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Site-Specific Classification of *N*-Linked Oligosaccharides of the Extracellular Regions of Fcγ Receptor IIIb Expressed in Baby Hamster Kidney Cells

Nana Kawasaki[1], Terumi Okumoto[2], Yoshiki Yamaguchi[2,3], Noriko Takahashi[2], Wolf Herman Fridman[4], Catherine Sautès-Fridman[4], Hirokazu Yagi[2] and Koichi Kato[2,5,6,7,*]

[1]*Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kami-yoga, Setagaya-ku, Tokyo 158-8501, Japan*
[2]*Faculty and Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan*
[3]*Structural Glycobiology Team, Systems Glycobiology Research Group, RIKEN-Max Planck Joint Research Center, RIKEN Global Research Cluster, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*
[4]*UMRS1138, University Paris Descartes, 15, rue de l'Ecole de Medecine 75270 Paris, France*
[5]*Institute for Molecular Science and Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, 5-1 Higashiyama Myodaiji, Okazaki 444-8787, Japan*
[6]*The Glycoscience Institute, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-ku, Tokyo 112-8610, Japan*
[7]*GLYENCE Co., Ltd., 2-22-8 Chikusa, Chikusa-ku, Nagoya 464-0858, Japan*

Abstract

Human Fcγ receptor III (FcγRIII) consists of two isoforms that are encoded by two individual genes: transmembrane FcγRIIIa and glycosylphosphatidylinositol-linked FcγRIIIb. Both isoforms can exist as a soluble form (sFcγRIII), which is composed of their extracellular region produced by proteolytic cleavage. FcγRIII-mediated immunological functions such as antibody-dependent cell-mediated cytotoxicity and phagocytosis critically depend on the *N*-glycosylation of FcγRIII molecules. In our previous study, high-performance liquid chromatography-based profiling indicated that *N*-linked oligosaccharides released from the NA2 allele of human sFcγRIIIb expressed in baby hamster kidney cells are composed of high-mannose-type oligosaccharides and core-fucosylated complex-type oligosaccharides. Here we successfully classified the *N*-glycans of this glycoprotein into these two types at each of the six *N*-glycosylation sites by liquid chromatography (LC)-electrospray tandem mass spectrometry analysis combined with endoglycosidase treatments. Our results indicated that four sites of sFcγRIIIb, Asn38, Asn74, Asn162, and Asn169, expressed only complex-type oligosaccharides, while the remaining two sites, Asn45 and Asn64 (both are not conserved in the NA1 allele), were occupied by not only complex-type oligosaccharides but also high-mannose-type oligosaccharides, which are thought to be involved in the interaction of FcγRIIIb with complement receptor type 3. Together with the previously reported site-specific *N*-glycosylation profiling of recombinant sFcγRIIIa, this study underlines that both sFcγRIIIa and sFcγRIIIb produced in different production vehicles express core-fucosylated complex-type oligosaccharides as the major glycoforms at Asn74 and Asn162. These finding provide insights into the design and development of therapeutic antibodies because the Asn162 *N*-glycan significantly contributes to immunoglobulin G binding.

Keywords: Fcγ receptor III; *N*-glycosylation; Mass spectrometry; High-mannose-type oligosaccharide; Complex-type oligosaccharide; Immunoglobulin G; Glycosidase treatment

Introduction

Receptors for the Fc portion of IgG (FcγRs) are a heterogeneous family of membrane-associated glycoproteins, which mediate various effector and regulatory functions such as the release of inflammatory regulator, endocytosis of the immune complex, and antibody-dependent cell-mediated cytotoxicity (ADCC) [1-3]. Human FcγRs are divided into three classes: FcγRI, FcγRII, and FcγRIII. These three FcγR classes bind different IgG isotypes with varying affinity and are expressed on different types of immune cells. Moreover, there are various isoforms and polymorphisms within each FcγR class [4]. For example, FcγRIII contains two isoforms that are encoded by two individual genes: transmembrane FcγRIIIa and glycosylphosphatidylinositol-linked FcγRIIIb. Both FcγRIII isoforms can exist as a soluble form (sFcγRIII) that consists of an extracellular region proteolytically cleaved from the transmembrane segment [5-7].

