Structural and Functional Insights of RANKL–RANK Interaction and Signaling

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*J Immunol* 2010; 184:6910-6919; Prepublished online 14 May 2010; doi: 10.4049/jimmunol.0904033

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Bone remodeling involves bone resorption by osteoclasts and synthesis by osteoblasts and is tightly regulated by the receptor activator of the NF-κB ligand (RANKL)/receptor activator of the NF-κB (RANK)/osteoprotegerin molecular triad. RANKL, a member of the TNF superfamily, induces osteoclast differentiation, activation and survival upon interaction with its receptor RANK. The decoy receptor osteoprotegerin inhibits osteoclast formation by binding to RANKL. Imbalance in this molecular triad can result in diseases, including osteoporosis and rheumatoid arthritis. In this study, we report the crystal structures of unliganded RANK and its complex with RANKL and elucidation of critical residues for the function of the receptor pair. RANK represents the longest TNFR with four full cysteine-rich domains (CRDs) in which the CRD4 is stabilized by a sodium ion and a rigid linkage with CRD3. On association, RANK moves via a hinge region between the CRD2 and CRD3 to make close contact with RANKL; a significant structural change previously unseen in the engagement of TNFR superfamily 1A with its ligand. The high-affinity interaction between RANK and RANKL, maintained by continuous contact between the pair rather than the patched interaction commonly observed, is necessary for the function because a slightly reduced affinity induced by mutation produces significant disruption of osteoclast formation. The structures of RANK and RANKL–RANK complex and the biological data presented in the paper are essential for not only our understanding of the specific nature of the signaling mechanism and of disease-related mutations found in patients but also structure based drug design. The Journal of Immunology, 2010, 184: 6910–6919.

The receptor activator of the NF-κB (RANK; TNF superfamily [TNFRSF] 11A), and its cognate ligand, RANKL, play a pivotal role in bone remodeling, immune function and mammary gland development in conjunction with various cytokines and hormones (1–5). Recently the pair was also found to be important for thermoregulation (6), demonstrating them to be one of the most versatile physiological modulators in the body. RANKL is a type I transmembrane protein, consisting of around 620 aas with ∼85% homology between mouse and human homologs (7). The extracellular region (residues 30–194) is comprised of four tandem cysteine-rich pseudo-repeat domains (CRDs) that are characteristic of the TNFRSF (8). The C-terminal 383 residues form one of the largest cytoplasmic domains in the TNFRSF. Like other members of the family this region of the protein lacks intrinsic enzymatic activity, therefore it transduces intracellular signals by the recruitment of various adaptor proteins, including TNFR-associated factors (TRAFs), leading to the activation of JNK, ERK, p38, NFATc1, AKT, and NF-κB signaling pathways (9–13). RANKL (TNF superfamily [TNFSF] 11), the only ligand binding to the extracellular portion of RANK (14) was cloned respectively by four different groups (7, 15–17) and identified as a member of the TNFRSF. It is a type II transmembrane protein, primarily expressed on the surface of activated T-cells, bone marrow stromal cells, and osteoblasts. Soluble forms of RANKL that arise from either proteolytic processing or alternative mRNA splicing have also been observed (18). Both the membrane-spanning and soluble forms of RANKL are assembled into functional homotrimers like other members of the TNFRSF. The binding of RANKL to RANK causes trimerisation of the receptor, which activates the signaling pathway and results in osteoclastogenesis from progenitor cells and the activation of mature osteoclasts (19–21).
conditions. Various pathological conditions characterized as deregulated bone remodeling are associated with an imbalance between OPG and RANKL. Thus, RANKL, RANK, and OPG provide a ligand/receptor/receptor antagonist system for controlling bone homeostasis and other related biological processes. OPG-deficient mice exhibit a decrease in total bone density and develop osteoporosis (23). Mice with a genetic mutation of *rank*, phenotypically exactly like *rankl−/−* knock-out mice (24), have severely defective osteoclast development (25), which can be restored by the reintroduction of *rank* cDNA into bone marrow progenitor cells (26). In humans, mutations in the genes encoding RANKL and RANK have been found to dramatically reduce the number of osteoclasts and cause osteopetrosis, a disease associated with a high density of bone, resulting in blindness, facial paralysis, and deafness due to the increased pressure put on the nerves by the extra bone (27, 28). Presumably the mutations disrupt the association of RANKL to its receptor (29). A human mAb to RANKL has been developed for treatments of post-menopausal osteoporosis and rheumatoid arthritis (30, 31).

Members of the TNFSF, although structurally related, show significant sequence diversity. Structures of several ligands, receptors, and ligand–receptor complexes have been resolved, including TNF-α (32), RANKL (33, 34), CD40L (35), CmE (36), TNFRSF1A (37), TNF-β–TNFRSF1A (38), and TRAIL–DR5 (39, 40) complexes. Members of the TNFRSF are homotrimERIC with a core scaffold of β-sandwich jellyroll topology, whereas members of the TNFRSF consist of variable numbers of CRDs, the majority of which comprise five irregular β-strands linked by three disulfides (41). Receptor molecules bind to the clefts between the subunits of the ligand trimer to form a heterohexamer. The published structures of ligand–receptor complexes have provided detailed information about receptor–ligand interactions and the functional mechanism at atomic resolution for these pairs of molecules. Although the three-dimensional structure of mouse RANKL shows an overall fold characteristic of TNFSF molecules, there are large structural and conformational differences in the loops that form the receptor binding cleft (33, 34). In addition, the structures of TNFR molecules have shown great domain flexibility between the CRDs as well as structural flexibility within each CRD, and there is little sequence homology among the members of the TNFRSF. The structures of RANK and the RANKL–RANK complex are therefore essential for our understanding of the basis of ligand-receptor specificity in this system and the mechanism of molecular signaling.

