

Structure of FcγRI in complex with Fc reveals the importance of glycan recognition for high-affinity IgG binding

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Fc gamma receptor I (FcγRI) contributes to protective immunity against bacterial infections, but exacerbates certain autoimmune diseases. The sole high-affinity IgG receptor, FcγRI plays a significant role in immunotherapy. To elucidate the molecular mechanism of its high-affinity IgG binding, we determined the crystal structure of the extracellular domains of human FcγRI in complex with the Fc domain of human IgG₁. FcγRI binds to the Fc in a similar mode as the low-affinity FcγRII and FcγRIII receptors. In addition to many conserved contacts, FcγRI forms additional hydrogen bonds and salt bridges with the lower hinge region of Fc. Unique to the high-affinity receptor-Fc complex, however, is the conformation of the receptor D2 domain FG loop, which enables a charged KHR motif to interact with proximal carbohydrate units of the Fc glycans. Both the length and the charge of the FcγRI FG loop are well conserved among mammalian species. Ala and Glu mutations of the FG loop KHR residues showed significant contributions of His-174 and Arg-175 to antibody binding, and the loss of the FG loop-glycan interaction resulted in an ~20- to 30-fold decrease in FcγRI affinity to all three subclasses of IgGs. Furthermore, deglycosylation of IgG₁ resulted in a 40-fold loss in FcγRI binding, demonstrating involvement of the receptor FG loop in glycan recognition. These results highlight a unique glycan recognition in FcγRI function and open potential therapeutic avenues based on antibody glycan engineering or small molecular glycan mimics to target FcγRI for certain autoimmune diseases.

CD64 | FcγRI | IgG recognition | crystal structure | glycan recognition

IgGs and pentraxins are circulating immune components that directly recognize pathogens. On formation of immune complexes or opsonization, they activate cellular response through Fc receptors (FcRs) (1, 2). The FcRs for IgGs include FcγRI (CD64); FcγRII (CD32) with A, B, and C isoforms; and FcγRIII (CD16) with two isoforms (3). Most of these are activating receptors either containing an intracellular immunoreceptor tyrosine-based activation motif or associated with an FcR common γ chain (4). FcγRIIB is an inhibitory receptor that contains an intracellular immunoreceptor tyrosine-based inhibitory motif. FcγRIIB does not have a cytosolic domain and is anchored to the plasma membrane through glycosylphosphatidylinositol linkage. The binding affinity to IgG ranges from 10^{-8} M for FcγRI to 10^{-5} – 10^{-7} M for FcγRII and III (3).

FcγRI plays an important role in the protection against bacterial infections, but also exacerbates certain autoimmune diseases (5). Owing to its high-affinity antibody binding, FcγRI is important in antibody therapy as well (6, 7). To date, the structure of the ligand-bound high-affinity receptor has not been determined, however. Consequently, the mechanism of its high-affinity antibody recognition remains to be elucidated. The role of glycan in antibody function has been a subject of intense study. Differential glycosylation of Fc, notably fucosylated Fc, is known to affect Fc receptor binding (8, 9). Furthermore, sialylated IgGs have been shown to be anti-inflammatory components of intravenous immunoglobulin (10, 11), and glycosylation affects

their binding to the low-affinity FcγRIIB and FcγRIII (11–13). Structural evidence suggests that the conserved glycosylation at Asn-297 of the constant region of IgG₁ is important to maintain the conformation of Fc for receptor binding (12, 14). Whether Fc receptors make significant glycan contacts for their IgG affinity is not clear, however.

Structures of the low-affinity Fcγ receptors have been determined with bound IgG-Fc (15–18). Unlike the other two-domain FcγRs, FcγRI contains three extracellular Ig-like domains, designated D1, D2, and D3. Earlier mutational analysis suggests that D2 and D3 domains are important to confer high-affinity antibody binding (19). Recently, the structure of human FcγRI showed a close packing of the FcγRI D1 and D2 domains resembling that of FcεRI, and mutations in the FG loop of the FcγRI D2 domain reduced its IgG binding affinity (20). The mechanism of the high-affinity FcγRI ligand recognition remains unresolved. To provide further insight into the high-affinity antibody recognition by FcγRI, and to facilitate the development of FcγRI-mediated immunotherapy, we determined the structure of the extracellular domains of human FcγRI in complex with the Fc domain of human IgG₁. Our study identifies a structural mechanism for high-affinity IgG binding by the receptor.

Results and Discussions

Overall Structure of the FcγRI-Fc Complex. The nonglycosylated extracellular region of human FcγRI (residues 16–289) was expressed in *Escherichia coli* as described previously, and then

Significance

Fc gamma receptor I (FcγRI) contributes to protective immunity against bacterial infections, but exacerbates certain autoimmune diseases. It is the sole high-affinity receptor for IgG and plays a significant role in immunotherapy. To date, there is no structural information available on how the receptor recognizes its antibody ligands, however. Consequently, the mechanism of its high-affinity IgG binding remains unclear. We report the first structure of the high-affinity Fc receptor in complex with IgG-Fc. The structural work reveals a direct receptor recognition of Fc glycan as a major factor in receptor affinity. This is the first example of Fc receptor making direct glycan contact through protein residues. The results have implications for the use of glycan engineering in immunotherapy.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4X4M).

