Functional Dissection of Osteoprotegerin and Its Interaction with Receptor Activator of NF-κB Ligand

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The receptor activator of NF-κB (RANK) belongs to the neuregulin/tumor necrosis factor (TNF) receptor superfamily and is activated by RANK ligand (RANK-L), a homotrimeric, TNF-like cytokine. RANK is present on the surface of osteoclast cell precursors, where its interaction with RANK-L induces their terminal differentiation into osteoclasts, thus increasing bone breakdown. The secreted, soluble receptor osteoprotegerin (OPG) interrupts this activation by binding directly to RANK-L. Therefore, osteoclast maturation (and bone homeostasis) is regulated in vivo by OPG levels of expression. We have studied the assembly state and affinity of OPG for RANK-L by sedimentation analyses and surface plasmon resonance (Biacore). Full-length, homodimeric OPG binds to RANK-L with a KD of 10 nM. OPG is also a member of the TNF receptor superfamily and contains four disulfide-rich ligand-binding domains, yet lacks a transmembrane region separating the ligand-binding region from the two death domains, as observed for other receptor family members. We showed that dimerization of OPG results from noncovalent interactions mediated by the death domains and to a lesser extent by a C-terminal heparin-binding region. In contrast, a C-terminal intermolecular disulfide bond does not contribute to the formation or stability of OPG dimers. A truncate of osteoprotegerin, containing the ligand-binding domains but lacking the dimerization domains, bound RANK-L with a KD of ∼3 μM, indicating that monomer oligomerization for the OPG provides an increase of 3 orders of magnitude in the affinity for RANK-L. Therefore, OPG dimer formation is required for the mechanism of inhibition of the RANK-L/RANK receptor interaction.

Bone remodeling, a coordinated equilibrium between bone synthesis and bone resorption, is a continuously occurring process that maintains skeletal integrity in vertebrates (1). The cellular differential signals responsible for the regulation of bone remodeling processes are ultimately determined by binding interactions between proteins localized at the cell surface of osteoblasts and osteoclasts (1). Osteoblasts are cells derived from bone marrow stem cells, responsible for bone formation (2, 3). Osteoclasts mature from cells of the innate immune system and cause bone resorption (4, 5).

Receptor activator of NF-κB ligand (RANK-L)3 is a TNF ligand superfamily member (6, 7). RANK-L present on the surface of osteoblasts binds to its receptor, RANK, on the surface of immature osteoclasts, inducing their maturation, thus shifting the balance toward bone breakdown (5). This interaction requires cell-cell contact between, or in close proximity of, osteoblasts and osteoclasts (3). RANK-L is also an essential factor for the survival of mature osteoclasts (3). Over the last 8 years, dramatic progress has been made toward understanding the pathophysiology of bone resorption because of the discovery of RANK-L and its two receptors, a signaling receptor that mediates osteoclast differentiation, function, and survival (RANK), and a soluble receptor, OPG. The regulation of the balance between bone breakdown and reformation is modulated to a large extent by the secreted, soluble receptor OPG (7, 8). It has been postulated that, by binding to RANK-L, OPG makes RANK-L unavailable to RANK, blocking osteoclast differentiation and survival (5, 7, 9). This discovery provided a conceptual framework for understanding bone remodeling under physiological conditions and in several pathological states, including osteoporosis (a benign form of osteolysis common in post-menopausal women) (10–12), osteopetrosis (bone hyperplasia caused by the lack of functional RANK-L or its receptor, RANK) (13, 14), and rheumatoid arthritis (a common autoimmune disease where chronic inflammation results in joint destruction) (15, 16).

RANK-L is displayed as an intrinsic protein on the surface of osteoblasts, but it is also post-translationally cleaved. The physiological meaning of this cleavage event is not yet understood (6, 7, 17). The ectodomain or receptor-binding domain of RANK-L has been demonstrated to be the only region necessary for binding to the soluble receptor OPG and for activation of osteoclastogenesis through binding to RANK (7, 9). The domain structure of each protein is illustrated in Fig. 1. RANK-L is a 61-kDa homotrimer with equilibrium association constant for trimerization of 6.5 × 1020 M−2 (18), but it is likely to be even higher, as this affinity is at the limit of detection for an association that can be measured by sedimentation equilibrium using absorbance optics. The structure of the murine RANK-L homotrimeric ectodomain has been solved by x-ray crystallography and shown to have a “jelly roll” architecture comparable with other TNF-like cytokines (19). In similarity to other neuregulin/TNF receptor (TNFR) superfamily members, the extracellular domain of RANK is characterized by four C-terminal

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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental equations 1–4 and supplemental Table 7.

† Deceased. This paper is dedicated to the memory of Derril Willard, a dedicated scientist and loyal friend.

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3 The abbreviations used are: RANK-L, receptor activator of NF-κB ligand; RANK, receptor activator of NF-κB; OPG, osteoprotegerin; OPG-Fc, OPGFc-fusion; OPG-H, OPGHis6 fusion; OPG400A-H, OPG cysteine 400 to alanine-His6 fusion; OPGΔB-H, OPG basic domain truncated construct-His6 fusion; OPGΔCRD-H, OPG cysteine-rich domains truncated construct-His6 fusion; DD, death domain, CRD, cysteine-rich domains; TNF, tumor necrosis factor; TNFR, TNF receptor; NTA, nitritotriacetic acid; RU, response units.

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cysteine-rich domains (CRDs). These TNFR-like domains are followed by a single transmembrane helix and a large cytoplasmic domain containing three TNFR-associated factor-binding sites (20).

