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Crystal Structure of Human RANKL Complexed with Its Decoy Receptor Osteoprotegerin

Xudong Luan,*1 Qingyu Lu,*1 Yinan Jiang,* Senyan Zhang,* Qing Wang,* Huihui Yuan,‡ Wenming Zhao,‡ Jiawei Wang,* and Xinquan Wang* 

Receptor activator of NF-κB ligand (RANKL), its signaling receptor RANK, and its decoy receptor osteoprotegerin (OPG) constitute a molecular triad that is critical in regulating bone remodeling, and also plays multiple roles in the immune system. OPG binds RANKL directly to block its interaction with RANK. In this article, we report the 2.7-Å crystal structure of human RANKL trimer in complex with the N-terminal fragment of human OPG containing four cysteine-rich TNFR homologous domains (OPG-CRD). The structure shows that RANKL trimer uses three equivalent grooves between two neighboring monomers to interact with three OPG-CRD monomers symmetrically. A loop from the CRD3 domain of OPG-CRD inserts into the shallow groove of RANKL, providing the major binding determinant that is further confirmed by affinity measurement and osteoclast differentiation assay. These results, together with a previously reported mouse RANKL/RANK complex structure, reveal that OPG exerts its decoy receptor function by directly blocking the accessibility of important interacting residues of RANKL for RANK recognition. Structural comparison with TRAIL/death receptor 5 complex also reveals structural basis for the cross-reactivity of OPG to TRAIL. The Journal of Immunology, 2012, 189: 245–252.

Bone remodeling is a dynamic and balanced progress that involves bone matrix formation mediated by osteoblasts and bone resorption mediated by osteoclasts. Osteoblasts are cells that arise from bone marrow stem cells, whereas osteoclasts are multinucleated cells derived from hematopoietic precursors of the monocyte/macrophage lineage. Receptor activator of NF-κB ligand (RANKL), its signaling receptor RANK, and its decoy receptor osteoprotegerin (OPG) play key roles in regulating bone remodeling (1–3). Experiments using knockout mice have unanimously established their pivotal roles as central regulators of osteoclast function (4–7). Specifically, the interaction of RANKL expressed by osteoblasts with RANK expressed on osteoclast precursors is essential for the differentiation, activation, and survival of osteoclasts. The decoy receptor OPG expressed and secreted by osteoblasts inhibits osteoclastogenesis by binding RANKL to prevent RANKL/RANK interaction. In the bone microenvironment, the local ratio of RANKL and OPG determines the level of osteoclast activation. The normal osteoclast activation results in physiological bone resorption in bone remodeling, whereas overactivated osteoclasts result in various bone diseases such as osteoporosis and rheumatoid arthritis (8, 9). Besides the best-known critical roles in bone remodeling, RANKL also plays multiple roles in immune system (10), mammary gland development (11), cancer bone metastasis (12), hormone-derived breast cancer development (13), and thermal regulation (14). Therefore, the RANKL–RANK–OPG molecular triad is an attractive target to develop rational therapy for many osteopenic conditions and prevent bone destruction in osteoporosis and arthritis (15).

RANKL is a member of the TNF superfamily (TNFSF), consisting of a membrane-anchor domain, a connecting stalk, and a receptor-binding ectodomain. RANKL also exists in a soluble form from proteolytic cleavage of the transmembrane form or alternative mRNA splicing (16, 17). The ectodomain of RANKL adopts the characteristic jellyroll β-sandwich fold in TNFSF cytokines and assembles into a homotrimer in either membrane-bound or soluble form (18, 19). The receptor RANK belongs to the TNFR superfamily (TNFRSF), consisting of four extracellular cysteine-rich TNFR homologous domains (CRD1–CRD4), a transmembrane helix, and a cytoplasmic region. RANKL/RANK interaction induces RANK receptor trimerization, triggering the association of RANK cytoplasmic region with TNFR-associated factors and subsequent multiple signaling pathways (20, 21). The decoy receptor OPG represents an atypical member in the TNFRSF because it lacks the transmembrane region and is secreted as a soluble protein. The OPG has four CRD domains in the N-terminal region that are necessary and sufficient for the inhibition of osteoclastogenesis (7, 22), and its C-terminal region contains two death domain homologous regions, a heparin-binding domain, and a cysteine residue at the C-terminal end necessary for dimer formation (7, 22, 23).
The crystal structure of mouse RANKL was determined 10 years ago (18, 19), and residues in RANKL that are important for RANK recognition have also been proposed based on structural modeling and mutational studies (18, 19, 24, 25). The crystal structure of the mouse RANKL/RANK complex was recently solved independently by two groups, providing precise molecular details of RANKL/RANK interaction (26, 27). In this article, we report the complex structure of human RANKL ectodomain with N-terminal four CRD domains of human OPG (OPG-CRD). Key residues in OPG-CRD for RANKL binding were identified by structure-based mutagenesis of OPG-CRD and surface plasmon resonance (SPR) analyses, which was further collaborated by osteoclast differentiation assay. These results, together with previous reported mouse RANKL/RANK complex structure, provide a complete picture of ligand/receptor interactions in the RANKL-RANK-OPG molecular triad.