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purified using sequential Ni-NTA and IgG₁ affinity columns. Human IgG₁-Fc (residues 216–446) was expressed in stable transfected CHO cells (7). The Fc γ RI and IgG₁-Fc complex was crystallized in a P1 space group and diffracted to 3.5-Å resolution. The structure was solved by the molecular replacement method using Phaser and refined using Phenix to final *R* factors of 0.245 for *R*_{cryst} and 0.295 for *R*_{free} (Table S1).

There are two complexes in each crystallographic unit cell. Electron densities are well defined for the two N-terminal domains of the receptor and for both chains of Fc (Fig. S1). The densities for the C-terminal D3 domain of Fc γ RI are less well defined, indicating flexibility in the receptor D3 domain orientation. The conformation of the Fc-bound Fc γ RI is essentially identical to that of the ligand-free receptor, resulting in a root mean square difference (rmsd) of 0.7 Å among all C α atoms. The human IgG₁-Fc (residues 224–446) contains the lower hinge peptide (Cys-229–Pro-238), C γ 2 (Ser-239–Lys-338) and C γ 3 (Ala-339–Gly-446) domains. Both the C γ 2 and C γ 3 domains of Fc have well defined electron densities, including the glycosylation associated with Asn-297. The overall conformation of Fc resembles that of Fc γ RIII bound Fc with a small but asymmetric rotation induced by the bound receptor to the C γ 2 domains, resulting in a loss of the twofold symmetry observed in the receptor-free Fc structure.

Fc γ RI bound to the lower hinge region of the Fc with its D1 and D2 domains resembling the conformation of the two-domain Fc γ RII, Fc γ RIII, and Fc ϵ RI ligand complexes (15–17, 21) (Fig. 1). Except for a \sim 10° rotation in the C γ 2 domains of each Fc to accommodate receptors, the entire complexes are superimposable, resulting in an rmsd of 1.0 Å between Fc γ RI-Fc and Fc γ RII

and an rmsd of 1.3 Å between Fc γ RI-Fc and Fc γ RIII. The Fc γ RI-Fc complex buries a total solvent-accessible surface area of 2,161 Å², significantly greater than the 1,800 Å² buried in the low-affinity receptor-Fc complexes (15). Previous work has suggested the importance of Fc γ RI D3 domain in IgG binding (19). The D3 domain is not in direct contact with Fc in the current complex structure; however, the receptor D3 domain packs against the D2 domain, forming a hydrophobic hinge core, suggesting that the D3 domain is important for maintaining receptor conformation and stability.

Interface of Fc γ RI with the Lower Hinge Fc Region Is Conserved. The interface between the receptor and the two Fc chains can be divided into three regions: between the Fc γ RI D2 domain C-strand (Tyr-133–Leu-136), C'-strand (Lys-142–His-148), and C'E loop (His-148–Trp-149) and the lower hinge region from the Fc A-chain; between the receptor D1-D2 interdomain hinge (Arg-102–Trp-104), D2 domain BC loop (Trp-127–Tyr-133), and lower hinge from the Fc B-chain; and between the Fc γ RI D2 domain FG loop (Met-171–Tyr-176) and the Fc glycans.

At the Fc A-chain lower hinge region, the receptor contacts are primarily salt bridges and hydrogen bonds. Specifically, Lys-142 and Lys-145 of Fc γ RI form two pairs of salt bridges with Fc residues, Glu-294 and Glu-269, respectively (Fig. 24). Both lysine residues are also present in the low-affinity Fc γ RIIA, Fc γ RIIB, and Fc γ RIII (Fig. 2C). Two hydrogen bonds are observed, between Asn-134 of the receptor and Asp-265 of Fc and between His-148 of the receptor and the carbonyl group of Gly-236 of Fc. Asn-134 is replaced with a Lys in the low-affinity Fc γ receptors. His-148 is conserved in Fc γ RIIA and Fc γ RIII, but replaced with

