly revealed binding mode accurately reflects previous and a new deletion analyses of the loop and enables us to correctly highlight molecular strategies for controlling specificity between members of the TNF and TNFR superfamilies.

EXPERIMENTAL PROCEDURES

Purification and Crystallization—Active human TRAIL (residues 114–281) was expressed and purified as a soluble form from Escherichia coli (9). sDR5 gene (encoding residues 1-130) was amplified by polymerase chain reaction from human fetal cDNA library. The gene was inserted into the downstream of the T7 promoter on the expression plasmid pET-21b (+), and the plasmid was introduced in E. coli strain BL21 (DE3). Cells were grown to an A600 of approximately 0.4 in Luria-Bertani medium containing 0.1 mg/ml ampicillin at 37 °C, and the expression of sDR5 was induced by 0.5 mM isopropyl-β-thiogalactoside. After 7 h induction at 27 °C, cells were harvested and resuspended in a 20 mM sodium phosphate buffer (pH 7.5) and disrupted by sonication. After centrifugation, supernatants were consecutively loaded on nickel-nitrilotriacetic acid (Qiagen) and Hi-Trap Q (Amersham Pharmacia Biotech) columns. The purified TRAIL and sDR5 were mixed in 1:1 molar ratio. After 12 h of incubation at 4 °C, the mixture was loaded on Superdex 200 HR 10/30 sizing column (Amersham Pharmacia Biotech) columns. The purified TRAIL and sDR5 were mixed in 1:1 molar ratio. After 12 h of incubation at 4 °C, the mixture was loaded on Superdex 200 HR 10/30 sizing column (Amersham Pharmacia Biotech). The fractions containing the TRAIL-sDR5 complex were collected and used for crystallization. Crystals of the complex were obtained using the hanging drop vapor diffusion method containing 16% polyethylene glycol 3000, 0.05 M sodium acetate (pH 4.5), 0.55 M sodium acetate, and 0.6 M NaCl. The crystals belonged to the space group P21, with cell dimensions a = 68.63, b = 124.81, c = 128.37 Å, and β = 104.49°. The asymmetric unit contained two trimeric complexes of TRAIL and sDR5.

Structure Determination and Refinement—A 2.2 Å data set was collected using x-ray beam (λ = 1 Å) from the BL6A beamline at the Photon Factory (Japan) and processed with the programs DENZO and SCALEPACK (32) (see Table I). For data collection, the crystal was frozen at 100 K after being briefly immersed in a cryoprotectant solution containing 15% glycerol, 16% polyethylene glycol 3000, 0.05 M sodium acetate, 0.3 M sodium acetate, and 0.3 M NaCl. With the uncomplexed TRAIL structure (9) as a search model, two promising solutions were found using the CCP4 program. The initial electron density map was calculated with 5% of the data.

| Table I Crystal structure determination and refinement statistics |
|-------------------|-----------------|
| Resolution (Å)    | 2.20            |
| R refinement (%)  | 6.1             |
| Completeness (F > 1σ) | 93.1         |
| R factor          | 21.2            |
| Rfree             | 29.1            |
| Number of protein atoms | 12,837      |
| Water molecules   | 162             |
| Metal ion         | 2               |
| Average B-factor (Å²) | 21.8        |
| Rmsd bond lengths (Å) | 0.911         |
| Rmsd bond angles (degree) | 1.772      |
| Ramachandran plot (%) | 77            |
| Most favored region | 23           |
| Additionally allowed region | 0          |
| Disallowed region | 0               |

sensor chip and the other flow cell containing an underviridated chip. For kinetic measurements, TRAIL samples, ranging from 300 to 900 ng/ml were prepared by dilution with the HBS buffer containing 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate, and 10 mM HEPES (pH 7.4). Injection of each of 80 μl of TRAIL solution into the flow cells (association phase) was followed by the flow of the HBS buffer (dissociation phase). Protein concentrations (0–1 μg/ml) of both at 30 μl/min. Between cycles, the immobilized ligand was regenerated by injecting 30 μl of 10 mM sodium phosphate buffer (pH 7.5) containing 1.0 mM NaCl at 10 μl/min. All experiments were performed at 25 °C. The kinetic parameters were determined by nonlinear regression analysis according to 1:1 binding model using the BLAevaluation version 2.1 software provided by the manufacturer.

Measurement of in Vitro Cytotoxic Activity—The cytotoxic activity of TRAIL variants were measured on human SK-HEP-1 hepatoma cells (American Type Culture Collection HTB-52). Briefly, a total of 50 μl of the cells in 10% fetal bovine serum and Dulbecco’s modified Eagle’s medium was transferred to individual wells of 96-well microplates at a density of 1 x 10^4 cells/well. Wild type or the mutant TRAIL diluted in the 50 μl of the same medium was added to the wells at various concentrations (0–1 μg/ml), and the cells were incubated for 24 h at 37 °C. Cell viability was determined by measuring the cellular metabolic activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (thiazolyl blue, Sigma-Aldrich) added to the medium at a concentration of 0.5 mg/ml. Absorbance was measured at 540 nm.