OPG is also a TNFR family secreted, soluble homodimeric receptor (50-kDa monomer) composed of four N-terminal CRDs, followed by two modules homologous to the death domains (DDs) of the TNFR that are extracellular because OPG lacks a transmembrane domain (21). DDs on all other known TNF receptor superfamily members are exposed only to the cytoplasmic environment (22). There is a C-terminal basic region involved in heparin binding, ending in a single cysteine (Cys-400), adjacent to the C-terminal leucine (Leu-401), known to form a disulfide bond in the intact homodimeric form of OPG (23, 24). Other TNFR superfamily members also exhibit soluble decoy forms, but they are generated by cleavage of the membrane-anchored receptor. So far, OPG is the only reported soluble decoy receptor in this superfamily with extracellular DDs stemming from the lack of a transmembrane domain (22). The function of the DDs and the C-terminal basic region of OPG is presently unknown. OPG has been observed \textit{in vivo} as both a covalent, intermolecular disulfide cross-linked dimer and as a species lacking the intermolecular disulfide bond because of C-terminal truncation (23, 25–27). Both the truncated monomer and the dimer appeared to have similar IC$_{50}$ values in osteoclastogenesis inhibition assays (23).

Several lines of evidence support the notion of direct competition between RANK and OPG for association with RANK-L. The binding of RANK-L to the RANK receptor has been demonstrated through immunoprecipitation assays using a soluble RANK-L ectodomain form and osteoclast progenitors as a source of RANK (28). Ligation of RANK-L to the soluble OPG receptor was observed functionally through an \textit{in vitro} osteoclastogenesis assay. In this assay, the induction of osteoclast maturation by RANK-L was detected by the production of tartrate-resistant phosphatase and cellular morphological changes. Osteoclastogenesis as measured in such a way was inhibited in a competitive manner by OPG (5, 23). Direct binding of RANK-L to a osteoprotegerin-IgG Fc domain fusion (OPG-Fc) has also been measured and reported to exhibit a $K_{d}$ of 6.7 nM, using surface plasmon resalation (18).

Although these studies provide convincing evidence of the cellular function and tight affinity of binding of OPG to RANK-L, much is still unknown about the mechanism of complex formation and the resulting inhibition of osteoclastogenesis. In fact, the high affinity interaction between OPG and RANK-L suggests an avidity model for their interaction. We hypothesize that the domain structure of OPG is important for the proper oligomerization and orientation of these multiple binding sites. In this study, we investigate the strength and stoichiometry of the OPG-RANK-L complex and the structural aspects of OPG determining its ability to ligate RANK-L. We also address the contribution of individual domains of OPG to its binding function. Therefore, our results provide for the first time a molecular context for previous functional analyses of the effect of OPG on osteoclast differentiation.

All of our studies used the globular ectodomain of RANK-L, a trimer at concentrations above 40 pM (18). This form has been shown to contain the receptor-binding module and to fold independently of the remainder of the protein (7, 9). This truncated form is physiologically relevant, because membrane-bound RANK-L has been shown to undergo regulated shedding from the cell surface in a metalloproteinase-dependent manner to yield this ectodomain (17).

**MATERIALS AND METHODS**

\textbf{Production of Recombinant Viruses and Protein Expression}—All constructs were prepared using the pFastBac1 (pFB; Invitrogen) expression vector. The pFB-RANK-L truncate was a generous gift from GlaxoSmithKline, and its preparation and characterization were published previously (18). The wild type pFB-OPG-H (His$_{6}$) was a generous gift from GlaxoSmithKline, and its preparation was similar to that published for the pFB-OPG-Fc (18). The pFB-C + RANK-L construct was engineered with an N-terminal cysteine for Biacore surface coupling using PCR to insert the additional codon. The OPG constructs are depicted in Fig. 1 and are the same as those of Yamaguchi et al. (29) so that functional comparisons can be made. A cassette containing the C400A mutation was subcloned into the BstEII and NotI sites of pFB-OPG-H after a SalI/XbaI digest to remove a NotI site in the multiple cloning site. EcoNI/NotI digestion of pFB-OPG-C400A-H followed by a Pfu polymerase fill-in reaction generated pFB-OPGADDB-H ending at residue 201 with a non-native tryptophan as residue 202, followed by the linker AAKKG and the His$_{6}$ tag. The sequence GLMHALKSHKTYHPFPTVTQSL365, followed by the linker and the His$_{6}$ tag, was subcloned between the Eco0109I and NotI sites of pFB-OPG-C400A-H to generate pFB-OPGAD-B-H, a truncate ending at residue 365. The end of the signal peptide (ALVFLDISIKWTTQ) and reaching into the first four residues...
of the first death domain (DVTL) were subcloned into the BsmHII and EcoNI sites of pFB-OPG-H to generate pFB-OPGΔCRD-H. All restriction enzymes were obtained from New England Biolabs (Beverly, MA).

Recombinant bacmids were produced by transformation of DH10Bac Escherichia coli cells (Invitrogen) with pFB plasmids. Bacmid DNA was isolated and purified. Sf9 insect cells (Spodoptera frugiperda; Invitrogen) were transfected by using FuGENE (1 μg of bacmid) to generate baculovirus as described (30). Viruses were amplified and titered using an agarose plaque-forming assay. T. ni insect cells (Trichoplusia ni; Invitrogen) were infected and growing logarithmically with each virus at a multiplicity of infection of 1. Cells were harvested at 24 h for OPG-C400A-H, 72 h for OPGΔB-H, and 48 h for all other constructs.

Protein Purification—The truncates OPG-C400A-H, OPGΔCRD-H, and OPGΔB-H were adsorbed over Ni²⁺-NTA-Sepharose and then eluted with stepwise imidazole washes. OPG-C400A-H and OPGΔCRD-H eluted at 1 M imidazole, and OPGΔB-H eluted at 300 mM. A Q-Sepharose column was used to purify OPGΔDDB-H, which elutes in the flow-through and was then adsorbed to Ni²⁺-NTA-Sepharose and eluted with 100 mM imidazole. C + RANK-L was purified over Q-Sepharose washed stepwise with NaCl. Elution occurred at 200–300 mM NaCl. T. ni-derived human OPG-IgG fusion (OPG-Fc), RANK-L, and OPG-H pure proteins were purified as described (18) and were provided as a generous gift from D. Willard, GlaxoSmithKline, Research Triangle Park, NC.