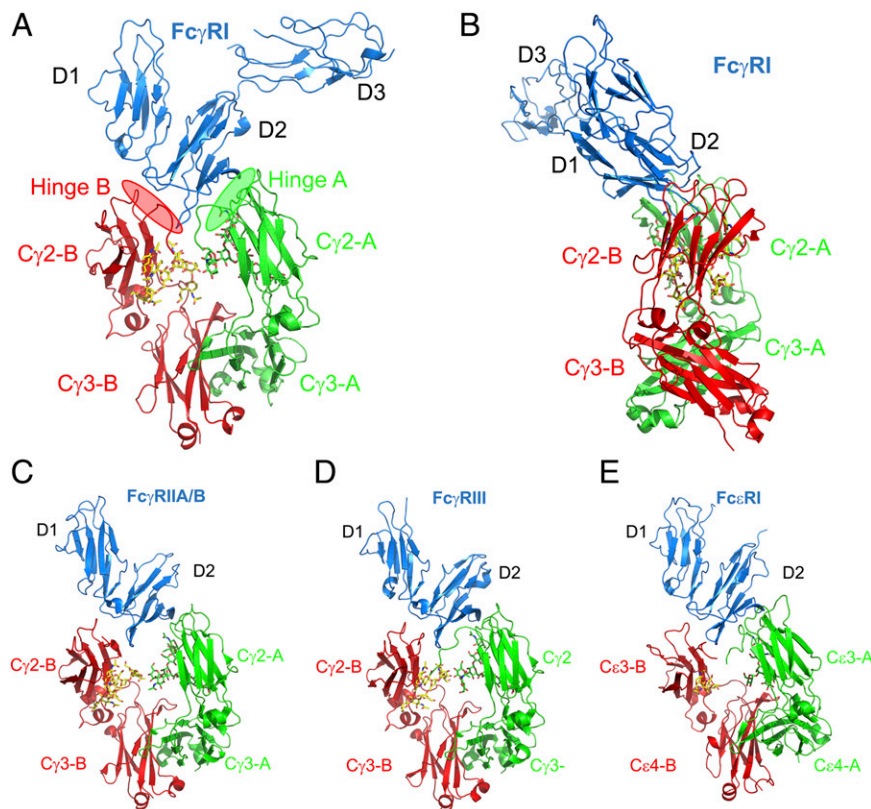


Fig. 1. (A and B) Ribbon representations of the overall structure of Fc γ RI (blue; A) and IgG₁-Fc complex (B) in two orthogonal views. (C–E) Structures of Fc γ RIIA/B (PDB ID codes 3WJJ and 3RY6) (C), Fc γ RIII (PDB 1T83) (D), and Fc ϵ RI (PDB ID code 1F6A) (E) complexes with their Fc. The two chains of Fc are shown in green (chain A) and red (chain B). Carbohydrates associated with Asn-297 on both the A-chain and B-chain of Fc are shown as stick models. Fc γ RI contact regions with the hinge A and B of Fc are shaded in green and red, respectively.