In this study, we report crystal structures of the extracellular region of mouse RANK alone and in complex with the ectodomain of RANKL. The structure of RANK contains four full-length CRDs and folds into an elongated shape. There are distinct features in CRD disulfide topology and domain connectivity. The structure of the RANKL–RANK complex, when compared with the TNF-β–TNFRSF1A and TRAIL–DR5 complexes, reveals that both the position and orientation of the bound receptor differ significantly, and there is little conservation in the ligand–receptor interface contacts. A sodium ion bound between CRD3 and CRD4 of RANK may be crucial for maintaining the structural integrity of the receptor and explains some of the disease-related mutations. The affinity between RANKL and RANK has been determined using Biacore analysis, and the results (Kₐ up to 10⁻¹¹ M) indicate that the pair is bound strongly together. Structure-guided mutations of RANKL show that the contribution of the individual residues tested to the binding of RANKL to RANK is directly related to RANKL signaling-dependent osteoclast formation. A slight disruption of binding between RANK and RANKL would have a dramatic effect on osteoclast formation.

### Materials and Methods

Oligonucleotides were prepared by Sangon Biotech (Shanghai, China). Restriction enzymes, T4 DNA ligase, and First Strand cDNA Synthesis Kit were purchased from Fermentas (Burlington, Ontario, Canada). Pfu DNA polymerase was obtained from Taigen Biotech (Beijing, China). Glutathione (reduced and oxidized) was purchased from Sigma-Aldrich (St. Louis, MO).

The cDNA coding for the extracellular domain of murine RANK (residues 26–210) was obtained by RT-PCR from the mRNA of mouse RAW264.7 cells and cloned into pET28a vector (Novagen, Madison, WI). The expression plasmid for GST–RANKL (encoding residues 159–361 of mouse RANKL as a fusion with Glutathione S-transferase) was a gift from Prof. Fremont (Washington University School of Medicine, St. Louis, MO). RANKL was expressed as a His₆ tag at each terminus of the protein. Site-directed mutagenesis of the *rankl* was performed using QuickChange Kit supplied by Stratagene (Agilent Technologies, Palo Alto, CA). The mutants were verified by DNA sequencing. *Escherichia coli* strain BL21-Gold (DE3) was used to express the recombinant proteins.

The recombinant RANK was produced as inclusion bodies that were dissolved by sonication in 6 M guanidine hydrochloride, 50 mM Tris (pH 8.5), 1 mM EDTA, 150 mM NaCl, and 10 mM DTT to a protein concentration of ~30 mg/ml at room temperature. The refolding of recombinant RANK was performed at 4°C by diluting the solubilized protein in 20 mM Na₂HPO₄ (pH 7.3), 1 M t-ariginine, 20% glycerol, 10 mM reduced glutathione, and 1 mM oxidized glutathione, followed by sequential dialysis against 20 mM Na₂HPO₄ (pH 7.3), 0.5 M t-ariginine, and 10% glycerol for 12 h, 20 mM Na₂HPO₄ (pH 7.3), 0.2 M NaCl, 5% glycerol for 12 h, and finally twice against 20 mM Na₂HPO₄ (pH 7.3) for 12 h. After centrifugation at 20,000 g for 10 min, the supernatant was further purified by size exclusion chromatography (Superdex 200, GE Healthcare) and the correctly refolded RANK was collected and analyzed by SDS-PAGE.

The soluble extracellular domain of mouse RANKL was expressed as a GST fusion protein, purified by affinity purification with glutathione-Sepharose fast flow 4B beads (GE Healthcare) according to the manufacturer’s protocol and the tag cleaved with PreScission protease (GE healthcare). The cleaved RANK was further purified by size exclusion chromatography (Superdex 200) in Tris pH 7.0.

### Crystallization and data collection

Purified RANKL and RANK were concentrated to 10 mg/ml in with 0.1 M Tris at pH 7.0. Crystallization screens of RANK and RANKL–RANK complex were performed at a temperature of 294 K using nanoliter sitting drop vapor diffusion in the crystallization facility of the Oxford Protein Production Facility (42). The best RANK crystals were grown in 10% polyethylene glycol 3350, 15% polyethylene glycol 5000, 0.1 M ammonium sulfate, 0.1 M sodium tartrate, and 0.05 M MES at pH 6.5. Crystals of RANKL–RANK complex were grown in 0.1 M sodium dihydrogen phosphate, 2 M sodium chloride, 0.1 M potassium dihydrogen phosphate, and 0.1 M MES (pH 6.5), using a 1:1 molar ratio of RANKL and RANK (39), 20 mg/ml. Details of protein purification and crystallization have been published elsewhere (43).