Materials and Methods

Constructs

The coding sequence of human RANKL ectodomain (residues 162–317) was cloned into a modified pET30a vector (Novagen) with NusA/His tag followed by a tobacco etch virus protease cleavage site. Constructs expressing wild type human RANKL and its mutants for CRD pull-down experiments were made by using vector pGEX-6P-1 vector (GE Healthcare). The coding sequences of human OPG-CRD containing four CRD domains (residues 1–165) and human RANK (residues 1–166) were cloned into pFastBac vector (Invitrogen) with C-terminal 6×His tag. The residues in OPG-CRD and RANK ectodomain were numbered without counting the cleaved signal peptide in the mature receptors. Mutations on OPG-CRD were made by overlap extension PCR using synthetic oligonucleotide primers.

Protein expression and purification

The OPG-CRD and RANK ectodomains were expressed and purified from baculovirus-infected insect cells. In brief, the pFastBac vector containing gene of interest was transformed into DH10bac competent cells, and high m.w. mini-prep DNA was prepared from selected Escherichia coli colonies containing the recombinant bacmid. This DNA was then used to transfect S9 insect cells. After two cycles of amplification in S9 cells, the recombinant virus was used to infect Hi5 cells at a density of 2×10^6 cells/ml in HyQ SFX medium (HyClone). The cell culture medium was collected after 48 h, and the secreted protein was purified using Ni-NTA resin (Qiagen) followed by size exclusion chromatography (Superdex 200 10/300; GE Healthcare). The OPG-CRD mutants were expressed and purified in a similar way. The RANKL was overexpressed in E. coli strain BL21(DE3) at 16°C for 20 h with 0.5 mM isopropyl β-D-1-thiogalactopyranoside induction. The cells were collected and lysed in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, pH 8.0. The fusion protein NusA-His-RANKL in the soluble fraction was purified using Ni-NTA resin (Qiagen), followed by the cleavage with tobacco etch virus protease to remove NusA and His tag. The released RANKL was further purified by size exclusion chromatography (Superdex 200 10/300; GE Healthcare). GST-tagged wild type RANKL and its mutants were also expressed in E. coli strain BL21(DE3) and purified by glutathione-Sepharose beads (GE Healthcare) and size exclusion chromatography (Superdex 200 10/300; GE Healthcare).

The RANKL/OPG-CRD complex was reconstituted by mixing OPG-CRD and RANKL in 1:1 molar ratio and incubated at 4°C for 10 h. The complex was further purified by size exclusion chromatography (Superdex 200 10/300; GE Healthcare) and ion exchange chromatography (Resource S; GE Healthcare).

Crystallization and x-ray data collection

Crystals were grown using the hanging-drop vapor-diffusion method by mixing RANKL/OPG-CRD complex (10 mg ml^-1 in 10 mM HEPES sodium, pH 7.2 and 150 mM NaCl) with an equal volume of reservoir solution containing 100 mM sodium phosphate monobasic, 100 mM potassium phosphate monobasic, 100 mM MES monohydrate pH 6.5, and 2.0 mM sodium chloride. Crystals appeared in 1 wk at 18°C. X-ray diffraction data were collected at Shanghai Synchrotron Research Facility beamline BL17U1 at a wavelength of 0.97 Å. Data images were indexed and integrated using program MOSFLM (28) and scaled using program SCALA from the CCP4 suite (29). The final statistics of data processing are listed in Table I.