FcγRIIIa is primarily expressed on natural killer cells and promotes ADCC activity through an interaction with IgG in complex with antigens [3,8]. This interaction depends upon *N*-glycosylation of both FcγRIIIa and IgG–Fc. *N*-glycans at Asn162 and Asn45 of FcγRIIIa have positive and negative effects, respectively, on its reactivity with IgG [9,10]. X-ray crystallographic data indicate that the Asn162 glycan directly mediates the IgG–FcγRIIIa complex formation, primarily through interaction with the *N*-glycan at Asn297 of Fc, while the Asn45 glycan negatively affects IgG binding through steric hindrance [11,12]. The positive carbohydrate–carbohydrate interaction between the FcγRIIIa Asn162 glycan and the Fc Asn297 glycan is sterically hindered by core fucosylation of this Fc glycan. Hence, removal of the fucose residue from the IgG–Fc *N*-glycan results in increased affinity for the Asn162-glycosylated FcγRIIIa glycoprotein, which dramatically improves ADCC activity [13-15]. These findings demonstrate that the *N*-glycosylation status of FcγRs and IgG–Fc can be a crucial factor in the design and development of therapeutic antibodies.

In contrast to FcγRIIIa, FcγRIIIb is exclusively expressed on neutrophils and triggers the degranulation and phagocytosis of the IgG-labeled target cell [16-18]. The interaction between IgG and FcγRIIIb is also impaired by core fucosylation of IgG–Fc [19]. FcγRIIIb

***Corresponding author:** Koichi Kato, Faculty and Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan, Tel: 81-564-59-5225; Fax: +81-564-59-5224; E-mail: kkatonmr@ims.ac.jp

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and its soluble counterpart also interact with complement receptor type 3 (CR3; CD11b/CD18) and thereby modulate activation of CR3-displaying cells [20,21]. This receptor–receptor interaction is thought to be mediated by the glycan(s) of FcγRIIIb and the lectin-like region of CD11b. There exist two alleles of human FcγRIIIb, NA1 and NA2, which differ at four amino acid positions. This results in different numbers of potential *N*-glycosylation sites between them: four in NA1 and six in NA2 (Figure 1) [22]. Homozygous NA2 individuals express a lower capacity to mediate phagocytosis than NA1 individuals, suggesting that FcγRIIIb-mediated immunological function is also influenced by the *N*-glycans at the unconserved glycosylation sites Asn45 and/or Asn64 [23]. The remaining four potential *N*-glycosylation sites, Asn38, Asn74, Asn162, and Asn169, are all conserved between FcγRIIIa and FcγRIIIb.

To gain deeper insight into the *N*-glycosylation-dependent defense mechanisms mediated by the FcγRIII glycoproteins, site-specific information about their *N*-glycan structure is necessary. The site-directed *N*-glycosylation profiles of human sFcγRIIIa proteins produced in Chinese hamster ovary (CHO) and human embryonic kidney (HEK) cells have been reported [24]. Although the site-specific *N*-glycosylation profile of FcγRIIIb has not been available, our high-performance liquid chromatography (HPLC)-based *N*-glycosylation profiling of human sFcγRIIIb (NA2) expressed in baby hamster kidney (BHK) cells indicated that this glycoprotein possesses 14% high-mannose-type oligosaccharides and 86% complex-type oligosaccharides [25]. Intriguingly, the complex-type oligosaccharides were almost completely fucosylated at their innermost GlcNAc residue. This prompted us to perform site-specific classification of the *N*-linked oligosaccharides displayed on human sFcγRIIIb into high-mannose- and complex-types using liquid chromatography (LC)-electrospray tandem mass spectrometry (MS/MS) analysis in conjunction with endoglycosidase treatments.