Sedimentation Analyses—Analytical ultracentrifugation of all proteins was performed at 20 °C using interference optics (for sedimentation velocity analysis of OPG-Fc and its complexes) in an XL-1 ultracentrifuge, employing an 8-hole rotor, or using absorbance optics (for sedimentation equilibrium analysis) in an XL-I ultracentrifuge, employing a 4-hole rotor. Samples were buffer-matched in 10 mM Tris, pH 7.5, 150 mM NaCl. The partial specific volumes (ρ) of the protein were estimated from the amino acid composition plus a glycosylation stepwise imidazole washes. OPG-C400A-H, OPGΔCRD-H, and OPGΔB-H eluted at 300 mM NaCl. A Q-Sepharose column was used to purify OPGΔDDB-H, which elutes in the flow-through and was then adsorbed to Ni²⁺-NTA-Sepharose and eluted with 100 mM imidazole. C + RANK-L was purified over Q-Sepharose washed stepwise with NaCl. Elution occurred at 200–300 mM NaCl. T. ni-derived human OPG-IgG fusion (OPG-Fc), RANK-L, and OPG-H pure proteins were purified as described (18) and were provided as a generous gift from D. Willard, GlaxoSmithKline, Research Triangle Park, NC.

Circular Dichroism Spectroscopy—CD spectra were collected at 25 °C using an Aviv circular dichroism spectrometer (model 202) from 300 to 190 nm in 2-nm increments using a 1-nm bandwidth and an averaging time of 30 s. The buffer-subtracted spectra were converted to mean residue ellipticity using the path length, number of residues, and molar concentration as measured by absorbance at 280 nm.

Surface Plasmon Resonance (Biacore)—Kinetic analysis was performed on a Biacore X, Biacore 1000, or a Biacore 2000 (Biacore, Inc., Piscataway, NJ) SPR instrument. Assays to measure OPG-Fc binding to RANK-L used a CM5 carboxymethyl-dextran sensor chip with protein A/G (Pierce) immobilized via amine coupling at 500 RU per flow cell. Approximately 150 RU’s of OPG-Fc were captured (10 mM Tris, 150 mM NaCl, pH 7.5) during a 1-min injection at 10 μl/min for each cycle. RANK-L at varying concentrations was then injected for 3.3 or 4 min at 30 μl/min followed by 10 min of dissociation using kinjet. The surface was regenerated back to the base-line level of immobilized protein A/G with a 1.5-min injection of 50 mM NaOH and a 1.5-min injection of 0.1 M H3PO4. Global analysis of at least six concentrations around the Kd was performed using BiaEvaluation 3.1 using a 1:1 Langmuir model that fits for the integrated version of these association and dissociation Equations 1 and 2 (36).

\[
\frac{dR}{dt} = k_d \cdot C \cdot (R_{\text{max}} - R) - k_a \cdot R 
\]  
(Eq. 1)

\[
\frac{dR}{dt} = -k_d \cdot R 
\]  
(Eq. 2)

Biacore assays for binding of the His₆-tagged wild type OPG and its truncates were done using a CM5 sensor chip with C + RANK-L immobilized through its free cysteine residues. This was done by amine coupling 2-(pyridyldisulfanyl)ethylamine-linked immobilization using the thiol coupling kit from Biacore. The assay was run under higher ionic strength conditions (350 mM NaCl) to prevent nonspecific interactions between OPG or its truncates (which are positively charged) and the carboxyl groups on the sensor surface matrix. OPG binding in the absence of RANK-L to the CM5 surface at 150 mM was eliminated with 350 mM NaCl. Binding of OPG constructs (including OPG-Fc as a control) was measured by a 4-min injection at 30 μl/min, followed by 10 min of dissociation using kinjet. The surface was regenerated using a 1.5-min pulse of 0.1 M H3PO4. Global analysis of at least six concentrations around the Kd was performed using BiaEvaluation 3.1 using a 1:1 Langmuir model for the association (Equation 1).
RESULTS

Tight Binding of RANK-L to OPG—Based on the equilibrium binding studies of Willard et al. (18) and other functional studies of OPG and RANK-L, the high affinity of this interaction is well established. We seek to understand the mechanism of this high affinity. We expanded on the work of Willard et al. (18) to include kinetic analysis of the interaction using surface plasmon resonance. The kinetics of RANK-L binding to OPG-Fc captured on a protein A/G/CM5 surface was measured over a 2.5 order of magnitude concentration range of RANK-L. OPG-Fc presents a covalent dimer in an oriented fashion on the sensor surface. RANK-L binds to OPG-Fc and forms a stable complex. The binding curves shown in Fig. 2 fit well to a 1:1 Langmuir binding model. The excellent fits to such a simple model indicate that in the low nanomolar range, the binding interaction of OPG and RANK-L is 1:1 (or some multiple thereof). This also indicates that the surface presented by the immobilized OPG homodimer is correctly displayed for binding to the RANK-L trimer. The result is a tight equilibrium binding constant of 1.7 ± 0.7 nM. This value was determined from the kinetic values from a 1:1 Langmuir binding model, $k_d = 1.33 ± 0.15 \times 10^{-3} \text{s}^{-1}$, $k_a = 7.62 ± 0.01 \times 10^5 \text{M}^{-1}\text{s}^{-1}$, and $K_d = 1.7 ± 0.7 \text{nM}$.