X-ray diffraction data for RANK were collected at beamline BM14 at the ESRF (Grenoble, France). A total of 180 images of 1° oscillation were collected from a single crystal at a wavelength of 0.954 Å. X-ray diffraction data of the RANKL–RANK complex were collected at two ESRF beamlines; 130 images of 1° oscillation from one crystal were collected at beamline ID14-4 at a wavelength of 0.940 Å, and 180 images of 1° oscillation were collected from two positions of a single crystal at ID23-2 operated at a wavelength of 0.873 Å. In all cases, 25% glycerol was added to the crystallization drops as cryoprotectant, and crystals were frozen and maintained at 100 K by a stream of nitrogen gas during data collections. Data images were indexed, integrated, and merged using HKL2000 (44). The statistics for x-ray data are given in Table I.

### Structure solution and refinement

The space group of the RANK crystals is P2₁2₁2₁ with unit cell dimensions of a = 39.8 Å, b = 94.3 Å, and c = 102.4 Å. The RANKL–RANK complex belongs to a hexagonal space group of P6₃ with unit cell dimensions of a and b = 121.2 Å and c = 94.7 Å. The structure of the complex was solved first using RANKL monomer (33) as a search model for molecular replacement with MOLREP (45) of the CCP4 program suite (46). There is one RANKL subunit and one RANK molecule in the crystal asymmetric unit, giving a solvent content of 74%. The 3-fold axis of the heterohexameric RANKL–RANK complex is aligned with the crystallographic 3-fold axis. The initial difference electron density map calculated from this partial...
model clearly showed the RANK polypeptide and was of sufficient quality to allow the RANK molecule of four CRDs to be built. The structure of RANK in the complex was then used to solve the crystal structure of RANK alone. However, structure solution using molecular replacement was not straightforward because of the thin elongated shape and the flexibility of the molecule. The correct solution was only found by using the central two CRDs with the program PHASER (47). The initial R factor was 0.54 for data from 30 to 4.0 Å. There are two molecules in one crystal asymmetric unit related by a local 2-fold rotation axis with the N terminus of one molecule interacting with the C terminus of the other. Superposition of the RANK model from the complex onto the molecular replacement solution resulted in a large number of clashes between the first and fourth CRDs, indicating large conformational changes. Nevertheless, the first and fourth CRDs were built after a round of refinement using all data to 2.0 Å resolution. Both structures were refined with the crystallography and NMR system (48) using simulated annealing, conjugate gradient minimization, and individual isotropic B factor refinement, followed by model rebuilding and solvent molecule addition with COOT (49). Because of the large conformational differences between the two molecules of the RANK, no non-crystallographic symmetry restraints were applied during refinement. The final refined structures of both RANK and RANKL–RANK complex have good crystallographic R factors and stereochemistry as shown in Table I.

Surface plasmon resonance

The affinities of RANKL and its mutants for the receptor, RANK, were measured using Biacore 3000 (GE Healthcare) according to the published protocol (50). Briefly, an NTA chip (GE Healthcare) was charged with 0.3 M NiSO₄, and then RANK (50 nM, 1 μl) was injected into the channel to load. Recombinant TNFRSF9 with two His-tags was injected into a different channel as a control. Different concentrations of RANKL or its mutants (0, 1.88, 3.75, 7.5, 15, and 30 nM, 30 μl) were injected into both channels. All steps were performed at 25°C, and signals were recorded as sensorgrams. Sensorgrams were fitted into the 1:1 binding model using BIA evaluation software 4.1 (Biacore, GE Healthcare), and the equilibrium dissociation constants (Kd) calculated.

Osteoclast formation and tartrate-resistant acid phosphatase staining

The murine monocytic cell line RAW264.7 (American Type Culture Collection, Manassas, VA) was cultured in a humidified incubator (5% CO₂ in air) at 37°C, and maintained in α-MEM containing 10% (v/v) heat-inactivated FCS. For osteoclastogenesis experiments (20), cells were seeded into a 24-well tissue culture plate (2 × 10³/well) in the presence or absence of 50 ng/ml RANKL or its mutants for 4 d. The cells were then fixed and stained using the Acid Phosphatase, Leukocyte (tartrate-resistant acid phosphatase [TRAP]) Kit (Sigma-Aldrich, 387A) according to the manufacturer’s instructions. The numbers of TRAP-positive, multinucleated (>3 cells per well) were counted under a light microscope as described (51). Mouse bone marrow-derived monocytes were isolated from 7-wk-old BALB/c mice, cultured in α-MEM containing 10% FCS, and plated in a 10-cm petri dish overnight (52). The following day, nonadherent cells were collected, washed, and seeded into a 24-well tissue culture plate (5 × 10⁵/well) with 20 ng/ml macrophage-CSF (M-CSF) in the presence or absence of 50 ng/ml RANKL or its mutants. From the fourth day, the medium was changed daily with fresh α-MEM containing 10% FCS, 20 ng/ml M-CSF, and 50 ng/ml RANKL or its mutants. Cells were then fixed on the eighth day and stained using the TRAP staining kit as before.

