Site-specific N-glycosylation analysis of soluble Fcγ receptor IIIb in human serum

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Fc-receptors for immunoglobulin G (FcγRs) mediate a variety of effector and regulatory mechanisms in the immune system. N-glycosylation of FcγRs critically affects their functions which is well exemplified by antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis mediated by homologous FcγRIa and FcγRIIb, respectively. Although several reports describe N-glycosylation profiles of recombinant FcγRIII glycoproteins, much remains unknown regarding their native glycoforms. Here we performed site-specific N-glycosylation profiling of a soluble form of FcγRIIIb purified from human serum based on mass spectrometric analysis. Our data indicate a distinct and common tendency of the glycoforms exhibited at each N-glycosylation site between the native and the previously reported recombinant FcγRIII glycoproteins. Among the six N-glycosylation sites of serum soluble FcγRIIIb, Asn45 was shown to be exclusively occupied by high-mannose-type oligosaccharides, whereas the remaining sites were solely modified by the complex-type oligosaccharides with sialic acid and fucose residues. The results of our endogenous FcγRIII glycoform analyses are important for the optimization of therapeutic antibody efficacy.

Various effector and regulatory mechanisms in the immune system are mediated through the interactions between immunoglobulins (Igs) and their cognate receptors that specifically recognize their Fc portions1–3. Fc-receptors for IgG (FcγRs) are categorized into three classes: FcγRI, FcγRII, and FcγRIII, which exhibit different binding affinities to IgG isotypes and distinct expression profiles on immune cells. In humans, each Fcγ class shows structural variations resulting from multi-genes, alternative splicing, and genetic polymorphisms4. Human FcγRIII has two isoforms, transmembrane FcγRIIia and glycosylphosphatidylinositol-linked FcγRIIib, encoded by two individual genes, and share 96% amino acid sequence identity in their extracellular Fc-binding regions (Fig. 1). FcγRIIia is primarily expressed on natural killer cells and promotes antibody-dependent cell-mediated cytotoxicity (ADCC) by interacting with the IgG of the antigen–antibody complex5, whereas FcγRIIib is exclusively expressed on neutrophils and mediates the degranulation and phagocytosis of the antibody-labeled target cells6–8. These receptors exist not only as membrane proteins but also in soluble forms, designated as sFcγRIIia and sFcγRIIib, each comprising two extracellular Ig-fold-domains proteolytically cleaved from the transmembrane segment6–11.

FcγRs are modified with N-glycans that significantly affect their interactions with IgGs12–14, and human FcγRIIia molecules with different N-glycosylation patterns exhibit different affinities for IgG15. This is well exemplified by the effects of glycosylation at Asn45 and Asn162 of human FcγRIIia on its interactions with IgG112,16. Crystallographic data suggest that the Asn162 glycan has the potential to interact with the N-glycan of IgG1–Fc, thereby reinforcing the IgG1–FcγRIIia interactions, whereas the Asn45 glycan cause steric hindrance to the Fc of IgG117–19. The intermolecular carbohydrate–carbohydrate interactions involving the Asn162 glycan can be
optimized to increase the Fc-RIIIa-binding affinity of IgG1 by removing the core fucose of the Fc glycan, which offers a promising strategy for the improvement of therapeutic antibody efficacy20–23. Hence, the glycosylation of Fc-γRs is now considered a critical factor in the design and development of antibody therapeutics3,4,24.

N-glycosylation profiling of Fc-γRs has been performed using high-performance liquid chromatography (HPLC) and mass spectrometry (MS) in a total or site-specific manner24–29. However, to date, the structural information on the Fc-γR glycosylation has been obtained using recombinant proteins produced by mammalian cell lines. Furthermore, the glycosylation patterns of the recombinant Fc-γRs depend on the expression vehicles14,25. To understand the molecular mechanisms of the Fc-γR-mediated functions better, from both immunological and therapeutic perspectives, it is crucial to elucidate the glycosylation profiles of the endogenous Fc-γRs.

To address this issue, as a first step, we performed site-specific N-glycosylation profiling of sFc-γRIII purified from human serum. Plasma level of sFc-γRIII is approximately 1 μg/mL in healthy individuals30. The vast majority of sFc-γRII molecules present in plasma are derived from neutrophils31, indicating that sFc-γRIII is a dominant isoform in the serum. Using liquid chromatography (LC)-electrospray tandem mass spectrometry (MS/MS) analysis, we analyzed the site-specific N-glycosylation profile of the endogenous sFc-γRIII, consisting of the extracellular domains released from the immune cell membranes by proteolytic cleavage.