Testing the Avidity Model for OPG/RANK-L Interaction—The tight binding constant observed and the oligomeric states of OPG (homodimer) and RANK-L (homotrimer) suggest that avidity plays a key role in this interaction. To explore further the structural source of this avidity, analytical ultracentrifugation studies were performed. All species that have been studied by sedimentation velocity are diagrammed in Fig. 3. These symbols are used throughout the sedimentation velocity figures to highlight the different observed species. Sedimentation velocity profiles show the rate of sedimentation for each protein or complex in Svedberg units ($10^{-13}$). The sedimentation velocity profiles of OPG-Fc and RANK-L alone (Fig. 4, A and B, respectively, and quantitated in TABLE ONE) are consistent with a trimer for RANK-L and a dimer for OPG-Fc. These results are confirmed by the sedimentation equilibrium analysis in TABLE TWO. OPG-Fc does have a small amount of aggregated material that is likely to occur through association of the Fc domains. These aggregates form in a concentration-dependent fashion, and therefore consistent amounts were always observed by sedimentation velocity under these conditions. OPG-Fc contained in these aggregates appears to still be capable of binding.
### TABLE ONE
#### Sedimentation velocity analysis of individual proteins

| Species            | Protein | \(s_{20,w}\) [I] (S) | Co\(^{b}\) [I] | \(\chi^2\) |
|--------------------|---------|-----------------------|----------------|-----------|
| Wild type          | RANK-L  | 4.26 ± 0.01           | 0.66 ± 0.01\(^d\) | 162       |
|                   | OPG-Fc  | 5.81 ± 0.01           | 1.12 ± 0.01\(^d\) | 475       |
|                   | OPG-H   | 3.51 ± 0.01           | 0.52 ± 0.01\(^d\) | 56        |
| OPG-H variants    | OPG-HC400A | 3.17 ± 0.02              | 0.24 ± 0.02       | 75        |
|                   | OPG-HAB | 2.78 ± 0.03           | 0.17 ± 0.01       | 91        |
|                   | OPG-HΔDB| 2.05 ± 0.05           | 0.10 ± 0.01       | 70        |
|                   | OPG-HΔCRD| 2.53 ± 0.08            | 0.09 ± 0.01       | 111       |

\(^a\) \(s_{20,w}\) indicates sedimentation coefficient corrected for 20 °C in water.
\(^b\) Co indicates amplitude coefficient.
\(^c\) \(\chi^2\) indicates the goodness of the fit.
\(^d\) Detection was by interference optics rather than absorbance optics (280 nm) for all other measurements in this table.

### TABLE TWO
#### Sedimentation equilibrium analysis of individual proteins

The basic domain and the death domains both contribute to OPG dimerization, whereas cysteine 400 has no effect. The abbreviations used are as follows: NA indicates not applicable; M/D indicates monomer and dimer both observed; r.m.s.d. indicates root mean square deviation.

| Protein species | Protein | Masses | Error values | Summary |
|-----------------|---------|--------|--------------|---------|
|                 |         | Sequence mass\(^a\) | Solution mass\(^{b}\) [I] | Solution mass\(^{b}\) [II] | r.m.s.d.\(^{d}\) (\(\times 10^3\)) | SRS\(^{b}\) (\(\times 10^3\)) | \(K_{1,2}\) | Solution species |
|                 |         | kDa (monomer) | kDa | kDa | |
| Wild type       | RANK-L  | 20.0 | 61.0 | NA | NA | 1.94 | NA | Trimer |
|                 | OPG-Fc  | 72.3 | 146.9 | Aggregate | NA | 5.55 | NA | Dimer |
|                 | OPG-H   | 45.0 | 95.0 | 240 | NA | 15.0 | NA | Dimer |
| OPG-H variants  | OPG-HC400A | 45.0 | 92.0 | NA | NA | 0.14 | NA | Dimer |
|                 | OPG-HAB | 40.3 | 50.4 | 101 | 9.7 | 335 nm | M/D |       |
|                 | OPG-HΔDB| 21.9 | 32.4 | 64.8 | 3.6 | NA | 11 μM | M/D |
|                 | OPG-HΔCRD| 25.0 | 50.4 | NA | NA | 9.4 | NA | Dimer |

\(^a\) Sequence mass does not include glycosylation. Glycosylation is estimated to add 4–10 kDa to each monomer mass.
\(^b\) Solution mass is the average molecular weight if only one mass is provided or is determined by the fit to an interacting species model for covalent dimer/aggregate systems in OPG-Fc and OPG-H or a monomer–dimer interaction model for OPG-HAB and OPG-HΔDB.
\(^d\) r.m.s.d. values are from analysis by Igor Pro Macro (J. Cole) analysis.
\(^c\) Sum of residuals squared values are from nonlinear (M. Johnson) analysis (31).

### TABLE THREE
#### Sedimentation velocity results for wild type OPG (Fc and His) binding RANK-L

Only major species are presented in this table. Complete traces and fits are plotted in Figs. 4 and 5. An excess of OPG-Fc or OPG-H shifts the complex to a higher S value indicating a 2 OPG to 1 RANK-L complex. The abbreviations used are as follows: O-Fc is OPG-Fc; O-H is OPG-H; R is RANK-L; NP indicates that there was no major peak 2 and/or 3 for these ratios.