Structural determination and refinement

The position of human RANKL monomer in the asymmetric unit was determined by using molecular replacement program PHASER (30) with mouse RANKL monomer (Protein Data Bank code: 1IQ2) as search model. Starting with initial phases provided by RANKL, the OPG-CRD model was built gradually by cycles of iterative manual model building with program COOT (31) and structural refinement with program PHENIX (32) refinement module. In the final model, 80.4, 16.4, 2.1, and 1.1% of the amino acids residue in the most favorable, additional allowed, generously allowed, and disallowed regions of the Ramachandran plots, respectively. The final refinement statistics are listed in Table I.

SPR experiment

The binding affinity between RANKL and OPG-CRD and its mutants was determined by SPR using BIACore 3000 at 25°C. The RANKL was immobilized to ∼50 response units on a research-grade CM5 sensor chip in 10 mM sodium acetate, pH 5.5 by standard amine coupling method. The flow cell 1 was left blank as a reference. OPG-CRD in 10 mM HEPES, pH 7.2, 150 mM NaCl, and 0.005% Tween 20 was injected over the flow cells at different concentrations at a flow rate of 30 μl min^-1 to measure binding affinity of OPG-CRD or its mutants by RANKL. The RANKL/OPG-CRD complexes were allowed to associate for 90 s and dissociate for 150 s. The surfaces were regenerated with 5 mM NaOH between each cycle if needed. Data were analyzed with BIACore 3000 evaluation software BIAevaluation 4.1 by globally fitting to a 1:1 Langmuir binding model.

Osteoclast differentiation assay

The biological activities of RANKL and OPG proteins were estimated using an osteoclast differentiation assay. Mouse osteoclast precursors were extracted from the tibia and femur of 7-week-old male mice, cultured in RPMI 1640 containing 10% FBS and 30 ng ml^-1 M-CSF and 100 ng ml^-1 RANKL. Then osteoclast precursors were seeded in 96-well plates (2×10^4 cells/well) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Three days later, the medium was changed with fresh RPMI 1640 containing 10% FBS and 30 ng ml^-1 M-CSF, and 100 ng ml^-1 RANKL. The inhibitory effects of OPG-CRD and its mutants were tested by adding them into wells with a final concentration of 500 ng ml^-1.

Differentiation of osteoclast precursors was measured in vitro by quantitation of tartrate-resistant acid phosphatase (TRAP) activity. In the seventh day, cells were fixed and stained using the TRAP staining kit (Sigma Aldrich no. 387), and the reaction product was quantified by spectrophotometric measurement at 405 nm. TRAP-positive multinucleated cells were evaluated under the microscope, and each experiment was repeated three times for statistical analysis.