### Results and Discussion

As previously reported8, sFc-γRIIIb was purified from a pool of human serum by a series of chromatographic procedures. Consistent with the previous report7, we obtained a smear, instead of a band, of the sFc-γRIIIb, which was stained with Coomassie Brilliant Blue (CBB), indicating that the serum sFc-γRIIIb is highly glycosylated with considerable heterogeneity (Supplementary Fig. 1). According to the sandwich ELISA results, we obtained 100 μg of sFc-γRIIIb from 2 L of human serum. Due to the limited sample availability, we performed only one round of sFc-γRIIIb purification and site-specific glycosylation profiling.

We analyzed the purified serum sFc-γRIIIb using LC-MS/MS after the GluC and chymotrypsin digestions, and identified and semiquantified (i) 14 glycoforms on Asn38, (ii) 6 glycoforms on Asn45, (iii) 30 glycoforms on Asn48, and (iv) 30 glycoforms on Asn51. Our data revealed that each domain released from the immune cell membranes by proteolytic cleavage.

![Figure 1](image-url) Sequence alignments of the extracellular regions of human Fc-γRIIIa and sFc-γRIIIb NA1 and NA2 forms. N-glycosylation sites are shown in red. The residues substituted between NA1 and NA2 are shown in blue.

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Site-specific glycosylation information has been reported for recombinant sFcγRIIIb produced by BHK cells, as well as for the recombinant sFcγRIIIa produced by HEK293T and CHO cells. In Table 3, site-specific N-glycan classification of the human serum sFcγRIII is compared with those of the recombinant sFcγRIII glycoproteins. In the serum sFcγRIIIb, the Asn45 glycosylation site exclusively displays high-mannose-type glycans, whereas other glycosylation sites solely exhibit complex-type glycans. The glycans at Asn38, Asn74, Asn162, and Asn169 apt to be modified with complex-type oligosaccharides in the recombinant sFcγRIIIs as well. In the BHK-generated sFcγRIIIb, the major glycans associated with the Asn45 site were consistently shown to be of the high-mannose type, whereas Asn64 is inconsistently modified also by high-mannose-type oligosaccharides. Considerable fractions of the Asn45 glycans were shown to be occupied by hybrid-type oligosaccharides in the recombinant sFcγRIIIa glycoproteins.

In general, the progression of N-glycan processing depends on the degree of exposure of the individual oligosaccharide moiety to the solvent, as demonstrated by the statistical analysis. Highly accessible asparagine residues tend to be occupied by complex-type glycans, while less-exposed sites are frequently occupied by high-mannose-type and/or hybrid-type glycans. The solvent accessibility to the N-glycosylated asparagine residues in the crystal...
structure of sFcγRIIIb (PDB code: 1FNL)33 is as follows: Asn162 > Asn64 > Asn169 > Asn38 > Asn45 > Asn74. Therefore, the magnitude of N-glycan maturation cannot be simply ascribed to the solvent exposure of glycosylation sites in the native tertiary structure of a carrier protein estimated from the crystal structure.

Two alleles of human FcγRIIIb, NA1 and NA2, have been identified, and they differ in four amino-acid positions, which results in differences in the potential N-glycosylation site numbers: Asn45 and Asn64 glycosylation sites are not found in NA1 (Fig. 1)34. In agreement with the data presented here, FcγRIIIb on neutrophils from the NA2 donors was shown to be more reactive with concanavalin A than that obtained from the NA1 donors 35. Homozygous NA2 individuals have a lower capacity to mediate phagocytosis than NA1 individuals, suggesting that FcγRIIIb-mediated immunological functions are negatively affected by the N-glycans at these non-conserved glycosylation sites36. Complement receptor 3 (CR3) was proposed to exhibit lectin-like activity and thereby interacts with the soluble and membrane-associated FcγRIIIb glycoproteins through their high-mannose-type oligosaccharides26,37. Based on our data, we propose that the interactions between FcγRIIIb and CR3 are exclusively mediated by the Asn45-associated glycans and specific for the NA2 allele.