| Ratio           | \(s_{20,w}\) [I] | Co\(^{a}\) [I] | \(s_{20,w}\) [II] | Co [II] | \(s_{20,w}\) [III] | Co [III] | \(\chi^2\) |
|-----------------|-----------------|---------------|-----------------|--------|-----------------|----------|-----------|
| 1 O-Fc: 1 R     | 8.07 ± 0.01     | 0.99 ± 0.07   | NP              | NP     | NP              | NP       | 2811      |
| 1 O-Fc: 2 R     | 4.12 ± 0.01     | 0.87 ± 0.01   | 7.90 ± 0.03     | 1.14 ± 0.15 | NP              | NP       | 8680      |
| 2 O-Fc: 1 R     | 5.28 ± 0.01     | 0.51 ± 0.01   | 10.13 ± 0.01    | 1.47 ± 0.02 | NP              | NP       | 4418      |
| 1 O-H: 1 R      | 7.03 ± 0.01     | 1.23 ± 0.01   | 11.57 ± 0.01    | 0.21 ± 0.01 | NP              | NP       | 1121      |
| 1 O-H: 2 R      | 4.265\(^d\)     | 0.40 ± 0.01   | 6.81\(^d\)      | 0.82 ± 0.01 | NP              | NP       | 2338      |
| 2 O-H: 1 R      | 8.74 ± 0.01     | 0.29 ± 0.01   | 11.42 ± 0.03    | 0.04 ± 0.01 | 13.10 ± 0.05   | 0.15 ± 0.01 | 438       |

\(^a\) \(s_{20,w}\) indicates sedimentation coefficient corrected for 20 °C in water.
\(^b\) Co indicates amplitude coefficient.
\(^c\) \(\chi^2\) indicates the goodness of the fit.
\(^d\) These values were held constant during the fitting analysis because the peaks were not resolved.
indicated by the presence of a species at 5.3 S, which corresponds to free OPG-Fc dimer (Fig. 4E). The free OPG-Fc boundary accounts for approximately half of the signal of the 1 excess molar equivalent of OPG-Fc in the sample. This suggests that the affinity of the second OPG-Fc dimer binding to RANK-L is close to the protein concentration of OPG-Fc in the sample. This represents a decrease of 3 orders of magnitude for the strength of binding of the second OPG-Fc molecule to RANK-L, relative to the first one. Such reduced affinity is likely because of the use of two of the three available RANK-L trimer-binding sites for binding the first OPG-Fc dimer, leaving a one-site to one-site interaction for the second OPG-Fc dimer, which therefore does not benefit from avidity. Thus, the 2-site to 2-site interaction is roughly 3 orders of magnitude tighter than the 1-site to 1-site interaction.

For OPG-H, the same trends were observed in its interaction with RANK-L, although the data shown in Fig. 5 and in TABLE THREE had more complex sedimentation patterns because of the closer s values of the individual proteins and the complexes and the presence of aggregates and larger multimers. OPG-H, without the Fc domain to “fuse” the dimer still forms a stable 1:1 complex at 7 s and a 2 OPG-H to 1 RANK-L complex at 8.7 S in the presence of an excess of OPG-H. The large multimers observed around 11–12 s are likely 4 OPG-H-2 RANK-L or larger complexes.

**Functional Dissection of Domain Structure of OPG**—To determine which domains contribute to OPG dimerization and their impact on the RANK-L interaction, a series of OPG variants was prepared in the context of the OPG-H construct. These variants, for which the gene constructs are shown in Fig. 1 and the structural features are shown in Fig. 3, were generated to match those reported in the literature for biological activity measurements employing cell-based assays (29). We studied the homodimerization of these truncates and OPGC400A-H by equilibrium sedimentation analysis. TABLE TWO shows the equilibrium dissociation constants ($K_{D}$, dimer, a measure of the thermodynamic stability of the homodimer) for the OPGC400A-H mutant and truncates in comparison to OPG-H. Although Cys-400 does form inter-monomer disulfide bonds detectable by nonreducing SDS-PAGE, its absence in the OPGC400A-H point mutant has no apparent effect on the oligomeric state or thermodynamic stability of the OPG homodimer over the range of concentrations detectable by analytical ultracentrifugation. Cys-400 is located in the basic region. Therefore, for C-terminal truncated forms lacking the basic region ($\Delta B$, $\Delta DDB$), the Cys-400 is also truncated. For the OPGACRD-H construct, the Cys-400 is the wild type amino acid. The OPG$\Delta B$-H truncate (no basic region or C-terminal Cys-400); it was monomeric up to 11–12 S in the presence of an excess of OPG-H. The large multimers observed around 11–12 s are likely 4 OPG-H-2 RANK-L or larger complexes.

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TABLE FOUR

Sedimentation velocity results for OPG truncates and mutant binding RANK-L

Only major species are presented in this table. Complete traces and fits are plotted in Figs 6–9. The abbreviations used are as follows: OCA is OPGC400A; OΔB is OPGΔB; OΔΔDB is OPGΔΔDB; OΔCRD is OPGΔCRD; and R is RANK-L. OPGC400A and OPGΔΔB both show a 2 OPG to 1 RANK-L complex in the presence of excess OPG, whereas OPGΔΔDB does not; OPGΔCRD does not form a complex with RANK-L.