Results

Overall structure

We have determined the structure of the human RANKL in complex with human OPG-CRD at a resolution of 2.7 Å using a combination of molecular replacement method with mouse RANKL as search model with subsequent manual model building of the OPG-CRD region (Table I, Supplemental Fig. 1). We also tried the molecular replacement method to search the position of OPG-CRD with the available TNF family receptor structures including TNFR1, TNFR2, and others. However, the extensive search did not result in a clear solution, reflecting structural flexibility within each CRD domain and domain flexibility between the CRD domains in the TNFRSF. The final model in the asymmetric unit consists of one RANKL monomer (Ala^{162}–Asp^{317}) and one human OPG-CRD monomer (Pro^{3}–Ser^{165}), with additional three histidine residues (His^{166}–His^{168}) at the C-terminal end of OPG-CRD (Fig. 1A). We were also able to build a glycan attached to residue Asn^{123} of OPG-CRD (Fig. 1A). In the RANKL/OPG-CRD complex structure, functional RANKL trimer bound with three OPG-CRD monomers can be generated by rotating the monomeric RANKL/OPG-CRD along the 3-fold rotational axis of the symmetry (space group P₄₁32) (Fig. 1B, 1C).
The OPG-CRD fragment, corresponding to the extracellular four CRD domains of RANK (Fig. 2A), has been shown to be necessary and sufficient for the inhibition of osteoclastogenesis (7, 22). The CRD1-CRD4 domains of OPG-CRD adopt the so-called A1-B1, A1-B2, A1-B1, and A1-B1 module, respectively (Fig. 2A), where A and B denote the type of topology and 1 and 2 the number of interstrand disulfide bond within the module (33). The CRD1-CRD4 domains of RANK adopt the A1-B2, A1-B1, A1-B1, and A1-B1 module, respectively (Fig. 2A). Besides the difference of interstrand disulfide bond distribution in CRD1 and CRD2 domains, an additional intrastrand disulfide bond (Cys 125-Cys127) in the CRD3 domain of RANK is also absent in that of OPG-CRD. Structural alignment of each CRD domain between OPG-CRD and RANK results in a root mean square deviation of 1.16, 1.36, 1.49, and 1.69 Å for CRD1 (33 Ca atoms), CRD2 (41 Ca atoms), CRD3 (37 Ca atoms), and CRD4 (45 Ca atoms), respectively. There are apparent rigid-body movements in CRD1 and CRD4 after overall alignment based on CRD2 and CRD3 responsible for ligand binding (Fig. 2B). Similar rigid-body movements were also observed among different states of RANK including unbound and bound with RANKL (27). The RANKL monomer in the complex is composed of 10 β-strands, forming the outer β-sheet (B’, B, G, D, and E) and inner β-sheet (A’, A, H, C, and F) (Fig. 1A). The most significant conformation changes of RANKL on OPG-CRD binding are at AA’ and CD loops that are involved in the direct interaction, revealed by structural comparison of RANKL in unbound and OPG-CRD bound states (Supplemental Fig. 2).

A and B denote the type of topology and 1 and 2 the number of interstrand disulfide bond within the module (33). The CRD1-CRD4 domains of RANK adopt the A1-B2, A1-B1, A1-B1, and A1-B1 module, respectively (Fig. 2A). Besides the difference of interstrand disulfide bond distribution in CRD1 and CRD2 domains, an additional intrastrand disulfide bond (Cys125-Cys127) in the CRD3 domain of RANK is also absent in that of OPG-CRD. Structural alignment of each CRD domain between OPG-CRD and RANK results in a root mean square deviation of 1.16, 1.36, 1.49, and 1.69 Å for CRD1 (33 Ca atoms), CRD2 (41 Ca atoms), CRD3 (37 Ca atoms), and CRD4 (45 Ca atoms), respectively. There are apparent rigid-body movements in CRD1 and CRD4 after overall alignment based on CRD2 and CRD3 responsible for ligand binding (Fig. 2B). Similar rigid-body movements were also observed among different states of RANK including unbound and bound with RANKL (27). The RANKL monomer in the complex is composed of 10 β-strands, forming the outer β-sheet (B’, B, G, D, and E) and inner β-sheet (A’, A, H, C, and F) (Fig. 1A). The most significant conformation changes of RANKL on OPG-CRD binding are at AA’ and CD loops that are involved in the direct interaction, revealed by structural comparison of RANKL in unbound and OPG-CRD bound states (Supplemental Fig. 2).

In our protein purification experiment, the OPG-CRD containing the N-terminal four CRD domains was purified as a monomer. The complex structure also shows that trimeric RANKL interacts with monomeric OPG-CRD to form a stable 1:3 trimer/monomer complex. In contrast, the full-length OPG is a disulfide-bond linker dimer, and it was reported that trimeric RANKL interacts with dimeric OPG to mainly form a stable 1:1 trimer/dimer complex in solution (34). We will further discuss the binding stoichiometry between RANKL and OPG later.

**FIGURE 1.** Overall structure of RANKL/OPG-CRD complex. (A) Ribbon diagram of one RANKL (green) with one OPG-CRD in the asymmetric unit (magenta). The disulfide bonds (magenta) and N-linked glycan (cyan) in OPG-CRD are shown in sticks. The β-strands in RANKL are labeled according to standard TNF-β–sandwich nomenclature. (B) Side view of the RANKL trimer (green) bound with three OPG-CRD (magenta) monomers. (C) View down the 3-fold axis of the RANKL/OPG-CRD complex, perpendicular to (B). All figures were made with the PyMol program (http://www.PyMol.org).