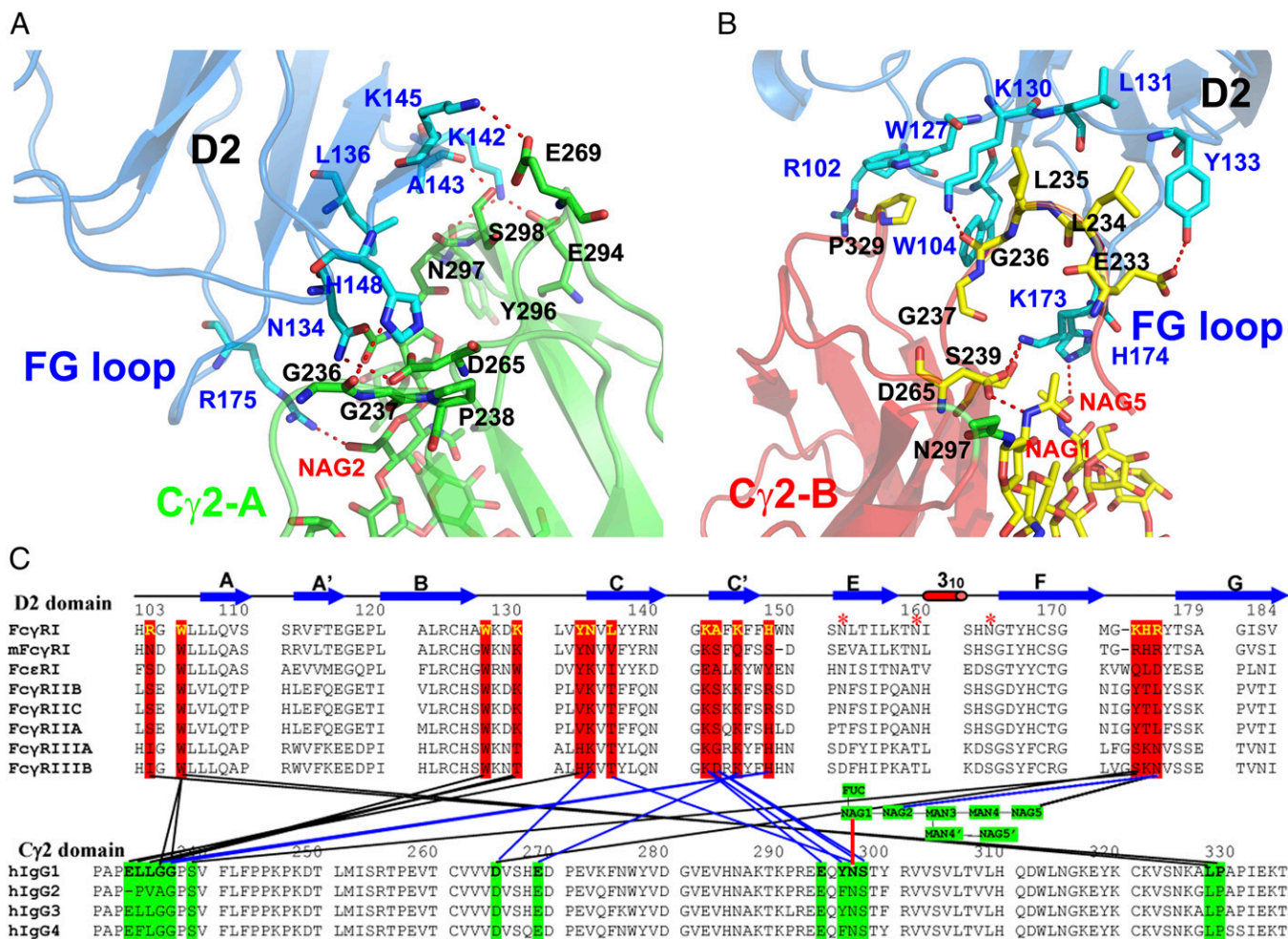


Fig. 2. Detailed receptor-Fc contacts at the hinge A (A) and hinge B (B) interface regions. FcγRI residues are shown as stick models in blue, and residues from the A and B chains of Fc are shown in green and yellow, respectively. Glycans associated with the A and B chains of Fc are shown in green and yellow, respectively. (C) Sequences of Fc receptors and IgG-Fc, with contacts colored in blue and black for the receptor interactions with chain A and chain B Fc, respectively.

an Arg in FcγRIIB. In addition, Leu-136 and Phe-146 of FcγRI form van der Waals interactions with the lower hinge LLGG motif of Fc (15). The majority of FcγRI contacts with the A chain of Fc are preserved in the low-affinity Fcγ receptors. Likewise, Asp-265, Glu-269, and Glu-294 are conserved among subclasses of IgGs.

At the Fc B-chain interface, FcγRI forms primarily van der Waals interactions with the Fc (Fig. 2B). Trp-104 and Trp-127 from FcγRI form a hydrophobic cluster with a pair of conserved leucine and proline residues, Leu-328 and Pro-329, from the Cy2 domain of the Fc. This cluster, also referred to as a Trp-Pro-Trp sandwich, is present in all low-affinity receptor complexes, including that of the IgE-Fc-FcεRI complex (15, 16, 21). Unique to FcγRI is a hydrogen bond between Arg-102 of the receptor and the carbonyl group of Pro-329 of Fc. A second hydrophobic cluster is observed between the side chains of Lys-130, Leu-131, and Tyr-133 of FcγRI and the lower hinge residues Leu-234 and Leu-235 of the Fc. This second hydrophobic cluster is smaller in the low affinity receptor-Fc complexes as both Leu-131 and Tyr-133 of FcγRI are replaced by smaller Pro, Ala, Val, and His residues in FcγRII and FcγRIII. Tyr-133 also forms a hydrogen bond with Glu-233 of Fc-B. Glu-233 is conserved in IgG₁, IgG₃, and IgG₄ but is replaced by a Pro residue in IgG₂, suggesting that the lack of this Glu-233–Tyr-133 hydrogen bond in IgG₂

contributes to its lower receptor binding affinity. Interestingly, this hydrogen bond is not present in FcγRIIA because the receptor contains a Val-133 in place of Tyr, suggesting that FcγRIIA is less discriminative against IgG₂ (22–24). As noted previously, other residues at the lower hinge of IgG₂ also differ from those of IgG₁ and may affect its receptor binding affinity (15).

The FcγRI D2 Domain FG Loop Forms Unique Contacts with Fc Glycan.

The third interface is from the receptor D2 domain FG loop, ¹⁷¹MGKHY¹⁷⁶, to Fc and its associated glycan. In particular, Lys-173 of the receptor forms a salt bridge with a conserved Asp-265 on the B-chain of Fc (Fig. 3A). A similar salt bridge is present between the Asp-265 on the B-chain of Fc and Lys-120 on the C-strand of FcγRIII in the low-affinity receptor complex structure (15); however, Lys-120 of FcγRIII is not conserved in FcγRI and is replaced by an asparagine. Instead, Lys-173 of FcγRI serves as a surrogate salt bridge partner.