Results

Structure determinations

The extracellular domain of RANK has been crystallized both alone and in complex with RANKL. The crystals of RANK and the RANK–RANKL complex diffracted to 2.0 Å and 2.8 Å, respectively, using synchrotron radiation. The structure of the complex was determined first using molecular replacement with the published structure of RANK as an initial model (33, 34). The crystallographic asymmetric unit contains one molecule of RANK (residues 35–199) and one subunit of RANKL (residues 161–316), which are assembled to form the biological heterohexameric complex through 3-fold crystallographic symmetry. The model has been refined to an R factor of 18.2% (Rfree of 21.2%) with root mean square deviations (rmsds) from ideal values of 0.007 Å for bond lengths and 1.0° for bond angles (Table I). The unliganded structure of RANK was solved using the receptor from the complex as the search model and has been refined to an R factor of 20.7% (Rfree of 23.7%) with rmsds of 0.007 Å for bond lengths and 1.1° for bond angles. There are two RANK monomers related by noncrystallographic 2-fold symmetry perpendicular to the long axis of the molecules in the asymmetric unit. The final model consists of residues 33–201 in one monomer and residues 36–176 and 186–194 in the second monomer (Fig. 1).

The structure of RANK

The extracellular regions of members of the TNFRSF adopt elongated structures of variable numbers of pseudorepeats of CRDs. A typical CRD, normally ~40 residues, consists of five irregular β-strands linked typically by three interstrand disulphides and can be further divided into two structural modules of various types defined by topology and number of disulphides (41). RANK contains
four such CRDs spanning a length of 100 Å, the longest among the structures of the TNFR family determined to date. RANK CRD1 (residues 35–71) is comprised of so-called A1-B2 modules, whereas CRDs 2–4 (residues 72–114, 115–154, and 155–197, respectively) are all made of A1-B1 modules, where A and B define the module topology and 1 and 2 the number of disulphides (41). Of the members of TNFR family with full-length CRD1, crystal structures of TNFRSF1A, OX40, and CrmE (53, 36, 54), either alone or in complex with ligand, have been determined. CRD1 is the most structurally conserved region among these receptors; >90% of Cα atoms can be overlapped with rmsds ranging from 1.0 Å to 1.4 Å despite the low sequence identity. Apart from the six conserved cysteines, Tyr41 and Gly54 of RANK are the only fully conserved residues among the CRD1 domains of these four proteins. Tyr41, positioned in the middle of the second strand of the A1 module, makes hydrophobic interactions with the first disulphide of the B2 module as well as a hydrogen bond to the highly conserved Ser67 (threonines in the other three structures) from the fifth strand. Ser67, in turn, binds to the carboxyl group of Ser49 positioned between the third and fourth cysteines in the third strand, indicating an important role of Tyr41 for stabilizing the relative position and orientation of the two modules. In contrast, Gly54 acts to strengthen the interactions between CRD2 and CRD3. It is the third residue of a tight turn linking strands 3 and 4 and makes both hydrophobic interactions with the first disulphide and main-chain hydrogen bonds to the amide group of Cys72 and the carbonyl group of Leu78 from CRD2. These interactions are conserved between these four receptors, and also observed in DR5 (40).

The A1-B1 modules of CRDs 2–4 in RANK do not have the third and fifth cysteines (the 3–5 disulphides); in contrast to the only previously experimentally observed A1-B1 module of CRD3 in OX40 that lacks the 4–6 disulphides (53). The two missing cysteines are substituted by aromatic and glycine residues in CRD3 (His90 and Gly105) and CRD4 (Trp173 and Gly187), replacing the disulphide constraint by ring-stacking hydrophobic and hydrogen bond interactions. As a result, the B1 modules in these two domains are structurally very similar to the B2 module (Fig. 2). In contrast, the B1 module in CRD3 of RANK is similar to the CRD3 B1 module of CrmE (36), in that the two cysteines are not replaced by aromatic and glycine residues so that the topological constraint by the disulphide is not compensated for, and the module adopts a much broader conformation (Fig. 2D). In addition, the β2β3 loop (residues 119–132) of CRD3 that makes the key contacts with the ligand (corresponding to the 90S loop of DR5 and residues 103–108 of TNFRSF1A) possesses an intrastrand disulphide formed by Cys125 and Cys127 (Fig. 2F). β2 (residues 119–122) and β3 (residues 127–130) form a regular antiparallel β-sheet linked by a four residue turn. Cys125, the third residue of the turn, is so close to Cys127 that the plane of the turn is almost perpendicular to the β-sheet. This unusual conformation is stabilized by hydrogen bonds from the side chain of Asn122 to the amide groups of residues 124 and 125, and a π-stacking of Trp121 with one side of the β-sheet (Fig. 2F).

Both the number and positions of cysteine residues in the extracellular regions of mouse and human RANKs are conserved, and it would be expected that all four CRDs in the human molecule are comprised of the same structural modules as found in mouse. The CRD3 domain of human RANK has, however, been wrongly predicted to contain A1-B1 modules lacking the 4–6 disulphides because of sequence misalignment (53), highlighting the limitations of sequence alignment.