**FIGURE 2.** Structure of OPG-CRD and its comparison with RANK. (A) OPG-CRD is composed of four CRDs (CRD1-CRD4), similar to the architecture of RANK. (B) The rigid-body movements of CRD1 and CRD4 between RANK (blue) and OPG-CRD (magenta) after structural superimposition based on CRD2 and CRD3.

**Table I.** Data collection and refinement statistics

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<tr>
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Values in parentheses are for highest resolution shell.

Statistics from data processing.

Statistics from structure refinement.
Interactions between RANKL and OPG-CRD

In the RANKL/OPG-CRD complex, the trimeric RANKL uses three spatially distinct but equivalent shallow grooves to bind three OPG-CRD molecules (Fig. 1B, 1C). One groove, formed by two neighboring RANKL monomers, accommodates one OPG-CRD monomer and the buried solvent-accessible surface area is 2200 Å² at the interface. The binding interface is composed of two binding sites (Fig. 3A, 3B). At site I, the OPG “50s loop” (His248, His253) from CRD2 domain is placed nearly parallel to the RANKL along the groove (Fig. 3A), whereas at site II, the OPG “90s loop” (Arg282, Arg284) and GH (Pro301, Asp302) of RANKL and the 50s loop (His47, Tyr48, Tyr49, Ser56, Asp57, Glu58, Tyr61, Pro64, Val65, Leu69) of OPG CRD2 domain (Supplemental Table I). Site II consists of continuous patches in both RANKL and OPG-CRD (Fig. 3B), including residues from loops AA’ (Arg191, Gly192), DE (Lys248, His253), FG (Lys282, Arg284), and GH (Pro301, Asp302, Glu303) of RANKL and the 50s loop (His47, Tyr48, Tyr49, Ser56, Asp57, Glu58, Tyr61, Pro64, Val65, Leu69) of OPG CRD2 domain (Supplemental Table I). Site II consists of relative small and separate contact patches in both RANKL and OPG-CRD (Fig. 3B). Residues involved in the site I interaction are from loops AA’ (Arg191, Gly192), DE (Lys248, His253), FG (Lys282, Arg284), and GH (Pro301, Asp302, Glu303) of RANKL and the 50s loop (His47, Tyr48, Tyr49, Ser56, Asp57, Glu58, Tyr61, Pro64, Val65, Leu69) of OPG CRD2 domain (Supplemental Table I). Site II consists of continuous patches in both RANKL and OPG-CRD (Fig. 3B), including residues from loops AA’ (Ser70, His80, Lys184), strands C (Arg223, His227, His225), D (Gln237, Met239, Tyr241), E (Lys257), and F (Phe272, His274, Phe276) of RANKL and the 90s loop (Arg90, Leu92, Glu93, Ile94, Glu95, Phe96, Cys97, Leu98, Glu160) of OPG CRD3 domain (Supplemental Table I).

Detailed inspection revealed obvious hydrophilic interactions including ionic and hydrogen bonds in both sites (Fig. 4A, 4B). Compared with separate interactions at site I (Fig. 4A), the more interactions at site II are focused around the tip of the 90s loop and form a network (Fig. 4B). Residues involved in the interaction network are Arg223, Glu257, Tyr241, and Lys257 of RANKL and Ile94, Glu95, and Phe96 of the OPG-CRD 90s loop. RANKL residues Arg223, Tyr241, and Lys257 all interact with central Glu95 residue in the 90s loop of OPG-CRD (Fig. 4B).

To confirm the roles of sites I and II in the binding of RANKL by OPG-CRD, residues in OPG-CRD involved in the binding through ionic and hydrogen bonds (Asp57 and Glu58 in site I and Glu95 in site II) were mutated to Ala. The SPR analyses showed that wild type OPG-CRD bound RANKL with an affinity of 0.65 nM (Fig. 4C). The binding affinity of RANKL by OPG-CRD Glu95Ala mutant was significantly reduced to 2.4 µM. Consistently, this OPG-CRD mutant lost most of the capacity to inhibit RANKL-induced osteoclast differentiation compared with wild type OPG-CRD (Fig. 4D). In contrast, the OPG-CRD Asp57Ala/Glu58Ala double-mutant bound RANKL with an affinity of 0.69 nM and still retained the same level of inhibition as wild type OPG-CRD (Fig. 4C, 4D). These data indicate that site II plays a more critical role in the binding than site I.