RESULTS AND DISCUSSION

We obtained the crystals of the TRAIL-sDR5 complex whose crystal packing is totally different from those of the other two crystals of the complex reported earlier (26, 27). The crystals contained six molecules of TRAIL and sDR5 (two trimeric complexes) in the asymmetric unit and provided six independent views. The structure of the complex was determined by molecular replacement (Table I) and refined to 2.2 Å resolution. The final model consists of TRAIL residues 120–135 and 146–281 in each monomer, sDR5 residues 21–128 of two copies, and residues 91–102 and 116–123 of the other four copies, two metal ions, and 162 water molecules. Residues 1–20 of sDR5 are completely disordered. In this section, we mainly describe the new findings obtained from this study and their biological implications. However, we refrain from describing structural features common in the three complex structures such as the interactions between TRAIL and sDR5 at the lower and upper contact regions (see below).

Structure of TRAIL—The overall structure of TRAIL in complex with sDR5 is virtually the same as that of the uncomplexed TRAIL structure we reported earlier (9). A TRAIL monomer contains two antiparallel β-pleated sheets that form a β-sandwich as a core scaffold and interacts with the adjacent subunits in a head-to-tail fashion to form a bell-shaped homotrimer. Structural variation between the uncomplexed and complexed TRAIL structures is concentrated in the loop re-
gions, with dramatic differences observed in the AA', CD, and EF loops (Fig. 1). The CD and EF loops that are disordered in the uncomplexed structure become ordered on binding to sDR5. The AA' loop (residues 130–160) displays the most remarkable structural changes. Although the conformation of residues 146–160, which interact heavily with the core scaffold, is well maintained, the flexible segment composed of residues 130–145 undergoes a drastic positional change (Fig. 1). The initial part of the segment penetrates into the receptor-binding site and interacts with sDR5 (Fig. 1B). This translocation is manifested by the strong electron densities observed for residues 130–135 of all six TRAIL molecules in the asymmetric unit.

A TRAIL monomer contains a single cysteine, Cys-230. The cysteine residues from the three monomers are close to each other and form a triangular geometry with the three cysteiny1 sulfur atoms as apices. This triangular arrangement is perpendicular to the molecular 3-fold axis. A strong electron density observed near the cysteiny1 sulfur atoms at the first cycle of the structure refinements indicated a metal binding. This is different from the structure of uncomplexed TRAIL (9) and that of TRAIL-sDR5 complex (Protein Data Bank code 1D4V) of Mongkolsapaya et al. (27), both of which were determined with refolded TRAIL. It is, however, consistent with the structure of TRAIL-sDR5 complex (Protein Data Bank code 1D0G) of Hymowitz et al. (26) in which the three cysteines chelate Zn$^{2+}$ with the fourth coordination arm, Cl$^-$. Removal of the Zn$^{2+}$ by chelating agents was shown to result in a 90-fold decrease in apoptotic activity and a marked decrease in melting temperature (28). Previously, we reported that refolded Zn$^{2+}$-unbound TRAIL slowly converted into an inactive monomeric or dimeric form (9). Although the zinc site is buried in the trimeric interface (Fig. 1), and thus Zn$^{2+}$ binding cannot directly influence the binding affinity of TRAIL for the receptors, it must be critical for maintaining the active trimeric structure of the protein (26).
Structure of sDR5—The TRAIL receptors contain two repeats of extracellular CRDs, whereas other TNFR family members contain three or more CRDs (29). sTNFR55 is composed of four CRDs; two central CRDs corresponding to the CRDs of TRAIL receptors, the N-terminal and the C-terminal CRDs. The basic repeating structural unit of the extracellular domain is a set of smaller modules that are the building blocks of CRDs (5, 30). sDR5 has modular composition of N1 (residues 28–41), A1 + B2 (CRD1; residues 44–84), and A1 + B2 (CRD2; residues 86–125) (Fig. 2A). A1 and B2 modules are composed of 12–17 and 21–24 amino acids, respectively. They are defined by the consensus sequence Cys1-Xaa2–5-Asn-Thr-Val-Cys4, respectively (30), where n is the number of intervening amino acids. The A1 modules exhibit a single disulfide bond between Cys1 and Cys2, while the B2 module has two disulfide bonds (Cys1-Cys3 and Cys2-Cys4). The N1 module has only one disulfide bond but is structurally related to the B2 module (Fig. 2B). Interestingly, in place of a N1 module in sDR5, a B2 module is located at the corresponding position in sTNFR55. Residues 28–34 of the N1 module, which is structurally homologous to the B2 module (Fig. 2B), are involved in tight backbone interactions with the A1 module in CRD1 of sDR5 (figure not shown), and these interactions are conserved in sTNFR55. Consequently, the domain conformation and the arrangement of CRD1 relative to the N1 module in sDR5 are virtually the same as those of the corresponding CRD relative to the B2 module in sTNFR55. A superposition of the structures of TRAIL-sDR5 and TNFβ-sTNFR55 discloses that the N1 module and CRD1 in sDR5 and the corresponding B2 and CRD in sTNFR55 adopt virtually the same geometry on complex formation (Fig. 3A). The structurally similar B2 and N1 modules appear to be functionally replaceable without affecting receptor-binding mode.