There are significant conformational differences and rigid-body movements apparent when the three independent copies of RANK are compared (two [A and B] from the unliganded crystal asymmetric unit and one [chain R] from the complex). The CXC motif linking the ligand binding CRD2 and CRD3 in both TNFR1 and DR5 has previously been identified as a hinge region that allows the two CRDs to orientate and position themselves onto the binding regions of the ligands (40). This motif is also conserved in RANK and the hinge region appears to extend into the C-terminal half of the CRD2 B1 module (Fig. 2A). The difference in relative orientation between CRD2 and CRD3 is 20˚ between the two unliganded copies, and these differ by 49˚ and 32˚ from the liganded molecule (Fig. 2A). In contrast, there is little rigid-body movement between CRD1 and CRD2, and between CRD3 and CRD4. It is interesting to note that the CRD1-CRD2 and CRD3-CRD4 junctions both have a CXXC motif, one residue longer than the CRD2-CRD3 linker. The two cysteine residues at the domain junctions are actually located in the same strand (β5) positioned on one side of the β3β4 loop. The longer CXXC linker enables the β2β3 loop of the second domain to contact the other side of the β3β4 loop and stabilize the two domains. In contrast, the CRD3 and CRD4 of TNFRSF1A (like CRD2 and CRD3 in RANK) are linked by a CXC motif such that the β2β3 loop is unable to closely interact with the β3β4 loop of CRD3 (Fig. 2F, 2H).
The structure of RANK. A. A diagram showing the structural differences and highlighting the flexibility of the three copies of RANK. The superposition was performed using CRD1 and CRD2 only. The RANK molecule in the complex is shown in red and the two copies in the crystal of RANK are shown in yellow and blue. B. The structure of TNFRSF1A CRD2 comprised of A1-B2 modules (pdb ID, 1ext), a typical CRD with five irregular β-strands linked by three interstrand disulphides. The side chains of key residues for structure stability are drawn as sticks with the position of each cystine marked and numbered. The backbone direction is indicated by arrows. C–E. The structures of RANK CRDs 2–4, which are all comprised of A1-B1 modules lacking a 3–5 disulphides. F. Close-up view of the β2β3 loop of CRD3, shows how the loop is stabilized by an intrasstrand disulphide and a network of hydrogen bonds (yellow broken lines). G, The coordination of the Na⁺ binding site. H and I. Comparison of the relative conformations between CRD3 and CRD4 in TNFRSF1A (H) and RANK (I). In TNFRSF1A, the CXC linker between the two domains positions, the β2β3 loop of CRD4 on the left side of the β3β4 loop of CRD4, allows little interaction between the two domains, whereas the CXC linker in RANK enables the β2β3 loop of CRD4 to be positioned on the right side of the β3β4 loop of CRD3 and makes a number of stabilizing interactions.

Superposition of the first two RANK CRDs of chain A with those of chain B results in an rmsd of 1.3 Å for 77 equivalent Cα atoms (1.5 Å for 75 Cα atoms and 1.2 Å for 78 Cα atoms, respectively, when the two chains are compared with the first two domains of chain R). Large conformational differences occur in the β4β5 loop (residues 60–67) of CRD1 and the hinge region of the B1 module (residues 90–114) of CRD2. Overlaps of CRDs 3–4 of chain R with the corresponding regions of chain A and B show rmsds of 0.9 Å for 75 Cα atoms (of 83) and 0.7 Å for 69 Cα atoms (of 73), respectively. The rigidity between CRD3 and CRD4 is further enhanced by a metal ion bound between the two domains. This metal ion exists in all three copies of RANK and has an octahedral coordination provided by the carboxyl oxygen atoms of Cys134, Ala135, and Phe138 from the β3β4 loop of CRD3, the hydroxyl group of Ser161, and the carboxyl oxygen of Val163 from the β2β3 loop of CRD4 and a water molecule. The bond distances range from 2.35 to 3.00 Å (Fig. 2G). This ion is assigned to be a Na⁺ based on the average calcium bond-valence sum of 1.2 calculated from the averaged bond lengths from the three binding sites (expected values: 1.6 for Na⁺, K⁺ 0.6, Ca²⁺ 2.0, Mn²⁺ 3.2, and Mg²⁺ 4.2) (55). This assignment is supported by the anomalous difference map calculated using data collected at a wavelength of 1.698 Å, which shows electron density peaks for all ordered sulfur atoms but not for this ion (f''; S 0.7e⁻, Na 0.2e⁻, K 1.4e⁻, Mg 0.2e⁻, Mn 3.5e⁻, and Ca 1.6e⁻). It is expected that this Na⁺ is conserved in human RANK based on the sequence similarity.

The structure of RANK

Each subunit of the trimeric RANKL adopts a typical β-sandwich with jellyroll topology consisting of two five-stranded antiparallel β-sheets. The first β-sheet, containing strands A”, A, H, C, and F, is involved in trimer formation by making intersubunit contacts. The second β-sheet contains strands B’, B, G, D, and E and forms the outer surface. The two β-sheets form a core scaffold that is highly structurally conserved, whereas the loops linking the β-strands are involved in receptor binding and highly variable within the TNF family. Superposition of the RANKL monomer from the complex with the previously published structure of RANKL gives an rmsd of 0.5 Å for 145 Cα atoms of 156 residues. As expected, conformational changes are only observed in the surface loops of the molecule, including the AA”, CD, DE, and EF loops, all of which are involved in interactions with the receptor (Fig. 1B). The largest conformational change observed is in the CD loop, where the Cα atom and the ring center of Tyr234 are shifted by 3.3 Å and 6.5 Å, respectively.