Comparison with RANKL/RANK complex

The overall architectures of the human RANKL/OPG-CRD and mouse RANKL/RANK complexes are similar with a trimeric RANKL bound with three monomeric OPG-CRD or RANK receptors. The buried surface-accessible area is 2660 Å² at the RANKL/RANK interface, similar to the value (2200 Å²) at the RANKL/OPG interface. Both ligand/receptor interfaces are also composed of two binding sites (I and II; Fig. 5A). The DE, FG, and GH loops of RANKL involved in site I interaction are well conserved in RANKL/RANK and RANKL/OPG-CRD complexes, and the CRD2 domain is also similar in its orientation at the binding interface (Fig. 5A). We have shown that the OPG-CRD Asp57Ala/Glu58Ala mutant retained the nearly same RANKL binding affinity and inhibition of osteoclast differentiation as wild type OPG-CRD (Fig. 4C, 4D). Similarly, it has also been shown that mutation of RANK residue Asp95 at site I did not significantly affect the RANKL binding and inhibition of osteoclast differentiation by soluble RANK (26). At site II, the general binding mode of an inserted loop from receptor into RANKL trimer is also conserved in the RANKL/OPG-CRD and RANKL/RANK complexes. There are significant conformation changes at the central part of the long AA’ and CD loops in RANKL (Fig. 5A), and the 90s loop of CRD3 also has positional shift resulting from the change of relative orientation between CRD3 and CRD2 in two different complexes (Fig. 5B). Therefore, the detailed interactions at site II are different in these two complexes, especially at the central part of the inserted 90s loop of OPG-CRD and loop3 of RANK, whose sequences are –Glu95–Ile244–Glu95–Phe96– and –Glu124–Cys125–Glu126–Cys127–, respectively. In the human RANKL/OPG-CRD complex, the Glu95 from the OPG-CRD has interactions with RANKL residues Arg223, Tyr241, and Lys257, forming a hydrophilic interaction network (Fig. 5B). In the mouse RANKL/RANK complex, Glu126 from loop3 of RANK keeps the interaction with RANKL Tyr240 with additional interaction with RANKL Lys180 and has no direct interactions with RANKL Arg222 and Lys256 (Fig. 5B). There is a close to 180° side-chain rotation of mouse RANKL Arg222 compared with human RANKL Arg223 (Fig. 5C), which enables the interaction with RANKL Asp94. Mouse RANKL Lys256 has interaction with RANK Lys97 at the interface. Mutation of either Glu126 or Lys97 at RANK completely abrogated the RANKL binding and inhibition of osteoclast differentiation by soluble RANK (26). We also performed a GST

FIGURE 3. Overall view of RANKL/OPG-CRD binding interface. (A) Sites I and II in the binding interface. Two neighboring RANKL monomers are shown in surface, colored with green and cyan, respectively. The OPG-CRD is shown in ribbon colored with magenta. The “50s loop” in site I and “90s loop” of OPG-CRD in site II are colored with orange. (B) An open-book view of the contact residues in binding sites I and II. The two neighboring RANKL monomers and OPG-CRD are presented in surface with gray color. The contact residues in binding sites I and II are colored with red and blue, respectively.
pull-down experiment using human GST-RANKL and its mutants with human OPG-CRD or human RANK. The mutation of residue Arg^223, Tyr^241, and Lys^257 reduced the binding of human RANKL by both human OPG-CRD and human RANK (Fig. 5C). All these structural and biochemical data suggest that site II is the major binding determinant of RANKL by both OPG-CRD and RANK, although there are some subtle differences in the specific interactions.