Recently, Hayes et al. 15 have reported a relationship between glycosylation profiles of human serum sFcγRIIIa produced by different vehicles and their IgG1-binding affinities, indicating that sialylated and/or multi-antennary N-glycans of sFcγRIIIa negatively contribute to the interactions with IgG1. Previously obtained structural and biochemical data suggest that the Asn45 and Asn162 glycans of FcγRIII are involved in the IgG1 interactions 12,17. The present and previous site-specific glycosylation profiling studies demonstrate that the Asn45 site tends to display high-mannose-type N-glycans, whereas Asn162 is occupied by the complex-type glycans. Therefore, it is possible that the sialylation and multi-branching of the Asn162 glycans impair the IgG-FcγRIII interactions due to the negative steric effects. Reciprocally, our molecular dynamics simulation and crystallographic data obtained for the complexes formed between IgG1-Fc and sFcγRIIIa indicate that the core fucosylation of the IgG1-Fc glycan repels the Asn162 glycan of sFcγRIII, resulting in an increased conformational fluctuation of this N-glycan 38. These data

Table 2. Major glycoforms of the individual N-glycosylation sites of human serum sFcγRIIIb. aHex, hexose; HexNAc, N-acetylhexosamine; NeuNAc, N-acetylneuraminic acid; dHex, deoxyhexose.
cells were shown to be exclusively modified by biantennary complex-type glycans with partial sialylation 25. In off-the-clot human serum (Access Biologicals) as previously described with modifications9. The initial purification percentage distribution of the glycopeptides was calculated using the peak area of extracted ion chromatogram tool software. The remaining glycoforms were identified using the mass intervals between the glycoforms. The commonly considered more intense. Monosaccharide glycoform compositions were deduced using GlycoMod glycopeptides were deduced from the molecular masses of the peptide ion carrying a single such as 204.09 (HexNAc) and 366.14 (HexNAc-Hex). The peptide and glycan masses of ion spectra of glycopeptides were manually selected based on the identification of oligosaccharide oxonium ions, range of 350–2000. Following every regular mass acquisition, we performed MS/MS acquisitions against the 10 70,000. Mass spectrometer was operated in positive ion mode and full mass spectra were acquired using an m/z with Nanospray Flex Ion Source (Thermo Scientific). The electrospray voltage was 2.0 kV, and the resolution was C18; Nikkyo Technos) at a flow rate of 0.3 μg.

Methods
Purification of sFcγRIIIb from human serum. The sFcγRIIIb glycoprotein was purified from 2L of pooled off-the-clot human serum (Access Biologicals) as previously described with modifications9. The initial purification step included precipitation with 40–60% saturated ammonium sulfate. The precipitate was re-solubilized in phosphate-buffered saline and then applied to a Blue Sepharose 6 Fast Flow column (GE Healthcare) to remove albumin. The flow-through fraction was initially fractionated using a Protein A Sepharose 4 Fast Flow column (GE Healthcare) and then by an anti-FcγRIII antibody (3G8)-conjugated sepharose column. The elution fraction was fractionated using a Protein G Sepharose 4 Fast Flow column followed by Superdex 200 16/60 GL Chromatographic Separation Column (GE Healthcare). Finally, the purified sFcγRIIIb glycoprotein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by CBB staining (Fig. 2) and quantitated by sandwich ELISA as previously described9.

Digestion and glycopeptide enrichment of serum sFcγRIIIb. The purified sFcγRIIIb glycoproteins (10μg) were reduced in dithiothreitol (DTT; 25 mM) for 45 min at 60 °C and S-carboxamidomethylated with iodoacetamide (42 mM) at room temperature for 30 min in the dark followed by quenching with DTT (10 mM). The denatured and S-carboxamidomethylated sFcγRIIIb glycoprotein was incubated with endoproteinase GluC (0.5 μg; Thermo Fisher Scientific) in ammonium bicarbonate buffer (100 mM; pH 8.0) or chymotrypsin (Thermo Fisher Scientific) in Tris–HCl buffer (100 mM) containing calcium chloride (2 mM) at 37 °C for 20h. Acetone-based glycopeptide enrichment method was used as described in a previous study39. The glycopeptides were precipitated with five-fold volume of ice-cold acetone followed by centrifugation at 12,000 × g for 10 min, and dissolved in formic acid (0.1%) for LC/MS.