The RANKL–RANK complex

RANKL and RANK form a heterohexameric complex with a receptor molecule bound along each of the three clefts formed by neighboring monomers of the ligand homotrimer. Of the four CRDs of RANK, only the middle two are involved in direct contacts with the ligand. Superposition of RANKL–RANK with TNF-β–TNFRSF1A and TRAIL–DR5 complexes reveal that, although the RANK CRD2 is bound in a similar orientation as its counterparts in TNFRSF1A and DR5, there is a large difference in the orientation of CRD3, with a tilt of some 45° and 11° away from the ligand, whereas the position of RANK as a whole is ~2 Å lower (Fig. 3A).

Each of the three interfaces buries 2660 Å² solvent accessible surface area, 1290 Å² from the ligand, and 1370 Å² from the receptor. Of the surface area buried on RANKL by each receptor, 540 Å² is on subunit A and 780 Å² on subunit B, whereas, of the area buried on the receptor, 840 Å² is from CRD2 and 530 Å² from...
CRD3 (Fig. 3B). The areas involved in the interactions in both receptor and ligand are significantly different from the two published structures of TNF family complexes. In the TRAIL–DR5 complex, the buried area on TRAIL is contributed not only equally by the two repeats of the receptor, but also shared equally between the two ligand subunits. In the case of TNF-β–TNFRSF1A complex, the CRD2 and CRD3 of TNFRSF1A contribute in a similar ratio to their counterparts of RANK, but they are made approximately equally to the two ligand subunits. A unique feature of the RANKL–RANK complex is that the solvent accessible areas buried on the receptor and on subunit B of the ligand are continuous, contrasting with the TNF-β–TNFRSF1A and TRAIL–DR5 complexes, where contact regions are discontinuous and form two distinct patches on both ligands and receptors.

All solvent accessible loops bridging the β-strands in RANKL, apart from A”B’, B’B, and BC, are involved in receptor binding. These loops are structurally unique in terms of length and conformation and there is little sequence conservation compared with other members of the TNF family. Residue contacts from the β2β3 loop, the β3 strand of CRD2 and the β2β3 loop of CRD3 contribute most of the interactions. Residues involved in the ligand-receptor interactions are mainly hydrophilic in nature (34 of 50), forming 9 hydrogen bonds and 12 salt bridges as well as the majority of hydrophobic contacts.

The top patch of RANKL subunit A interacts with the receptor through the AA” and CD loops to the β2β3 loop of CRD3 (Fig. 3C). The interactions between the AA” loop and the β2β3 loop are predominantly hydrophilic; Asp124 in the β2β3 loop makes a salt bridge to His179 and a hydrogen bond to the main chain of Lys180, whose side chain, in turn, forms a salt bridge with Glu126 and a hydrogen bond to the carbonyl oxygen of Ser123. The interactions from the CD loop are mainly van der Waals contacts; Tyr240 is fully buried, largely by Glu126 of the receptor. Tyr234 undergoes the largest conformational change on receptor binding, making side chain stacking interactions with Arg129, which, in turn, is stabilized by a salt bridge with Glu268 from the EF loop of subunit B. The unique intrastrand disulfide Cys125-Cys127 of the receptor is solvent inaccessible, contacting both the CD loop of subunit A and the EF loop of subunit B. The AA” loops of TNFSF and the β2β3 loops of TNFRSF members are both structurally the most divergent, having the greatest number of amino acid deletions and insertions. The AA” loop in RANKL folds toward the top third of the molecule and is positioned above the β2β3 loop of the receptor. In contrast, the much longer AA” loop of TRAIL runs across the middle surface of the ligand and lies below the β2β3 loop to make a salt bridge from Arg149 to Glu147 of DR5, whereas the same loop in TNF-β is very short and does not make any interaction with TNFRSF1A (38). Deletion mutation of the AA” loop in both RANKL and TRAIL completely abolishes biological activity (33, 40). The structural diversity between the members of the TNF family, charged interactions, and mutagenesis all suggest that the AA” loop confers specificity.

The contact area on the lower part of RANKL subunit A is mediated by residues Ile248 and His252 of the DE loop to Glu84 and Leu88 of the β2β3 loop of CRD2. Ile248 corresponds to Tyr108 in TNF-β and Tyr214 in TRAIL and has been predicted to make strong hydrophobic interactions with the receptor analogous to those in the TNF-β–TNFRSF1A and TRAIL–DR5 complexes.
RANK and RANKL is very high with a Kd of 6.8 x 10^{-11} M. An Ile248 Asp mutation in mouse RANKL, however, showed only an 8-fold decrease in activity (33). Ile248 has direct contact with a charged residue (Glu84) of RANK, at an equivalent position to Leu68 of TNFRSF1A. The interaction is centered on Leu89 of TNFRSF1A and Leu57 of DR5, and the reduction in activity is likely due to the introduction of an electrostatic repulsive force. The DE loop is one of the regions that has the highest B factors and the side chains of Lys247 and Ile248 do not have well-defined electron density. It is likely that in RANKL, unlike other members of the TNFSF, the DE loop is not critical for receptor binding. Arg283 of the FG loop in RANKL forms a salt bridge with Asp85 that is at an equivalent position to Arg68 of TNFRSF1A and Leu58 of DR5 (Fig. 4). The FG loop is not involved in receptor binding in either TNF-β or TRAIL.