**Structural basis for the cross-reactivity of OPG**

Besides binding RANK, OPG is able to interact with TRAIL (35–37), which is also a member in the TNFSF and induces apoptosis of tumor cells through the cell-surface receptors death receptor 4 (DR4) and DR5 in the TNFRSF family (38). It has been shown that OPG inhibits the TRAIL-induced cell apoptosis and TRAIL inhibits OPG-mediated inhibition of osteoclastogenesis (35, 37). The RANKL/OPG-CRD complex is similar to TRAIL/DR5
complex (39–41) in both ligand/receptor docking mode and the organization of ligand/receptor binding sites (Supplemental Fig. 3A). The TRAIL/DR5 complex also has two major binding sites in the interface, corresponding to the sites I and II in the RANKL/OPG-CRD complex (Supplemental Fig. 3A). The site I in TRAIL/DR5 complex is clustered around residue Tyr 216 that interacts with residues in a hydrophobic groove in the DR5 CRD2 domain (40). This tyrosine residue is critical for the bioactivity of TRAIL and its interaction with DR5 (42), and is also strictly conserved in some TNFSF members including TNF, LT, FasL, and Apo2L (40). In RANKL, this corresponding position is replaced by residue Ile249 (Supplemental Fig. 3B). However, in the RANKL/OPG-CRD site I, residue Ile249 does not have close interactions with OPG-CRD, neither with RANK in the site I of RANKL/RANK complex. The four major RANKL residues Arg223, Gln 237, Tyr241, and Lys257 for OPG-CRD interaction at site II are strictly conserved in both primary and three-dimensional structure in TRAIL (Supplemental Fig. 3B, 3C). TRAIL residues in Arg 223, Tyr241, and Lys257 positions also have interactions with Glu86 in the loop of DR5 CRD3 domain, corresponding to Glu 95 in the OPG 90s loop (Supplemental Fig. 3C). The highly conserved contact residues and specific interactions observed in site II provide structural basis for the cross-reactivity of OPG to TRAIL.

**Discussion**

Full-length OPG is a disulfide-bond–linked homodimer, and each monomer has N-terminal four CRD domains followed by two death domain homologous domain, and a cysteine residue at the C-terminal end forming interchain disulfide bond. The RANKL/OPG-CRD structure we report in this article shows that the N-terminal four CRD domains behave as an independent structural module, and CRD2 and CRD3 contribute to the interaction with RANKL. Although the C-terminal region of OPG does not participate in the direction interaction with RANKL, it has been reported that the C-terminal death domain homologous regions are essential for OPG dimer formation (34). The native dimeric OPG has stronger avidity for RANKL than monomer form, providing the structural basis for its ability to effectively compete with signaling receptor RANK for ligand binding (34). This also raises the question about the binding stoichiometry of trimeric RANKL with full-length dimeric OPG. Previous ultracentrifuge analyses showed that trimeric RANKL
interacts with dimeric OPG to mainly form a stable 1:1 trimer/dimer complex in solution (34). The two OPG monomers per dimer both bind to the same RANKL trimer, and each monomer of OPG cannot bind a separate RANKL trimer (34). Based on these biochemical data and the RANKL/OPG-CRD complex structure, we suggest that the shape of OPG dimer would be like a “Y” shape. The bottom part is the dimeric C-terminal region of OPG including death domain homologous regions, heparin-binding domain, and the C-terminal disulfide bond, and the top part is two separate N-terminal regions including four CRD domains. In the 1:1 trimer/dimer RANKL/OPG complex, the two separate N-terminal arms of OPG occupy two of three grooves in RANKL trimer. This would allow efficient inhibition of RANKL-induced RANK trimerization, which is essential for subsequent signaling and osteoclast maturation.

The human RANKL/OPG-CRD and mouse RANKL/RANK structural comparison showed that OPG exerts its decoy receptor function by directly occupying the major binding determinant site II and blocking the accessibility of key interacting residues such as Arg223, Tyr241, and Lys257 in human RANKL for RANK recognition. This information could help the development of therapeutic agents targeting the RANKL/OPG-CRD molecular triad for the treatment of bone-related diseases (46, 47). Current strategies under investigation include the usage of OPG-Fc, RANK-Fc, anti-RANK Ab, OPG peptidomimetics, RANK vaccination, and modulators of intracellular signaling pathways (47). Designing small molecules targeting protein–protein interface is an enormous challenge in drug development, but the existence of a small subset of residues that contribute to most of the free energy of binding (“hot spots”) in the interface still makes it possible (48). The existence of critical RANKL residues such as Arg223, Tyr241, and Lys257 at site II for RANK interaction suggests that designing small molecules that could target these “hot-spot” residues in RANKL is still a potential way in targeting the RANKL-RANK-RANKL molecular triad.

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Disclosures

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References