His-174 and Arg-175 of the receptor extend into the Fc opening toward the glycans, such that their closest contacts are the carbohydrates attached to Asn-297. The His-174 side chain is close to the C4 hydroxyl group on a branched mannose. Both Lys-173 and His-174 are close to the glycan attached to the B-chain of Fc. The side chain of Arg-175 is located within hydrogen-bonding distance to the O3-hydroxyl of the first N-acetyl glucosamine (GlcNAc) and to the O6-hydroxyl of the second

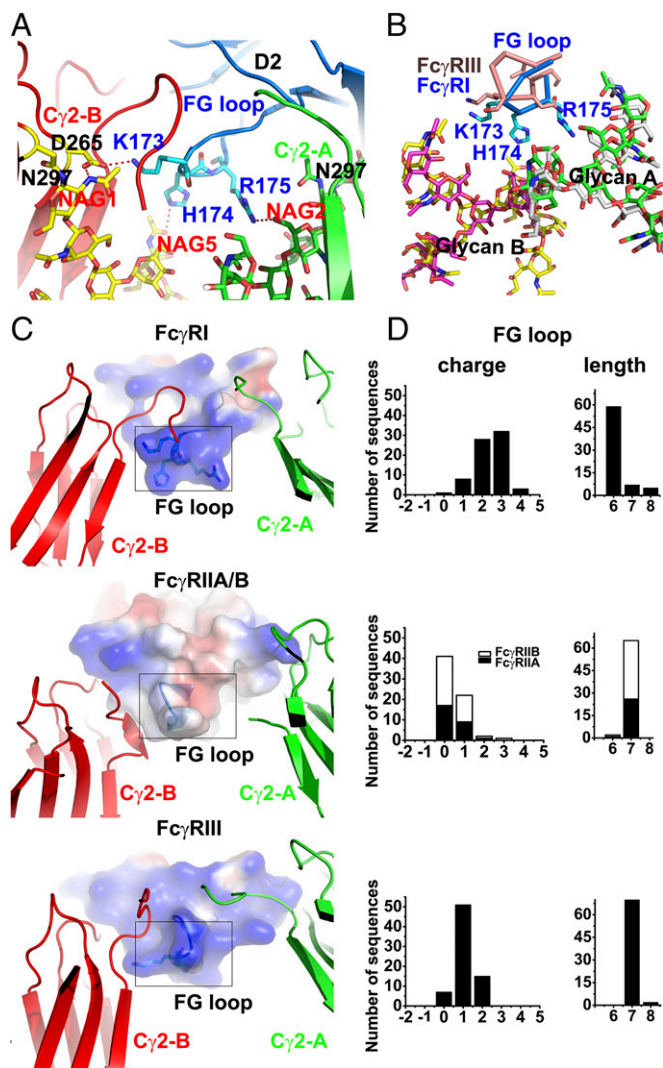


Fig. 3. (A) Detailed contacts between the Fc γ RI D2 domain FG loop and Fc with glycans associated with the Fc A and B chains in green and yellow, respectively. (B) Structural comparison of the FG loop conformation between Fc γ RI (blue) and Fc γ RIII (wheat). The structures are superimposed on their Fc A chain. The glycans (magenta and white) from Fc γ RIII-Fc complex (PDB ID code 1T83) and the glycans (yellow and green) from the current Fc γ RI-Fc complex are well superimposed. The FG loop of Fc γ RI is positioned closer to the carbohydrates. (C) Electrostatic potential [-50, 0, 50] of the D2 domain FG loop calculated for Fc γ RI (Top), Fc γ RIIA/B (Middle; PDB ID codes 3WJJ and 3RY6), and Fc γ RIII (Bottom; PDB ID code 1T83). (D) Distribution of the FG loop charge and length among homologous mammalian Fc γ RI (Top), Fc γ RIIA (Middle; solid bars), Fc γ RIIB (Middle, shaded bars), and Fc γ RIII (Bottom) receptors.

GlcNAc attached to the A-chain of Asn-297 (Fig. 3A). Thus, the FG loop links glycans from both chains of Fc. Interestingly, all three FG loop residues contact the proximal rather than distal carbohydrate units of the Fc glycan, suggesting that the receptor interacts with the conserved and less flexible part of the glycan. This is also consistent with the finding that EndoS-treated IgG₁, which leaves the first GlcNAc and its associated branched core fucose, retains better Fc γ RI binding than IgG₁ treated with peptide-N-glycosidase F, which removes all glycan attached to Asn-297 (23).

In comparison, the D2 domain FG loops of the low-affinity Fc γ Rs are 8–10 Å away from the Fc glycans and make little contact with Fc, except for a hydrogen bond between the Fc γ RIII

FG loop Lys N ζ and the backbone carbonyl of the lower hinge Gly-236 on Fc (Fig. 3B) (15, 17, 25). Whereas the Fc γ RI contacts to both hinge A and hinge B of Fc are largely conserved in the low-affinity Fc γ receptors, the contacts from the FG loop of Fc γ RI are unique to the high-affinity receptor, because the FG loop KHR motif is not conserved in other Fc γ receptors, except for the presence of a Lys in Fc γ RIII in the His-174 position of Fc γ RI (Fig. 2C).

Structural Mechanism for High-Affinity Fc γ RI Binding to Fc. Although the overall receptor-ligand docking mode is similar in all Fc γ receptors, Fc γ RI is the sole high-affinity receptor for monomeric IgG. Two of the three Fc γ RI-Fc contact sites involve receptor residues interacting with the lower hinge regions of the Fc chains. Both the hinge A and hinge B contacts are quite conserved in the low-affinity Fc γ RII and Fc γ RIII ligand complexes, suggesting that they are the critical contacts to define the common Fc γ R receptor and IgG binding mode. Interestingly, Fc γ RI forms a more extensive hydrophobic contact at hinge B, thus favoring its binding to IgG. In contrast, the interface between the FG loop of Fc γ RI and IgG is unique to the high-affinity receptor. The equivalent receptor FG loops in the low-affinity receptor-ligand complexes do not form significant contacts to Fc.