Materials and Methods

Preparation of sFcγRIIIb

The human FcγRIIIb ectodomains (194 amino acids long, including a seven-amino-acid C-terminal stop linker) encoded by NA2 cDNA were expressed in BHK cells. The sFcγRIIIb-transfected BHK cell were grown on hollow fibers in a perfusion cell culture system, which allows the continuous measurement and control of pH, dissolved oxygen and residual glucose throughout the culture. The expressed sFcγRIIIb were purified by ion exchange and affinity chromatography. The detailed culture and purification conditions were deceived previously [26].

```
                1                                        38        45
FcγRIIIa:       RTEDLPKAVV  FLEPQWYRVL  EKDSVTLKCQ  GAYSPEDNST  QWFHNESLIS
FcγRIIIb NA1:   RTEDLPKAVV  FLEPQWYRVL  EKDSVTLKCQ  GAYSPEDNST  QWFHNENLIS
FcγRIIIb NA2:   STEDLPKAVV  FLEPQWYSVL  EKDSVTLKCQ  GAYSPEDNST  QWFHNESLIS
               51          64          74
FcγRIIIa:       SQASSYFIDA  ATVDDSGEYR  CQTNLSTLSD  PVQLEVHIGW  LLLQAPRWVF
FcγRIIIb NA1:   SQASSYFIDA  ATVDDSGEYR  CQTNLSTLSD  PVQLEVHIGW  LLLQAPRWVF
FcγRIIIb NA2:   SQASSYFIDA  ATVNDSGEYR  CQTNLSTLSD  PVQLEVHIGW  LLLQAPRWIF
              101
FcγRIIIa:       KEEDPIHLRC  HSWKNTALHK  VTYQNGKGR   KYFHHNSDFY  IPKATLKDSG
FcγRIIIb NA1:   KEEDPIHLRC  HSWKNTALHK  VTYQNGKDR   KYFHHNSDFH  IPKATLKDSG
FcγRIIIb NA2:   KEEDPIHLRC  HSWKNTALHK  VTYQNGKDR   KYFHHNSDFH  IPKATLKDSG
               151         162  169
FcγRIIIa:       SYFCRGLFGS  KNVSSETVNI  TITQGLAVST  ISSFFPPGYQ  VSFCLVMVLL
FcγRIIIb NA1:   SYFCRGLFGS  KNVSSETVNI  TITQGLAVST  ISSFFPPGYD  PRLV
FcγRIIIb NA2:   SYFCRGLFGS  KNVSSETVNI  TITQGLAVST  ISSFFPPGYD  PRLV
              201
FcγRIIIa:       FAVDTGLYFS  VKTNIRSSTR  DWKDHKFKWR  KDPQDK
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Figure 1: Sequence alignment of the extracellular regions of human FcγRIIIa and sFcγRIIIb NA1 and NA2 forms *N*-glycosylation sites are shown in red. The residues substituted between NA1 and NA2 are shown in blue.

Glycosidase treatments

The sFcRγIIIb glycoprotein (200 μg) was incubated with a mixture containing 1 U of α-sialidase (Nacalai Tesque), 2.5 mU of β-galactosidase (Seikagaku Co.), 2 U of β-*N*-acetylglucosaminidase (Calbiochem), and 10 U of endoglycosidase Hf (Endo-Hf, Daiichi Pure Chemicals Co.) at 37°C for 48 h in 50 mM acetate buffer (pH 5.5). The efficiency of this glycosidase treatment was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Proteolytic digestion

Reduction and *S*-carboxymethylation of FcγRIIIb: After the glycosidase treatment, sFcRγIIIb (200 μg) was incubated with 2.6 μl of 2-mercaptoethanol at room temperature for 2 h in 540 μl of 0.5 M Tris–HCl buffer (pH 8.6) containing 8 M guanidine hydrochloride and 5 mM ethylenediaminetetraacetic acid (EDTA). In total, 11.3 mg of monoiodoacetic acid was added to this solution, and the mixture was incubated at room temperature for 2 h in the dark. The reaction mixture was desalted with a PD-10 column (Amersham Pharmacia Biotech) and then lyophilized.

