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Structural and Functional Insights of RANKL–RANK Interaction and Signaling

Changzhen Liu,*,†,‡, Thomas S. Walter,‡,§, Peng Huang,§, Shiqian Zhang,§, Xuekai Zhu,*‡, Ying Wu,*§, Lucy R. Wedderburn,‖, Peifu Tang,§, Raymond J. Owens,§, David I. Stuart,§, Jingshan Ren,‡, and Bin Gao*,‡,‖

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.

The binding of RANKL to RANK causes trimerisation of the receptor and promotes formation of functional homotrimers like other members of the TNFSF. membrane-spanning and soluble forms of RANKL are assembled by the interaction of its extracellular region (residues 24–194) with the receptor, which activates the signaling pathway and results in the activation of JNK, ERK, p38, NFATc1, AKT, and NF-κB proteins, including TNFR-associated factors (TRAFs), leading to intracellular signals by the recruitment of various adaptor proteins, including TNFR-associated factors (TRAFs), leading to transduction of intracellular signals by the recruitment of various adaptor proteins, including TNFR-associated factors (TRAFs), leading to cell death.

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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 control-
ing bone homeostasis and other related biological processes.

OGP-deficient mice exhibit a decrease in total bone density and
develop osteoporosis (23). Mice with a genetic mutation of rank,
phenotypically exactly like rank<sup>−/−</sup> knock-out mice (24), have
severely defective osteoclast development (25), which can be re-
stored 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 osteoporosis, a disease associ-
ated 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 dis-
rupt 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 sig-
nificant sequence diversity. Structures of several ligands, receptors,
and ligand–receptor complexes have been resolved, including
TNF-α (32), RANKL (33, 34), CD40L (35), Cmre3 (36), TNFRSF1A
(37), TNF-β–TNFRSF1A (38), and TRAIL–DR5 (39, 40) com-
plexes. Members of the TNFSF are homotrimers with a core scaf-
doll of β-sandwich jelly roll topology, whereas members of the
TNFRSF consist of variable numbers of CRDs, the majority of
which comprise five irregular β-strands linked by three disulphides
(41). Receptor molecules bind to the clefts between the subunits of
the ligand trimer to form a heterotrimer. The published structures
of ligand–receptor complexes have provided detailed information
about receptor–ligand interactions and the functional mechanism
at atomic resolution for those 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 bind-
ing cleft (33, 34). In addition, the structures of TNFR molecules have
shown great domain flexibility between the CRDs as well as struc-
tural flexibility within each CRD, and there is little sequence homol-
ogy 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 sys-
tem 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 fea-
tures in CRD disulphide 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 main-
taining 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 (KD up to 10<sup>−11</sup> 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
Cloning, protein expression, and purification

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 purchased from Tiangen Biotech (Beijing, China). Glu-
thionate (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 (encording 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 with a His<sub>6</sub> 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, 200 mM NaCl, and 10 mM DTT to a protein concentra-
tion 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 Na<sub>2</sub>HPO<sub>4</sub>
(pH 7.3), 1 M l-arginine, 10 mM reduced glutathione, and
1 mM oxidized glutathione, followed by sequential dialysis against 20 mM
Na<sub>2</sub>HPO<sub>4</sub> (pH 7.3), 0.5 M l-arginine, and 10% glycerol for 12 h, 20 mM
Na<sub>2</sub>HPO<sub>4</sub> (pH 7.3), 0.2 M l-arginine, and 50% glycerol for 12 h, and finally
twice against 20 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.3) for 12 h. After centrifugation
at 20,000g 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 manufactur-
er’s protocol and the tag cleaved with PreScission protease (GE Health-
care). The cleaved RANKL 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 nano-liter sitting
drop vapour diffusion in the crystallization plates of the Oxford Freezefac-
Production Facility (42). The best RANK crystals were grown in 10% poly-
ethylene glycol 3350, 15% polyethylene glycol 5000, 0.1 M ammon-
ium 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 RANK and RANKL
(10 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 1.0° 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 beam-
lines; 130 images of 1.0° oscillation from one crystal were collected at
beamline ID14-4 at a wavelength of 0.940 Å, and 180 images of 1.0° oscil-
lation were collected from two positions of a single crystal at ID23-2 oper-
atived 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 main-
tained 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 P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> with unit cell dimensions
of a = 39.8 Å, b = 94.3 Å, and c = 102.4 Å. The RANKL–RANK complex
were 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 heterohexa-
meric RANKL–RANK complex is aligned with the crystallographic 3-fold
axis. The initial difference electron density map calculated from this partial
structure solved using the Buccaneer program (47). The RANKL–RANK
complex was refined to R<sub>work</sub> = 24.9% and R<sub>free</sub> = 28.7% for the
expanded dataset.

Downloaded from www.jimmunol.org on January 24, 2011
CRDs were built after a round of refinement using all data to 2.0 Å resolution—indicating large conformational changes. Nevertheless, the first and fourth CRD involving a large number of clashes between the first and fourth CRDs, presumably one molecule interacting with the C terminus of the other. Superposition of a unit related by a local 2-fold rotation axis with the N terminus of one RANK molecule indicates 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 noncrystallographic 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 NaN3 and then RANK (50 nM, 15 μl) was injected into the channel to load. Recombinant TNFRSF17 with two His-tags was injected into a different channel to serve 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 × 104/well) in the presence or absence of 50 ng/ml RANKL or its mutants. 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 RANKL 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 hetero-hexameric 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