The domain structures of TNFR family members are very flexible, as was first observed for sTNFR55 that adapts its structure to changes in solvent conditions or upon binding to TNFβ (31). A superposition of the TRAIL-sDR5 and TNFβ-sTNFR55 structures shows a clear difference in the relative orientation between CRD2 and the corresponding CRD of sTNFR55 (Fig. 3A). A torsional angle change of ~30° occurs at the connection (Gln-85) between CRD1 and CRD2 of sDR5 compared with sTNFR55 bound to TNFβ (Fig. 3A). Apparently, the domain movement complements the geometric differences in the receptor-binding surfaces of the two ligands for specific interactions between pairs of residues. It probably is a common feature of the receptor family.

The six sDR5 molecules in the asymmetric unit exhibit a large structural disparity in the B2 module of CRD2. Although the B2 module and the three successive residues at the C terminus in two sDR5 molecules are well ordered, those in the other four molecules are highly disordered. Because of different crystal packing, the positions of the two ordered B2 modules in our structure are also different from those in the previously reported structures of the complex (Fig. 3B). The module is a link between the putative transmembrane helix and the other part of the extracellular domain. The connection region of sTNFR55 (the C-terminal CRD) in the TNFβ-sTNFR55 complex is also highly disordered. The conformational flexibility at the connection region appears to be a general feature of the TNFR family that may help the intracellular domains to form signaling complexes with cellular partners upon ligand binding.

Interface of the TRAIL-sDR5 Complex—TRAIL and sDR5 form a tight 3:3 complex. Three sDR5 molecules in a curved shape bind to the three identical grooves between neighboring subunits of TRAIL (Fig. 1A). The complex formation reduces the solvent-accessible surface areas by 4879 Å² in TRAIL and 5058 Å² in the three sDR5 molecules. The binding interface between TRAIL and sDR5 can be divided into three areas similarly to that of the TNFβ-sTNFR55 complex: a lower contact region, a central region, and an upper contact region (Fig. 1A). A critical feature distinguished from the two previously determined structures of TRAIL-sDR5 (26, 27), that of TNFβ-sTNFR55, and a model of TRAIL-sDR4 (9) is the existence of extensive interactions in the central region of the interface in our structure. The central contact region, otherwise a central cavity as in the other three structures of the complexes, involves residues 131–135 of the AA’ loop that penetrates into the central binding interface upon complex formation (Figs. 1A and 4A). The segment forms several specific polar interactions with sDR5, displaying strong electron densities even in an omit map (Fig. 4B). The guanidino group of Arg-132 makes a polar interaction with Tyr-50 of sDR5. Asn-134 and Thr-135 interacts with Glu-70 and Asn-81 of sDR5, respectively (Fig. 4C). The three interacting residues of DR5 are identical or homologously substituted in the other three TRAIL receptors. A significant decrease in solvent-accessible surface area of residues 131–135 upon the complex formation from 526 to 130 Å² also supports...
tight van der Waals’ contacts with sDR5. Intriguingly, several residues after Thr-135 that should exit out of the relatively small space between TRAIL and sDR5 exhibit barely observable electron densities. Putative courses of these residues starting from Leu-136 are surrounded by a negatively charged surface (Fig. 4A). Unfavorable contact between this region and Leu-136 and the flexible nature of the first part of the loop are likely to be responsible for preventing the formation of an ordered segment.