Site-specific glycosylation analysis by MS. Glycopeptides were analyzed by LC-MS/MS. HPLC was performed on an EASY-nLC 1000 (Thermo Fisher Scientific) equipped with an Acclaim PepMap 100 trapping column (75 × 20 mm, nanoViper; Thermo Scientific) and a Nano HPLC Capillary Column (75 × 120 mm, 3 μm, C18; Nikkyo Technos) at a flow rate of 0.3 μL/min. The eluents comprised 0.1% formic acid (A buffer) and 0.1% formic acid in acetonitrile (B buffer). The samples were eluted with a linear gradient from 0% to 35% B buffer over 60 min. MS analyses were performed using a Q Exactive mass spectrometer (Thermo Scientific) equipped with Nanospray Flex Ion Source (Thermo Scientific). The electrospray voltage was 2.0 kV, and the resolution was 70,000. Mass spectrometer was operated in positive ion mode and full mass spectra were acquired using an m/z range of 350–2000. Following every regular mass acquisition, we performed MS/MS acquisitions against the 10 most-intense ions using a data-dependent acquisition method with normalized collision energy of 27%. Product ion spectra of glycopeptides were manually selected based on the identification of oligosaccharide oxonium ions, with a characteristic m/z such as 204.09 (HexNAc) and 366.14 (HexNAc-Hex). The peptide and glycan masses of glycopeptides were deduced from the molecular masses of the peptide ion carrying a single N-acetylgalosamine, commonly considered more intense. Monosaccharide glycoform compositions were deduced using GlycoMod tool software. The remaining glycoforms were identified using the mass intervals between the glycoforms. The percentage distribution of the glycopeptides was calculated using the peak area of extracted ion chromatogram (XIC) and summed across all charge state of glycoforms. The most abundant ions were used for quantitative analysis.

| Sites | Serum sFcγRIIIb in this study | RHK-expressed sFcγRIIIb | HEK293T-expressed sFcγRIIIb | CHO-expressed sFcγRIIIb |
|-------|-------------------------------|------------------------|-----------------------------|-------------------------|
| Asn38 | Complex > 99%                 | Complex > 99%          | —                          | —                       |
| Asn45 | High-mannose > 99%            | High-mannose 82%       | Hybrid 16%                  | Hybrid 70%              |
|       |                               | Complex 12%            | Complex 84%                 | Complex 30%             |
| Asn64 | Complex > 99%                 | High-mannose 41%       | substitution                | substitution            |
| Asn74 | Complex > 99%                 | Complex > 99%          | Complex > 99%               | Complex > 99%           |
| Asn162| Complex > 99%                 | Complex > 99%          | Hybrid 25%                  | Complex > 99%           |
|       |                               | Complex 75%            |                             |                         |
| Asn169| Complex > 99%                 | Complex > 99%          | Complex > 99%               | —                       |

Table 3. Site-specific classification of N-glycans of the endogenous and recombinant sFcγRIIIb. The glycopeptides were not observed under the LC-MS/MS conditions used in this study.

underscore the therapeutic significance of FcγRIII glycosylation, best illustrated by its Asn162 glycans, which play an important role in the promotion of ADCC by enhancing the interactions with the non-fucosyl IgG112,16. In previous studies, Asn162 of the recombinant sFcγRIIa glycoproteins expressed by HEK293T and CHO cells were shown to be exclusively modified by biantennary complex-type glycans with partial sialylation25. In contrast, our results reveal that in human serum sFcγRIIIb, the major glycoforms at Asn162 are partially sialylated tri-antennary glycans, suggesting different functional glycosylation between native and recombinant FcγRIII glycoproteins. Moreover, an earlier study indicated that the endogenous FcγRIII exhibits cell-type-specific glycoforms based on the distinct lectin-binding properties35. Therefore, to optimize the design of therapeutic antibodies targeting FcγRIII displayed on specific cells, such possible variations of the receptor glycoforms should be considered. The data obtained in this study may assist the development of therapeutic antibodies with maximum efficacy in terms of effector functions mediated by the glycoforms of endogenous FcγRIII.
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Author Contributions
H.Y., N.K., and K.K. developed study concept and design. H.Y., L.R., W.F., and C.S. prepared serum sFc\(\gamma\)RIIIb. D.T. and N.K. performed MS analysis. H.Y., N.K., and K.K. wrote the manuscript.

Additional Information
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