There are two key interface areas between subunit B and the receptor. One, at the lower part of the interface, is mediated by the β1β2 loop and β3 of CRD2 of the receptor that interacts with the GH loop and the N and C termini of the AA" loop from the ligand. The interaction is centered on Leu89 that nests in a hydrophobic pocket formed by Tyr187, Arg190, and Glu302 of RANKL (Fig. 3D). This interface area, together with the two salt bridges formed from Asp94 and Lys97 of β3β4 loop at the C-terminal part of CRD2 to residues Arg222 and Asp229, appears to be the determinant for the position and orientation of CRD2. The second key interface area is centered on His224 of the CD loop that is located in a pocket formed between the two ligand-binding repeats of the receptor (Fig. 3E). Residues lining the pocket include Ala98 from the β3β4 loop of CRD2, Tyr119 from the β1β2 loop, the first disulphide Cys115-Cys128, and Cys128 of CRD3. The hydrophobic interactions centered on His224 are sandwiched by two clusters of charged interactions: the two salt bridges mentioned previously, and two additional salt bridges made from Glu225 of the CD loop and Glu268 of the EF loop to Arg129 and Arg130 of the β3 strand of CRD3. The CD and AA" loops, located opposite each other across the receptor binding cleft, act as two anchor points for the CRD3 of the receptor. The interactions centered on His224 are not observed in the TNF-β–TNFRSF1A or in the TRAIL–DR5 complexes, because of the different orientations of the receptors.

High-affinity binding is critical for functional osteoclast formation

The affinity of RANKL and RANK was measured by Biacore with RANK immobilized in a channel of a chelating NTA sensor chip. As seen in Fig. 3A, the affinity between RANK and RANKL is very high with a Kd of 6.8 x 10^{-11} M.

To elucidate the critical residues responsible for this very tight binding, and the contribution of the binding affinity to functional osteoclast formation, the following RANKL mutants were made according to the buried area on complex formation (Fig. 3B): Asn266Ala, 1–20%; Glu225Ala, 40–60%; Arg222Ala, 60–80%, and Asp299Ala 80–100%. All four of these residues interact with the receptor via either hydrogen bonds or salt bridges. The binding affinities of RANKL mutants for immobilized RANK were measured as before. The binding affinities of Glu225Ala, Arg222Ala, and Asp299Ala RANKL mutants for RANK are dramatically decreased by >100-fold (Fig. 5A) and these mutants have completely lost their ability to promote functional osteoclast formation (Fig. 5B). In contrast, amino acid Asn266 (marked in blue on Fig. 3B) contributes moderately to binding with <20% buried area. Its mutant Asn266Ala only marginally affects its binding to RANK (Kd 8.8 x 10^{-11} M compared with a Kd of 6.8 x 10^{-11} M for wild type). Interestingly, this slightly reduced affinity between RANKL and RANK significantly affects the ability of RANKL to promote osteoclast formation (Fig. 5B, 5C), demonstrating that a strong association between RANK and RANKL is prerequisite for proper RANK signaling and subsequent osteoclast formation.

Discussion

Members of the TNFSF adopt the same trimeric structural scaffold with each receptor-binding cleft formed between two neighboring ligand subunits. Receptor binding is mediated predominantly by surface loops with little sequence homology and much structural divergence between family members. The multidomain TNFRs possess two ligand-binding CRDs with a similar overall fold (but differing disulphide topology) and a flexible CXC domain junction. Thus, for a given TNF ligand-receptor pair, the structural diversity of the ligand surface loops is coupled to structural variations and domain flexibility of the receptor, leading to a distinct, specific, binding mode. It is therefore unsurprising that ligand–receptor interactions in the RANKL–RANK complex are significantly different to the structurally known complexes of TNF-β–TNFRSF1A and TRAIL–DR5.

The majority of residues involved in complex formation are hydrophilic in nature, achieving both surface and electrostatic complementarity. Of the three key interface areas identified in the RANKL–RANK complex (the AA" loop mediated interactions with the β3β3 loop of CRD3, the area centered on His224 of the ligand, and the hydrophobic contacts centered on Leu89 of the receptor) none is conserved in the other two complexes, giving an indication of the complexity of ligand–receptor binding in the superfamily. Our observations are in agreement with the notion that the interactions of the β3β3 loop of CRD3 with the ligand may have an important role in controlling the specificity and cross-reactivity among the superfamily members, but do not support the proposal that the hydrophobic interaction between the DE loop of the ligand and the β2β3 loop of CRD2 of the receptor, as observed in the complexes of TNF-β–TNFRSF1A and TRAIL–DR5, is a general feature important for binding in the superfamily (39).