| Table I.  X-ray data collection |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Data set        | RANK            | RANKL/RANK      |
| X-ray source    | BM14, ESRF      | ID23-2/1D14-4, ESRF |
| Wavelength (Å)  | 0.9537          | 0.8726/0.9395   |
| Space group     | P212121         | P63            |
| Unit cell (a, b, c [Å]) | 39.82, 94.25, 102.42 | 121.23, 121.23, 94.67 |
| Resolution range * (Å) | 30.0–2.00 (2.07–2.00) | 30.0–2.80 (2.90–2.80) |
| Unique reflections | 26,472 (2582)   | 19,756 (1970)   |
| Completeness (%) | 99.6 (99.2)     | 100 (100)      |
| Redundancy      | 6.9 (6.7)       | 16.3 (13.8)    |
| Average I/σI    | 17.7 (2.3)      | 18.6 (2.9)     |
| Rmerge          | 0.096 (0.644)   | 0.191 (0.995)  |
| Refinement statistics |
| Resolution range (Å) | 30.0–2.00 | 30.0–2.80 |
| No. of reflections (working/test) | 24896/1332 | 18581/995 |
| R factor a (Rwork/Rmerge) | 0.207/0.237 | 0.182/0.212 |
| No. of atoms (protein/water/others) | 2433/221/47 | 2487/48/5 |
| Rms bond length deviation (‘) | 0.007 | 0.007 |
| Rms bond angle deviation (‘) | 1.1 | 1.0 |
| Mean B factor b (Å²) | 31/35/49 | 50/41/49 |

*Numbers in parentheses are for the highest resolution shell.

aRwork and Rmerge are defined by R = Σhkl |Fobs| – |Fc(l)| / Σhkl |Fc(l)|, where h, k, and l are the indices of the reflections (used in refinement for Rwork, 5%, not used in refinement for Rmerge) and Fobs and Fcalc are the structure factors, deduced from measured intensities and calculated from the model, respectively.

bMean B factors for protein, water, and others, including ions and glycerol molecules.

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 × 104/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 RANKL 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).

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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 CRD1 and CRD2. 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 CRD2 (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. 2H, 2I).
The structure of 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 the ligand.

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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 receptors 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 60%, green; 60–80%, orange; 80–100%, red). C–E, Close up of three key interface areas with residues in RANK labeled in black; (C) residues Asp124 and Glu126 from the β2β3 loop of the receptor form salt bridges with residues His179 and Lys180 from the AA” loop of the ligand. D, Hydrophobic interactions centered at Leu89 of the receptor. E, Interface area centered at His224 of the ligand, where the His224 is fully buried, Glu225 and Glu268 make two salt bridges to Arg129 and Arg130 of the receptor. On receptor binding the ring center of Tyr234 from the ligand has moved 6.5 Å to make stacking interactions with Arg129 (from red to blue).

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 disulphide 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 Glu147 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 hydrophilic interactions with the receptor analogous to those in the TNF-β–TNFRSF1A and TRAIL–DR5 complexes.
(38–40). Tyr108 in TNF-β is fully buried in a hydrophobic depression formed by the β2β3 loop consisting of residues 60–71 of TNFRSF1A, and Tyr214 in TRAIL makes similar interactions with the β2β3 loop (residues 50–61, the 50S loop) of DR5; the only conserved ligand–receptor interactions in the two complexes. The importance of this tyrosine has been shown by mutagenesis in TNF-α, TNF-β, FasL, and TRAIL that abolishes receptor binding (39, 40, 56–58). 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 Leu67 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 has direct contact with a charged residue (Glu84) of RANK, 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 Gln302 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 RANKL immobilized in a channel of a chelating NTA sensor chip and RANKL as the mobile phase. Recombinant TNFRSF9, as a nonspecific protein control, was immobilized in a different channel in the same chip. As seen in Fig. 5A, the affinity between RANK and RANKL is very high with a 

$K_d$ of 6.8 × 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 ($K_d$ 8.8 × 10^{-11} M compared with a $K_d$ of 6.8 × 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 β2β3 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 β2β3 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.](image-url)
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 disulphide 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 disulphide constraint by stacking and hydrogen bond interactions. The CRD3 of OPG is thus expected to be structurally similar to the CRD2 of RANK (Fig. 4C). 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, Arg222A.
and Cys175Arg (27), which correspond to residues of Gly54, Arg130, Lys171, and Cys176, respectively, in the mouse RANK. Lys171 forms a salt bridge with the conserved Asp162, which lies between the Na⁺ binding residues Ser161 and Val163 (Fig. 2G). A mutation to glycine is likely to disturb the Na⁺ binding. Cys176 forms the 4–6 disulfide of CRD4 with Cys195; removing the disulfide constraint by an arginine mutation will result in conformational flexibility in the second structural module of CRD4, especially the β5 strand leading to the transmembrane helix. Gly54 is highly conserved in the TNFRSF, and its structural role has been discussed earlier. The Gly54Arg mutation is likely to cause conformational changes of the β1β2 loop of CRD2 and disrupt the interactions between Leu89 and the ligand. Arg130 is directly involved in ligand binding, forming a salt bridge with Glu225 and a hydrogen bond to Asn266. An Arg130Gly mutation would abolish these interactions.

Three RANKL mutations, an N-terminal deletion of residues 145–177, a C-terminal truncation starting at residue 277, and a Met199Lys substitution, have been identified in patients with autosomal-recessive osteopetrosis (28). The deletion of 145–177 results in loss of βA and half of the AA⁺ loop. The loop interacts with the CRD3 β2β3 loop and is crucial for receptor binding (33). The C-terminal deletion mutation causes loss of two central strands of the jellyroll scaffold, likely resulting in an unfolded ligand. The side chain of Met199 nests 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 TNFRSF, 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–RANKL 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.

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Disclosures
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References