| Ratio       | \( s_{20, w} \) (1) | \( Co^a \) (1) | \( \Delta s_{20, w} \) (2) | \( Co^b \) (2) | \( \chi^2 \) \( e \) |
|-------------|---------------------|----------------|-----------------|----------------|-----------------|
| 1 OCA: 1 R  | 7.34 ± 0.01         | 0.33 ± 0.01    | 7.03 ± 0.02     | 0.23 ± 0.01    | 36              |
| 1 OCA: 2 R  | 4.95               | 0.55 ± 0.01    |                 |               |                 |
| 2 OCA: 1 R  | 8.59 ± 0.01         | 0.57 ± 0.01    |                 |               |                 |
| 1 OΔB: 1 R  | 5.40 ± 0.17         | 0.25 ± 0.06    | 6.96 ± 0.10     | 0.17 ± 0.06    | 203             |
| 1 OΔB: 2 R  | 4.30 ± 0.22         | 0.31 ± 0.15    | 5.66 ± 0.16     | 0.49 ± 0.13    | 207             |
| 2 OΔB: 1 R  | 6.04 ± 0.25         | 0.20 ± 0.08    | 7.43 ± 0.07     | 0.43 ± 0.07    | 233             |
| 1 OΔΔDB: 1 R | 6.67 ± 0.01   | 0.59 ± 0.01    |                 |               |                 |
| 3 OΔΔDB: 1 R | 2.05              | 0.20 ± 0.01    | 6.59 ± 0.01     | 0.45 ± 0.01    | 360             |
| 1 O ΔCRD: 1 R | 2.61 ± 0.01 | 0.099 ± 0.001  | 4.12 ± 0.01     | 0.23 ± 0.01    | 5.4             |
| 1 O ΔCRD: 2 R | 2.14 ± 0.05 | 0.04 ± 0.01    | 4.07 ± 0.02     | 0.41 ± 0.01    | 230             |
| 2 O ΔCRD: 1 R | 2.44 ± 0.05 | 0.16 ± 0.02    | 4.14 ± 0.06     | 0.28 ± 0.02    | 251             |

\( a \) \( s_{20, w} \) indicates sedimentation coefficient corrected for 20 °C in water.

\( b \) Co indicates amplitude coefficient.

\( c \) \( \chi^2 \) indicates the goodness of the fit.

\( d \) NP indicates that there was no major peak 2 for these ratios.

\( e \) These values were held constant during the fitting analysis because the peaks were not resolved.

We also determined the oligomeric species formed upon complexing of these OPG variants with RANK-L by sedimentation velocity. First, the individual OPG-H variants were studied in isolation, and their individual \( s \) values are shown in TABLE ONE. OPGC400A-H and OPGΔB-H (Figs. 6 and 7, respectively, with the major peaks shown in TABLE FOUR) formed 1:1 complexes similar to wild type OPG-H (Fig. 5) and showed some ability to form 1 RANK-L to 2 OPG dimer complexes under conditions of excess OPG, but only a 1:1 complex with excess RANK-L, just like the wild type OPG-H and OPG-Fc (Figs. 5 and 4, respectively). Sedimentation velocity data were collected at an OPG concentration of 1–6 \( 4 \), respectively). Sedimentation velocity data were collected at an OPG protein concentration of 1–6 \( \mu M \), well above the dimerization constant of OPGΔB-H (335 nM). This explains its wild type behavior under these conditions; once dimerized, OPGΔB-H behaves like wild type OPG in terms of its interaction with RANK-L. It is worth noting that OPGC400A-H behaves like wild type OPG-H except that it does not have the aggregates present for the wild type. Mutation of cysteine 400 to alanine creates a clean dimer population without any larger aggregates. This allows us to use OPGC400A-H for shape analysis (below) to mimic the wild type OPG-H.

OPGΔΔDB-H also formed stable complexes with RANK-L (Fig. 8 and TABLE FOUR). OPGΔΔDB-H is mostly monomeric at concentrations detectable by sedimentation velocity. The complex observed has a mass and \( s \) value consistent with one RANK-L bound to 2–3 monomers of OPGΔΔDB-H. Excess OPGΔΔDB-H is observed (at an \( s \) value of 2) unbound to RANK-L. This result is consistent with a single site interaction between OPGΔΔDB-H and RANK-L with an equilibrium binding constant in the low micromolar range. OPGΔCRD-H did not form any complexes with RANK-L detectable by sedimentation velocity analyses (Fig. 5 and TABLE FOUR).

To determine the binding constant (\( K_D \) RANK-L) for the association of the OPG variants with RANK-L, Biacore kinetic analysis was used. The Biacore assay was redesigned by immobilizing RANK-L instead of the His_{6/-}tagged OPG variants. This was done because the OPG-His_{6/-} was not stably captured on a Ni^{2+}-NTA sensor surface (over the 10–15 min of the assay), and the His_{6/-} tag affinity had a strong dependence on the dimerization state of OPG. Therefore, capture of the OPG-H and the truncated forms at varying surface stabilities makes comparison studies difficult. For this modified Biacore assay, a cysteine residue was introduced before the N-terminal amino acid of the soluble, secreted protein (C + RANK-L). Therefore, the new N terminus of the mature, secreted C + RANK-L is Cys-1. It was coupled to a carboxymethyl-dextran sensor surface using 2-(pyridyldisulfanyl)ethylamine coupling to covalently bond Cys-1 of C + RANK-L. Based on the crystal structure of murine RANK-L, the only native cysteine residue in the human RANK-L monomer is buried within the core of the trimer. Therefore, it is not available for disulfide bonding to the newly engineered Cys-1 or to couple to the sensor surface. The N terminus of RANK-L is oriented toward the cell membrane of the osteoblast expressing RANK-L (7, 19). Therefore, the orientation of C + RANK-L on the Biacore sensor surface is similar to its presentation on the surface of osteoblasts. We determined through control experiments that the binding of the immobilized C + RANK-L to OPG-Fc is quantitatively similar to that of protein A/G-immobilized OPG-Fc binding to wild type RANK-L, at both 150 and 350 mM NaCl (data not shown). This indicates that the C + RANK-L assumes the native trimeric structure and that it is oriented on...
the sensor surface such that it can easily bind OPG. This C + RANK-L sensor surface has been used to compare the ability of OPG truncates to interact with it, relative to wild type OPG-H (TABLE FIVE).