Glu-C digestion: The denatured and *S*-carboxymethylated sFcRγIIIb protein (100 μg) was incubated with 25 μg of endoproteinase Glu-C (Roche Diagnostics GmbH) at 37°C for 20 h in 0.1 M ammonium acetate buffer (pH 8.0).

Chymotrypsin digestion: The denatured and *S*-carboxymethylated FcγRIIIb protein (50 μg) was incubated with 2 μg of chymotrypsin at 37°C for 5 days in 0.1 M ammonium bicarbonate buffer (pH 8.0).

Mass spectrometric peptide mapping

Tandem electrospray mass spectra were recorded using a hybrid quadrupole/time-of-flight spectrometer (Qstar pulsar I, Applied Biosystems) interfaced with CapLC (Paradigm MS4, Michrom BioResources). In total, 2 μg of sample dissolved in 0.1% formic acid was injected onto a C18 column (0.2 mm×50 mm, 5 μ, Magic C18, Michrom BioResources). Peptides were eluted with a 5%–59% acetonitrile gradient in 0.1% aqueous formic acid over 60 min at the flow rate of 2 μl/min. The capillary voltage was set to 2500 V, and data-dependent MS/MS acquisitions were performed on precursors with charge states of 2 and 3 over a mass range of 400-2000.

Results and Discussion

Our previous HPLC profiling revealed that pyridylaminated *N*-linked oligosaccharides released from human sFcγRIIIb (NA2) expressed in BHK cells could be classified into two types: high-mannose-type oligosaccharides and exclusively core-fucosylated complex-type oligosaccharides containing sialyl oligosaccharides [25]. The former can be cleaved by Endo-Hf leaving the innermost GlcNAc residue, while the latter can be converted into a fucosylated trimanosyl core by sequential digestion using α-sialidase, β-galactosidase, and β-*N*-acetylglucosaminidase. Hence, it was expected that after treatment with a mixture of these glycosidases, the sFcγRIIIb protein would be modified only with a mono-GlcNAc residue and the fucosylated trimannosyl core. These could be easily discriminated by conventional MS analysis as traces of high-mannose-type and complex-type glycans, respectively. To test this idea, we performed glycosidase digestion of the sFcγRIIIb glycoprotein, followed by proteolytic digestion with Glu-C or chymotrypsin and LC-MS/MS analyses.

Figures 2A and 3A show the total ion current chromatograms from LC/MS of the Glu-C and chymotrypsin digests. The product ion spectra of glycopeptides were sorted from all mass spectra using

the presence of a carbohydrate-specific oxonium fragment ion of *m*/*z* 204 $[GlcNAc+H]^+$ (Figures 2B and 3B). The MS data of the proteolytic peptides from sFcγRIIIb are summarized in Tables 1 and 2 and Supplementary Figure 1.