The decoy receptor OPG is a soluble protein containing four N-terminal CRDs, followed by two death domains and a C-terminal basic domain. It has been shown that in vivo the protein exists in two states: as a homodimer cross-linked via C-terminal cysteines or as a C-terminal truncated monomer, both of which appear to have

![FIGURE 4. Sequence alignment of the interface regions. The mouse RANK (mRANK) is aligned with its human equivalent (hRANK), the decoy receptors of both species (mOPG and hOPG) and human TNFRSF1A and DR5 (hTNR1, hDR5). Residues known to be directly involved in interactions are shown in red.](http://www.jimmunol.org/)

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80 90 100 120

mRANK: CG-POEYDLTVNEDKCLLH-VCDAGKALV
hRANK: CG-POEYDLTVNEDKCLLH-VCDAGKALV
mOPG: CF-DHSITDSHTDSDCYESCFVCEKVVLQV
hOPG: CF-DHSITDSHTDSDCYESCFVCEKVVLQV
mTR1: CE-SGGPTASENVLHSLGLCCK-CKRMADGVQ
hTR1: CE-SGGPTASENVLHSLGLCCK-CKRMADGVQ
mDR5: CHYCGQDYTHNMLLLFCRLCTR-CD--GEV
hDR5: CHYCGQDYTHNMLLLFCRLCTR-CD--GEV

CTAGYHRNS---CCRECRMTRCAPEGF
CTAGYHRWS---CCRECRMTRCAPEGF
CREEBYL1---IEIFCSDR-SCPDS
CREEBYL1---IEIFCSDR-SCPDS
CREEYKREVNNENY-FNC5-LCLNT2
CREEYKREVNNENY-FNC5-LCLNT2

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similar specific activities in the inhibition of osteoclastogenesis (59). However, in a more recent report it has been shown that the dimerization of OPG is a result of noncovalent interactions mediated by the two death domains, and the dimer binds RANKL with an affinity of three orders of magnitude tighter than the monomer lacking the death domains. One dimer interacts with one RANKL trimer by occupying two of the three binding sites on the ligand (60). Nevertheless, aligning the OPG sequence with our structure of RANKL–RANK complex suggests that OPG would bind RANKL via its CRD2-3 in a similar mode to RANK (Fig. 4). The CRD2 of OPG has the same disulfide connectivity as the first three CRDs of TNFRSF1A, comprising A1-B2 structural modules; whereas OPG CRD3 is made of A1-B1 modules. The Asn131 and Leu144 at the third and fifth cystine positions of RANK CRD3 are however substituted by a histidine and a glycine in OPG; replacing the disulfide constraint by stacking and hydrogen bond interactions. The CRD3 of OPG is thus expected to be structurally similar to the CRD2 of RANK (Fig. 2C). Most of the key structural features observed in the RANKL–RANK complex are expected to be conserved in the RANKL–OPG complex, despite the two receptors having only 30% sequence identity. The sodium ion bound between these two CRDs may play an important role in the RANKL–RANK signaling by maintaining the structural integrity of these two domains.

Bone remodeling is a dynamically equilibrated process regulated by the RANKL/RANK/OPG system. Perturbation of the process by mutations in genes of the molecular system results in various bone diseases. The structure of the RANKL–RANK complex is essential for our understanding of the structural mechanism of these disease related mutations. Four autosomal-recessive osteopetrosis-related mutations in the extracellular region of RANK have been reported recently: Gly53Arg, Arg129Cys, Arg170Gly,
forms of human osteopetrosis linked to mutations in the RANK.

RANK being continuous, whereas for the other examples, the presentation of the receptor and, notably, in the conformation of the overall architecture to that observed for other members of the plex between RANK and its cognate ligand RANKL is similar in the RANK–RANKL complex. Our data show that although the structures of the extracellular domain of mouse RANK and of the C-terminal deletion mutation causes loss of two central strands results in loss of the jellyroll scaffold, likely resulting in an unfolded ligand. The C-terminal deletion mutation causes loss of the ligand binding residues Ser161 and Val163 (Fig. 2). The side chain of Met199 in a hydrophobic core between the two β-sheets and has direct contact with the backbone of Phe165 that stacks against Phe213 and Phe280 from a neighboring subunit. Mutation of Met199 to a charged residue in a hydrophobic environment is expected to cause local conformational changes and disturb the trimer interface.

In summary, we have elucidated at atomic resolution the structures of the extracellular domain of mouse RANK and of the RANK–RANKL complex. Our data show that although the complex between RANK and its cognate ligand RANKL is similar in overall architecture to that observed for other members of the TNFSF, there are significant differences in the position and orientation of the receptor and, notably, in the conformation of the bound RANK. This leads to each interaction surface in RANK–RANK being continuous, whereas for the other examples, the interaction consists of two distinct patches. Mutations of individual residues of RANKL involved in receptor binding demonstrate their functional significance in terms of osteoclastogenesis. The structural information obtained additionally helps to explain some forms of human osteopetrosis linked to mutations in the RANK and RANKL genes.

Acknowledgments

We thank the staff at the United Kingdom beamline, BM14, and at ID14-EH4 and ID23-EH2 ESRF, Grenoble, France, for help with data collections and Prof. Fremont for GST–RANKL construct.

Disclosures

The authors have no financial conflicts of interest.

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