The importance of the FG loop to high-affinity receptor-IgG binding was demonstrated in previous mutational work (26). Placement of the FG loop of Fc γ RI into Fc γ RIII resulted in a 15-fold increase in IgG binding affinity, and the mutant Fc γ RIII bound to IgG only fivefold weaker than Fc γ RI. As noted previously, the FG loop of Fc γ RI is one residue shorter than the FG loops of other Fc γ receptors (Fig. 2); however, this affinity increase is lost when a Val is inserted into the FG loop to match the length of those in the low-affinity Fc γ receptors. Structurally, this shorter FG loop of Fc γ RI is positioned ~5 Å closer to the glycan in the center of the horseshoe opening than that of Fc γ RIII (Fig. 3B). Interestingly, the D2 domain FG loop of Fc γ RI is the most positively charged FG loop among all three Fc γ receptors, owing to the presence of the KHR motif (Fig. 3C).

To further address the significance of a shorter but positively charged FG loop to the high-affinity Fc γ receptor, we compared the length and the number of charged residues present in the FG loop sequences among homologous Fc γ receptors in mammalian species with Lys, Arg, and His each as +1 and Asp and Glu each as -1. The sequences of majority homologous Fc γ RI receptors have six amino acid FG loops and carry +2 or +3 charges (Fig. 3D). In contrast, most of the D2 domain FG loops in all homologous low-affinity receptors are seven residues long. The FG loop sequences of Fc γ RIIA and Fc γ RIIB carry +1 or less charge, whereas those of Fc γ RIII carry 0 to +2 charges, with majority +1. Furthermore, the D1 domain FG loop of Fc γ RI is also longer and carries a -1 net charge, similar to the corresponding loops in the low-affinity receptors. Thus, the shorter and positively charged FG loop is a unique feature of the high-affinity receptor. Structurally, the shorter FG loop enables it to move closer toward the Fc glycans and to adopt a glycan binding conformation.

Mutations in the FG Loop of Fc γ RI Reduce Its IgG Binding Affinity. To further investigate the contribution of the FG loop KHR motif to high-affinity receptor-IgG binding, we generated both single and triple alanine mutations of the KHR motif and measured the mutant receptor binding affinities to all three subclasses of IgGs. K173A and H174A mutant Fc γ RI exhibited only minor reductions in their binding affinities (twofold to fivefold) to IgG₁, IgG₃, and IgG₄ (Table 1). In contrast, R175A mutation, the most disruptive mutant, resulted in ~20 fold reductions to IgG₁, IgG₃, and IgG₄ binding. The triple AAA mutant (K173A/H174A/R175A) bound to all three IgGs with 0.18–0.3 μM affinity, similar to that of R175A.

Table 1. Dissociation constants for Fc γ RI and IgG binding

Fc γ RI	Affinity, K_D , nM		
	IgG $_1$	IgG $_3$	IgG $_4$
WT, CHO	8.8 \pm 7	3.3 \pm 3	26.2 \pm 2
WT, <i>E. coli</i>	7.0 \pm 1	8.1 \pm 7	16.7 \pm 7
K173A	25.9 \pm 4	40.3 \pm 10	49.4 \pm 10
H174A	16.8 \pm 8	29.4 \pm 3	32.0 \pm 10
R175A	166.0 \pm 35	157.0 \pm 37	314.0 \pm 74
AAA	180.0 \pm 42	189.0 \pm 67	320.0 \pm 83
K173E	44.0 \pm 6	45.6 \pm 22	200.0 \pm 15
H174E	250.6 \pm 115	285 \pm 89	303.0 \pm 152
R175E	26.0 \pm 2	27.0 \pm 8	51.3 \pm 7

To evaluate the effect of charge contribution to Fc γ RI and IgG binding, we replaced the positively charged KHR each with a negatively charged Glu in the FG loop. K173E and R175E resulted in mild reductions (threefold to 10-fold) in IgG affinity. In contrast to the Ala mutants, R175E is the least disruptive among the three mutations, and H174E is the most disruptive, with 15- to 30-fold reductions in IgG binding affinities. The differential mutational effects on IgG binding, in which Glu is the most disruptive at residue 174 and Ala is the least favored at position 175, suggest that charge interaction is more important to H174, whereas hydrogen bond interaction is more important to R175. Both Ala and Glu mutations showed that glycan contact by the FG loop contributes 20- to 30-fold in receptor-IgG binding affinity, consistent with the earlier results from the FG loop swapping (26).