The reduced affinity of wild type OPG-H for RANK-L (10 nM) relative to OPG-Fc (1.7 nM) is likely because of increased conformational freedom. Because all mutations and truncates are in the OPG-H construct, all reductions in affinity and oligomerization are determined by comparison to wild type OPG-H. The point mutant OPG400A-H bound to C + RANK-L with an affinity slightly weaker than that of wild type, as shown in TABLE FIVE, again likely because of increased conformational freedom. The truncate OPGΔB-H showed a reduced ability to bind C + RANK-L, probably because of destabilization of the high affinity homodimeric form at lower Biacore assay concentrations, at or below its dimerization constant of 335 nM. OPGΔDDDDB-H exhibited very weak binding to C + RANK-L, only observable at Biacore assay concentrations at or above its dimerization constant. Binding of OPGΔCRD was not measurable, because of significant nonspecific binding even under higher ionic strength conditions (350 mM NaCl). The analytical ultracentrifugation results discussed above clearly demonstrated that OPGΔCRD does not bind RANK-L.

It can be argued that the observed changes in OPG dimerization and RANK-L binding stem from pleiotropic effects caused by the truncations on the folding of these variants. In order to assess the overall folding of OPG and its variants, we analyzed their secondary structure content by CD spectroscopy (Fig. 10). The spectra show that OPGΔB-H and OPG400A-H contain similar secondary structural elements relative to wild type OPG-H. More importantly, OPGΔCRD-H also has a CD profile very similar to that of full-length OPG-H, suggesting that the overall fold of the region comprising the death domains, basic region, and C-terminal disulfide is wild type-like. Therefore, the failure of this truncate to bind to RANK-L is because of the lack of the ligand-binding domain and not because of gross misfolding. Finally, despite the ability of OPGΔDDDDB-H to bind RANK-L at high concentrations, and thus a native-like structure, its CD spectrum lacks the signatures expected for regular elements of secondary structure such as α-helices and β-sheets. That profile fits the predicted structure of the N-terminal half of OPG, which is likely to consist of a series of loops held in place by an intricate pattern of disulfide bonds, as observed for the TNF receptor ligand-binding domain (7, 37).

Both OPG and RANK-L are glycosylated; however, the precise number and structure of the glycosylation are not known. Therefore, the partial specific volumes used for the sedimentation analysis, although they have been corrected for the estimated glycosylation, add variability to the measurements. For this reason, as well as a small amount of aggregated OPG that forms at sedimentation concentrations, these results provide more of a qualitative assessment of the interaction and its variation with modifications to OPG, and not a rigorous quantitative measurement.

Shape and Flexibility of OPG—Those individual proteins and complexes that produced clearly defined distributions for sedimentation velocity and equilibrium measurements were further analyzed to assess the shape and flexibility of OPG (TABLE SIX). The sedimentation velocity $s$ values and the solution masses were used to calculate the axial ratio of each species using supplemental Equations 1–3 (with supplemental Equation 4 as a control). The α-carbon homology model was used to estimate the static axial ratio for each construct or complex. The experimental axial ratio was divided by the model predicted axial ratio to give the estimated degree of flexibility shown in the last column of TABLE SIX. The higher this flexibility value, the more dynamic the protein or complex. This calculation allows us to assess the dynamic motion of the proteins because both deviations from globular structure and dynamic fluctuations will impede the rate of sedimentation, but the static model predicted axial ratio only accounts for the deviations from spherical shape. TABLE SIX shows that Cys-400 and the basic domain stabilize the dynamic motions of wild type OPG as evidenced by the

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**TABLE FIVE**

Biacore kinetics and affinity results for OPG constructs binding RANK-L

Cysteine 400 has a small contribution to RANK-L binding affinity, whereas the basic domain and the death domains are significant contributors to RANK-L binding affinity.

| Protein | Surface/experiment | $k_*$ | $k_*$ | $K_D$ | Equilibrium RU* | $\chi^2$ |
|---------|--------------------|-------|-------|-------|---------------|---------|
| OPG-Fc  | Protein A/G, OPG-Fc captured, RANKL as analyte | $7.6 \times 10^2$ | $1.3 \times 10^3$ | $1.7$ nM | 12 | 0.037 |
| OPG-Fc  | C + RANK-L, OPG-Fc as analyte | $2.5 \times 10^3$ | $1.5 \times 10^3$ | $6.0$ nM | 55 | 0.112 |
| OPG-H   | C + RANK-L, OPG-H as analyte | $3.0 \times 10^3$ | $2.0 \times 10^3$ | $10$ nM | 110 | 0.350 |
| OPG400A | C + RANK-L, OPG400A as analyte | $1.1 \times 10^3$ | $4 \times 10^3$ | $40$ nM | 90 | 0.289 |
| OPGΔB   | C + RANK-L, OPGΔB as analyte | $1.0 \times 10^3$ | $3 \times 10^3$ | $200$ nM | 35 | 0.079 |
| OPGΔDDDDB | C + RANK-L, OPGΔDDDDB as analyte | $1.6 \times 10^3$ | $5 \times 10^3$ | $3$ μM | 25 | 0.034 |
| OPGΔCRD | C + RANK-L, OPGΔCRD as analyte | ND | ND | ND | ND | ND |

* $\chi^2$ indicates the goodness of the fit.

**ND** indicates values were not determined due to high nonspecific binding of OPGΔCRD to CMS surface.
increase in dynamic fluctuations in the absence of these features. The binding of OPG to RANK-L causes a significant reduction in dynamic fluctuations, indicating that RANK-L binding stabilizes the dynamic motions of OPG. Thus, OPG is an elongated, flexible, and dynamic protein, and its binding to RANK-L reduces its conformational freedom.