Detection of the fucosylated trimannosyl core structure ($[Fuc][GlcNAc]_2[Man]_3$) but not GlcNAc in the peptides containing *N*-glycosylation sites Asn38, Asn74, Asn162, and Asn169 of sFcγRIIIb indicates that these sites display only complex-type oligosaccharides (Table 3). In contrast, both Asn45 and Asn64 were occupied with high-mannose-type as well as complex-type glycans, judging from the presence of peptides attached to GlcNAc. Furthermore, a Hex–HexNAc carbohydrate moiety was detected in two peptides containing no asparagine residue (AVSTISSF and SPPGADPR), indicating that these segments (Ala177–Phe184 and Ser185–Arg193) underwent *O*-glycosylation.

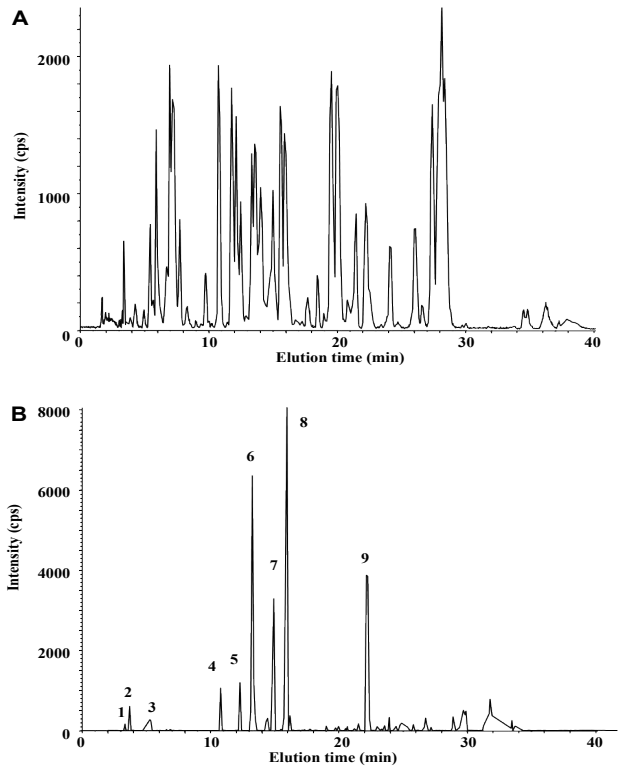

Figure 2: Detection of glycopeptides in the chromatogram of a Glu-C digest of recombinant human sFcγRIIIb (A) Total ion current chromatogram. (B) Mass chromatogram of product ion (*m/z* 204).

Our LC-MS/MS analysis thus demonstrated that the recombinant sFcγRIIIb displayed the specific glycosylation at individual asparagine residues. Generally, *N*-glycan structures are considerably associated with the degree of exposure of oligosaccharide moiety as exemplified by the statistical analysis reported by Thaysen-Anderson and Packer [27], indicating that incidence of *N*-glycan types, i.e. high-mannose-, hybrid-, and complex-types, is strongly correlated with solvent accessibility of the individual glycosylation sites: Highly accessible asparagine residues tend to be occupied by complex-type glycans, while less exposed sites are frequently occupied by high-mannose-type glycans. This tendency is presumably ascribed to the accessibility of the enzymes and chaperones involved in *N*-glycan processing. However, in the crystal structure of sFcγRIIIb, the solvent accessibility to the potentially *N*-glycosylated asparagine residues are as follows: Asn162>Asn64>Asn169>Asn38>Asn45>Asn74. This indicates that the rule could not explain the present results. The discrepancy may be explained if the solvent exposure of the glycosylation sites of sFcγRIIIb are significantly different between its native and folding intermediate states and the latter is critical in terms of interactions with the *N*-glycan processing factors.

The present data demonstrate that high-mannose-type oligosaccharides are expressed at a limited number of *N*-glycosylation sites of human sFcγRIIIb, Asn45 and Asn64, both of which are located in the membrane-distal extracellular domain (Figure 4 and Supplementary Figure 2). It has been suggested that the interaction between FcγRIIIb and CR3 is mediated by the high-mannose-type oligosaccharides characteristically exhibited by FcγRIII because the interaction was inhibited by α- or β-methyl mannoside [21,25]. Based on the present results, we suggest that the membrane-distal domain is primarily involved in CR3 binding through the high-mannose-type glycans principally located at Asn45 and Asn64. Intriguingly, these two *N*-glycosylation sites are not conserved in the NA1 allele. Indeed, FcγRIIIb expressed on neutrophils derived from NA1 and NA2 homozygous donors displayed different types of sugar chains [28]. Consistent with the present data, FcγRIIIb from NA2 donors was fully observed in the concanavalin A (Con A)-binding fraction, while