To further investigate the interaction of glycan with the FG loop, we measured Fc γ RI binding to IgG $_1$ treated with peptide-*N*-glycosidase F. Removal of the Fc glycan reduced receptor binding to 0.35 μ M, a 40-fold decrease in affinity. The greater loss in IgG $_1$ binding affinity by Fc γ RI deglycosylation compared with the R175A, H174E, and AAA mutations is likely related to an additional conformational destabilization in Fc as the result of glycan removal (12, 14). Thus, both the receptor mutations and glycan removal resulted in a loss of binding affinity, demonstrating the specific glycan binding by the FG loop KHR motif of Fc γ RI. Given that deglycosylation reduced the IgG $_1$ binding affinity of Fc γ RI to a level comparable to that of the lower-affinity Fc γ receptors, it is conceivable that the receptor can still recognize aglycosylated IgG in the context of multivalent immune complexes (23).

Potential Contribution of Fucosylated and Sialylated Fc Glycan to Fc γ RI Binding. Even though the electron densities at the current resolution of the complex contain significant ambiguities, there are sufficient densities for much of the Fc glycan, although the proximal carbohydrates are better defined than the distal carbohydrate units. The *N*-linked glycans associated with Asn-297 are fucosylated biantennae complex glycans. Receptor glycosylation on Asn-162 of Fc γ RIIIA was observed to interact with nonfucosylated glycan to stabilize the receptor complex, suggesting that a fucosylated glycan may inhibit such interaction (9, 18). Because Asn-162 is not present in Fc γ RI, the potential role of the branched fucose associated with the Asn-297 of Fc must be different for the high-affinity receptor. On both Fc chains, the branched fucose units form van der Waals contacts with Tyr-296 of Fc. On both Fc chains, the glycans extend along the Fc peptide, making multiple hydrogen bonds and van der Waals contacts with the C γ 2 domain residues, including Phe-241, Phe-243, Arg-301, and Lys-334 (Fig. 4). These interactions help reduce the conformational flexibility of the glycan. The direction of the glycan points from Asn-297 toward the C γ 3 domain of its Fc. The terminal sialic acid is visible only on one chain (Fig. 4), and is

located close to hydrogen bonding distance to the side chain of Ser-375 and Asp-376 from the C γ 3 domain of Fc, suggesting that sialylated glycans may reduce glycan conformational flexibility by contacting the C γ 3 domain of Fc.

In summary, the structure of the high-affinity human Fc γ RI bound to IgG $_1$ -Fc illustrates both a conserved receptor recognition to the lower hinge region of Fc that defines the common Fc γ receptor-IgG binding mode and a unique Fc γ RI FG loop interaction with Fc and its glycans that contributes to receptor high-affinity IgG binding. The contribution of Fc glycan to Fc γ RI high-affinity ligand binding opens potential new avenues in immunotherapy to apply glycan engineering in targeting Fc γ RI to achieve desired antibody efficacy. It also raises the possibility of using small-molecular glycan-based compounds or mimics to target Fc γ RI as effective treatment for certain autoimmune diseases.

Materials and Methods

Protein Expression, Purification, and Crystallization. The ectodomain of Fc γ RI (residues 16–289) was engineered with 19 mutations as described previously, including T20P, T25K, T38S, L46P, T63I, S69T, R71H, V77E, N78D, I100V, F114L, T160M, N163S, N195T, N206T, L207P, N240D, L283H, and L285Q. This engineered Fc γ RI ectodomain was synthesized and cloned into a pET26b vector by gene synthesis (Genscript), solubly expressed in *E. coli* cells, and purified via Ni-NTA affinity chromatography followed by IgG affinity chromatography. The purified Fc γ RI from *E. coli* bound to human IgG $_1$, IgG $_3$, and IgG $_4$ with nM affinities similar to those from recombinant mammalian expressed Fc γ RI (Table 1). Human IgG $_1$ -Fc protein (216–444) was expressed in CHO DXB-11 cells. The human tissue plasminogen activator prepro secretion signal sequence was used to direct secretion of the IgG $_1$ -Fc.

The recombinant Fc was purified by a protein A affinity column, followed by a Superdex 200 size exclusion column (GE Healthcare) in 50 mM sodium phosphate and 109 mM sodium chloride pH 7.3. The final product was filtered through a 0.22- μ m syringe filter, aliquotted into polypropylene vials, and stored at below -60 $^{\circ}$ C. Purified IgG $_1$ -Fc was verified by N-terminal sequencing, was at least 95% pure, and contained <0.27 endotoxin units/mg. Before crystallization, Fc γ RI was mixed with Fc dimer at a 1.2:1 molar ratio, and the Fc γ RI-Fc complex was further purified by gel filtration chromatography in 10 mM Hepes (pH 7.4) and 0.15 M NaCl. The crystals used for data collection were grown in hanging drops at 22 $^{\circ}$ C using 1 μ L of protein with a final OD $_{280\text{nm}}$ of 6.7 and 1 μ L of reservoir solution (10% PEG 8000, 10 mM Hepes