## DISCUSSION

The molecular dissection of OPG performed here provides a mechanistic understanding of the function of OPG in bone regulation. We show that homodimeric osteoprotegerin interacts strongly ($K_D = 10\text{ nM}$) with homotrimeric RANK-L to form stable 1:1 dimer/trimer complexes. Avidity seems to play a key role in the formation of OPG-RANK-L complexes as suggested by two complementary sedimentation velocity experiments: (a) 1:2 OPG-RANK-L complexes were not formed in mixtures containing a 2-fold molar excess of RANK-L over OPG, suggesting that each OPG monomer in the homodimer cannot ligate a separate RANK-L trimer; (b) 2:1 OPG-RANK-L complexes did form when OPG was present at a 2-fold molar excess over RANK-L. However, the second OPG dimer exhibited a dramatic loss of affinity ($K_D \approx 3\mu M$). The simplest interpretation consistent with both results is that high affinity binding of OPG to RANK-L relies on avidity; the two OPG monomers per dimer bind to two of the three monomers in the RANK-L trimer. Only one monomer in the second OPG molecule is able to interact with the third (and only) available RANK-L monomer, albeit weakly. As will be discussed below, it is the ability of OPG to homodimerize tightly that ultimately determines its high affinity for RANK-L. This is the basis of its ability to compete effectively with the RANK receptor for this cytokine ligand. It supports the theory that RANK oligomerization, as induced by the trimeric RANK-L, is necessary for activation. If OPG binds tightly to at least two of the RANK-L-binding surfaces, it is able to inhibit RANK oligomerization and thus osteoclast maturation.

Sedimentation and surface plasmon resonance studies of full-length OPG, the mutant C400A, and truncates provided further evidence that OPG must be a homodimer for high affinity binding to RANK-L. The affinity of dimeric OPG-H for RANK-L (10 nM) is 3 orders of magnitude greater than the one observed with monomeric OPG (OPGΔDDD-H, 3 $\mu M$). This confirms that the avidity due to the 2-site/2-site interaction between OPG and RANK-L is critical for OPG function. This avidity effect is only achieved if OPG can dimerize at physiological concentrations. Both the entire basic region, ending at the C-terminal Cys-400 residue, and the death domains of OPG are necessary for dimerization in the low nanomolar range. However, the absence of Cys-400 itself has no measurable effect on dimerization, arguing against a significant role for the Cys-400-mediated C-terminal disulfide bond in the dimer formation of OPG. Yet the inter-monomer cysteine does contribute to the stability of the OPG dimer at least in terms of its dynamic fluctuations, and it does have a small impact on the affinity of OPGC400A for RANK-L compared with OPG-H, which is likely due to these dynamic motions. In contrast, the absence of the basic region significantly reduces the ability of OPG to dimerize by shifting the $K_D$ value of dimerization to 335 nM and thus reduces its affinity for RANK-L. The loss of the entire C-terminal half, the two death domains and the basic region, nearly prevents dimerization altogether. The cysteine-rich domains of the N-terminal half only very weakly dimerize with a $K_D$ of 11 $\mu M$. Therefore, the C-terminal half of OPG is essential for proper dimerization and thus the high affinity binding to RANK-L. It is interesting that the death domains in other family members are involved in heteroassociations (22), but in OPG they serve to homodimerize the receptor and stabilize the avidity interaction with RANK-L. The N-terminal cysteine-rich domains are necessary for OPG binding to RANK-L, because they

### TABLE SIX

| Protein or complex | Major $s_{20, w}$ | Major solution mass | Perrin factor | Axial ratio | Model predicted axial ratio | Degree of flexibility $^a$ |
|-------------------|-------------------|---------------------|--------------|-------------|-----------------------------|--------------------------|
| RANK-L            | 4.25              | 61.0                | 1.270        | 5.85        | 2.11                        | 2.7                      |
| OPG-Fc            | 5.81              | 146.9               | 1.705        | 17.25       | 3.72                        | 4.6                      |
| OPGC400A          | 3.17              | 92.0                | 2.259        | 42.75       | 2.58                        | 16.6                     |
| OPGAB             | 2.78              | 100.8               | 2.772        | 80.6        | 2.10                        | 38.9                     |
| OPGΔDDD           | 2.05              | 32.4                | 1.764        | 19.3        | 2.27                        | 8.5                      |
| OPGΔCRD           | 2.53              | 50.4                | 1.875        | 25.5        | 2.58                        | 9.9                      |
| OPGΔCRD:1R        | 8.07              | 186.0               | 1.407        | 8.78        | 3.72                        | 2.4                      |
| OPGΔCRD:1R        | 7.03              | 140.2               | 1.338        | 7.24        | 2.58                        | 2.8                      |

$^a$ $s_{20, w}$ indicates sedimentation coefficient corrected for 20 °C in water.

$^b$ Degree of flexibility is a simple comparison value derived from dividing the $b/a$ experimental by the $b/a$ theoretical.
Functional Dissection of Osteoprotegerin

contain the ligand-binding site. Yet binding of the monomeric CRDs is not sufficient, because of the loss of avidity.

Our in vitro data have physiological significance. Our data correlate well with functional dissection experiments in which the ability of OPG truncates to block RANK-L activation of osteoclast precursors was measured in cell-based assays. Those studies showed that elimination of the dimerization domain severely reduced the ability of OPG to block osteoclast maturation (29). That same study also reported that cysteine 400 was responsible for dimerization. However, those studies used non-reducing SDS-PAGE and immunoblotting to characterize the dimeric and “monomeric” species. No solution measurements such as analytical ultracentrifugation were performed to characterize the oligomerization. Our results indicate that by AUC, both the “monomer” and dimer observed by Yamaguchi et al. (29) and others (23) would have been dimeric species. Therefore, the claims by Goto and co-workers (23) that the monomer and dimer have the same biological activity are inaccurate because we were measuring two different dimeric species. OPGC400A-H is a dimer and thus demonstrating the same activity as dimeric wild type is an expected result.

The ligand binding and dimerization domains of OPG seem to behave as independent modules not only functionally but also structurally. Analysis of the secondary structure of OPG truncates relative to the dimeric wild type is an expected result.

Because they were measuring two different dimeric species.

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