In the two previously determined TRAIL-sDR5 structures, the course of residues 131–135 in our structure is occupied by water molecules (26) or water molecules and the side chain of...
Arg-130 (27) that is located at the disallowed region in the Ramachandran plot. These residues plus the other residues of the first half of the AA\(^0\) loop in the two structures are not included (26) or poorly defined as reported (27). As a result, in the two preceding structures, a salt bridge between Arg-149 of TRAIL and Glu-94 of sDR5 is the only interaction between the AA\(^0\) loop and the receptor (26, 27). The distance between the two residues, which is >5.5 Å in all the three complex structures, precludes a strong ionic interaction. Accordingly, R149A mutant TRAIL showed only a slight reduction in the apoptotic activity (28). Therefore, the two previous structures preclude an assignment of any meaningful function of the novel insertion on the AA\(^0\) loop. The differences in the interpretation of the electron densities at the central contact region could be due to the different crystallization conditions, although the loops in the present and in the other two structures do not (26) or interact only weakly (27) with neighboring molecules in the crystals. We constructed a TRAIL mutant containing the deletion of residues 132–135. When assessed by surface plasmon resonance spectroscopy, the apparent \(K_D\) (dissociation constant) of the deletion mutant for sDR5, which was 2.94 \((\pm 1.61) \times 10^{-4}\) M, is 18-fold higher than that (1.59 \((\pm 0.25) \times 10^{-4}\) M) of wild type TRAIL (Fig. 5A). Consistently, the deletion mutant showed a profound decrease in the cytotoxic activity in an \textit{in vitro} assay using human hepatoma cells. Although wild type TRAIL reduced viability of culture cells by 50% at about 50 ng/ml concentration, the deletion mutant resulted in less than 50% cell death even at 1000 ng/ml (Fig. 5B). Therefore, the interactions at the central contact region bear direct correlation with the activity of TRAIL. These interactions also explain the activities of previously reported TRAIL variants containing a deletion on the AA\(^0\) loop, one containing the deletion of residues 137–152 and the other containing the Ser-Leu-Leu sequence instead of residues 135–155 to mimic the short AA\(^0\) loop of TNF\(\beta\). Both mutants exhibited a drastically reduced apoptotic activity (9) or affinity for sDR5 (27). The weak interaction of Arg-149 on its own cannot explain the defective activities. These, however, can be explained by the expected loss of the movable nature of the AA\(^0\) loop when it is curtailed. Although the shortened AA\(^0\) loops of the deletion mutants contain one fewer or all the residues that interact with the receptor, they could not be translocated into the central binding region, especially because the second half of the AA\(^0\) loop is held tight on the outer \(\beta\)-sheet of TRAIL. Together with the previous and the present deletion analyses, our structure underscores the critical role of the frame insertion in the receptor recognition played by providing the conformational flexibility of the AA\(^0\) loop.

**Major Determinants Conferring Specificity of Recognition—** The extracellular domains of the four TRAIL receptors show high sequence identity ranging from 45 to 81%. The residues of sDR5 involved in the TRAIL-sDR5 contacts are mostly identical or homologous to the corresponding residues in sDR4, sDCR1, and sDCR2 but not to those of other TNFR family members. A sequence comparison of sDR5 with sTNFR55 using noncysteinyl residues indicates that the sequence identity of CRD2 (12%) is lower than CRD1 (34%). CRD2 of sDR5 interacts much more extensively with TRAIL than CRD1 (data not shown). Although dominant interactions mediated by Tyr-216 of CRD1 in the TRAIL-sDR5 complex structure (data not shown) are commonly observed in the TNF\(\beta\)-sTNFR55 structure, many of the TRAIL-binding residues of CRD2 are distinctively different from the corresponding residues of sTNFR55. Therefore, it is not surprising that the receptor-binding surfaces of the TNF family members are quite different from each other, as can be appreciated by the electrostatic potentials of TRAIL, TNF\(\alpha\), TNF\(\beta\), and CD40L (figure not shown). The sequence diversity at the receptor-binding surface of the ligand family and complementary replacement of residues in the receptor family is definitely the major determinant controlling the binding specificity between the two family members. The unique interactions between TRAIL and sDR5 mediated by the AA\(^0\) loop at the central contact region, however, suggest that changes in the interacting pairs of residues may not be sufficient to warrant specific recognition in the two large superfamilies. The frame insertion can be a drastic means to alter the geometric and electrostatic properties of the receptor-binding surface as is observed in the TRAIL structure. Finally, the domain rearrangement of CRD2 appears to play a general and important role in accommodating geometric alterations arising from sequence variations in the receptor-binding sites of the TNF family ligands.

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**FIG. 5.** sDR5 binding and cytotoxic activity of wild type and Δ132–135 TRAIL. **A**, BIAcore analysis showing binding of sDR5 to wild type (thick line) or the deletion mutant (thin line) at 900 nM for both. Sensorgrams for the wild type and the mutant at 600 and 300 nM are not shown but were used to calculate \(K_D\) values. **B**, bioassay data showing decreased cytotoxic activity of Δ132–135 TRAIL (■) compared with wild type TRAIL (○). The measurements were done in triplicate, and the mean values are shown. Typical error bar is ~15% in the y scale.
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