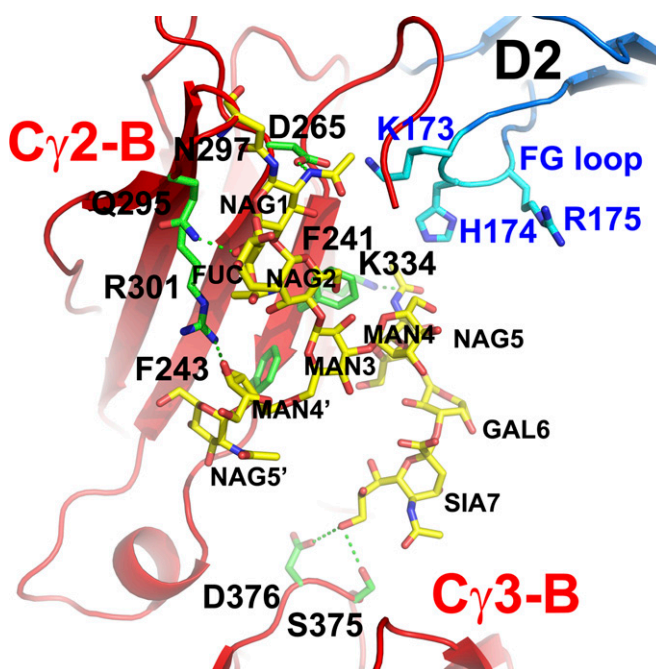


Fig. 4. Detailed interaction between carbohydrates associated with Asn-297 and Fc.

pH 7.5, and 50 mM Li₂SO₄). Human plasma IgG₁, IgG₃, and IgG₄ were purchased from Athens Research & Technology.

Diffraction Data Collection and Structure Determination. The crystals were immersed in cryoprotectant (20% PEG 8000, 10 mM Hepes pH 7.5, 50 mM Li₂SO₄, and 15% glycerol) before being flash-cooled in liquid nitrogen. Five X-ray datasets were collected to 3.5-Å resolution at SER-CAT beamlines, processed, and merged with HKL2000 (27) (Table S1). FcγRI-Fc complex crystals belong to space group P1 with a pseudomerohedral twofold twinning. The structure of the FcγRI-Fc complex was solved by a molecular replacement method with Phaser (28) in the CCP4 package (29) using FcγRI (PDB ID code 3RJD) and Fc (PDB ID code 3AY4), respectively, as the search model. Model building and refinement were carried out using Coot (30) and Phenix (31) with noncrystallography symmetry (NCS) restraints and the twin law of (−h, −k, l). The overall electron density of the complex is of decent quality (Fig. S1).

Carbohydrate molecules were added manually using (2Fo-Fc) electron density maps contoured at 1.0σ (SD of the map) and refined. The residues are numbered consistent with FCGR1_HUMAN in the Swiss-Prot entry. The final model includes two FcγRI-Fc complexes (PDB ID code 4X4M). The Ramachandran statistics were generated and verified by Procheck of CCP4. Hinge angles were calculated using HINGE (32), and the buried surface area was calculated using the CCP4 package. The electrostatic charges were converted from atom coordinates using the PDB2PQR server with the CHARMM force field and calculated by APBS and displayed between −50 and 50 kT/e (33). All structure figures were generated with Pymol (34).

Site-Directed Mutagenesis of FcγRI. FcγRI D2 domain FG loop (¹⁷¹MGKHRY¹⁷⁶) mutations were generated by site-directed mutagenesis using a QuikChange

II Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions. A total of eight mutants were generated, including three single Ala mutations (K173A, H174A, and R175A), a triple AAA mutant (K173A/H174A/R175A), three single Glu mutations (K173E, H174E, and R175E), and a triple EEE mutant (K173E/H174E/R175E). All mutations were confirmed by DNA sequencing (ACGT Inc.). The mutated FcγRI proteins were expressed as WT as described above and purified by Ni-NTA chromatography.

Surface Plasmon Resonance Solution Binding Experiments. Surface plasmon resonance measurements were performed using a Biacore 3000 instrument and analyzed with BIAevaluation 4.1 software (GE Healthcare). Different human IgG subclasses were obtained from Athens Research & Technology. For measuring the affinity to WT or mutant FcγRI proteins, human IgG₁, IgG₃, and IgG₄ were immobilized on Biacore carboxylated dextran CM5 chips (GE Healthcare) to 200–1,000 response units using primary amine coupling in 10 mM sodium acetate (pH 5.0). The analytes consisted of serial dilutions of WT or mutant FcγRI proteins between 2 μM and 63 nM in a buffer containing 10 mM Hepes (pH 7.4) and 0.15 M NaCl. The dissociation constants were obtained by kinetic curve fitting for the binding of FcγRI to IgG, and steady-state fitting for the binding of FcγRI mutants to IgG, using BIAevaluation 4.1 software. IgG₁ (100 μg) was treated with 2,500 U of peptide-N-glycosidase F (New England Biolabs) in sodium phosphate buffer (pH 7.5) at 37 °C for 3 h to remove the glycan on Fc. Representative binding sensorgrams are shown in Fig. S2.

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