Peak no. in Figure 2	Time (min)	Site	Fragment	Amino acid sequence[a]	Carbohydrate	Charge state	Observed *m/z*	Measured mass	Monoisotopic mass
1	3.3	64	60-65	AATVND	$[Fuc][GlcNAc]_2[Man]_3$	2^+	814.79	1627.58	1627.65
2	3.7	64	60-68	AATVNDSGE	$[Fuc][GlcNAc]_2[Man]_3$	2^+	951.35	1900.69	1900.74
3	5.4	162	157-166	LVGSKNVSSE	$[Fuc][GlcNAc]_2[Man]_3$	2^+	1029.43	2056.86	2056.91
4	10.8	-	149-156	SGSYFCRG	-	2^+	467.68	933.34	933.37
5	12.3	38, 45	37-46	DNSTQWFHNE	$[Fuc][GlcNAc]_2[Man]_3$ $[GlcNAc]_2[Man]_3$	3^+	1070.02	3207.06	3207.20
					$[Fuc][GlcNAc]_2[Man]_3$ $[Fuc][GlcNAc]_2[Man]_3$	3^+	1118.74	3353.22	3353.26
6	13.3	38, 45	37-46	DNSTQWFHNE	[GlcNAc] $[Fuc][GlcNAc]_2[Man]_3$	2^+	1259.93	2517.86	2517.97
7	14.9	169	167-175	TVNITITQG	$[Fuc][GlcNAc]_2[Man]_3$	2^+	992.92	1983.84	1983.89
8	16.0	162	149-166	SGSYFCRGLVGSKNVSSE	$[Fuc][GlcNAc]_2[Man]_3$	3^+	991.72	2972.16	2972.30
9	22.2	74	69-85	YRCQTNLSTLSDPVQLE	$[Fuc][GlcNAc]_2[Man]_3$	3^+	1021.75	3062.25	3062.33

[a]C: Carboxymethyl cysteine

Table 1: MS data for glycopeptides in the Glu-C digest of sFcγRIIIb.

Peak no. in Figure 3	Time (min)	Site	Fragment	Amino acid sequence[a]	Carbohydrate	Charge state	Observed *m/z*	Measured mass	Monoisotopic mass
1	3.8	185	185-193	SPPGADPR	[Hex][HexNAc]	2^+	581.24	1160.46	1160.52
2	5.7	74	70-75	RCQTNL	$[Fuc][GlcNAc]_2[Man]_3$	2^+	915.75	1829.50	1829.74
3	8.0	45	43-48	FHNESL	[GlcNAc]	2^+	475.18	948.36	948.42
4	10.4	38	34-42	SPEDNSTQW	$[Fuc][GlcNAc]_2[Man]_3$	2^+	1051.28	2100.56	2100.80
5	11.2	45	43-52	FHNESLISSQ	[GlcNAc]	2^+	682.76	1363.51	1363.63
6	11.8	74	70-78	RCQTNLSTL	$[Fuc][GlcNAc]_2[Man]_3$	2^+	1066.29	2130.58	2130.90
7	12.5	64	58-69	IDAATVNDSGEY	$[Fuc][GlcNAc]_2[Man]_3$	2^+	1146.84	2291.68	2291.92
8	13.1	64	58-69	IDAATVNDSGEY	[GlcNAc]	2^+	729.24	1456.48	1456.62
9	13.5	180	177-184	AVSTISSF	$[HexNAc]_2[Hex]_2$	2^+	771.27	1540.54	1540.68
10	14.2	45	43-56	FHNESLISSQASSY	$[Fuc][GlcNAc]_2[Man]_3$ $[GlcNAc]_2[Man]_3$	2^+ 2^+	1304.39 1231.34	2606.78 2460.68	2607.09 2461.03
11	15.1	180	177-184	AVSTISSF	[HexNAc][Hex]	2^+	588.74	1175.48	1175.54
12	17.1	64	57-69	FIDAATVNDSGEY	$[Fuc][GlcNAc]_2[Man]_3$	2^+	1220.34	2438.68	2438.99
13	18.0	64	57-69	FIDAATVNDSGEY	[GlcNAc]	2^+	802.76	1603.52	1603.69
14	20.3	162, 169	158-176	VGSKNVSSETVNITITQGL	$[Fuc][GlcNAc]_2[Man]_3$ $[Fuc][GlcNAc]_2[Man]_3$	3^+	1341.76	4022.28	4022.78

[a]C:Carboxymethyl cysteine

Table 2: MS data for glycopeptides in the chymotrypsin digest of sFcγRIIIb.

N-glycosylation sites	Site occupancy (rel. %)	GlcNAc-peptides (rel. %)	$Man_3GlcNAc_2$-containing peptides (rel. %)
38	>99	-	>99
45	>99	73	27
64	88	41	47
74	82	-	82
162	>99	-	>99
169	>99	-	>99

Site-specific glycosylation occupancy and relative ratios of two types of *N*-glycans were semi-quantitatively determined from the abundance of glycosylated and nonglycosylated versions of each given peptide

Table 3: Site-specific classification of sFcγRIIIb.

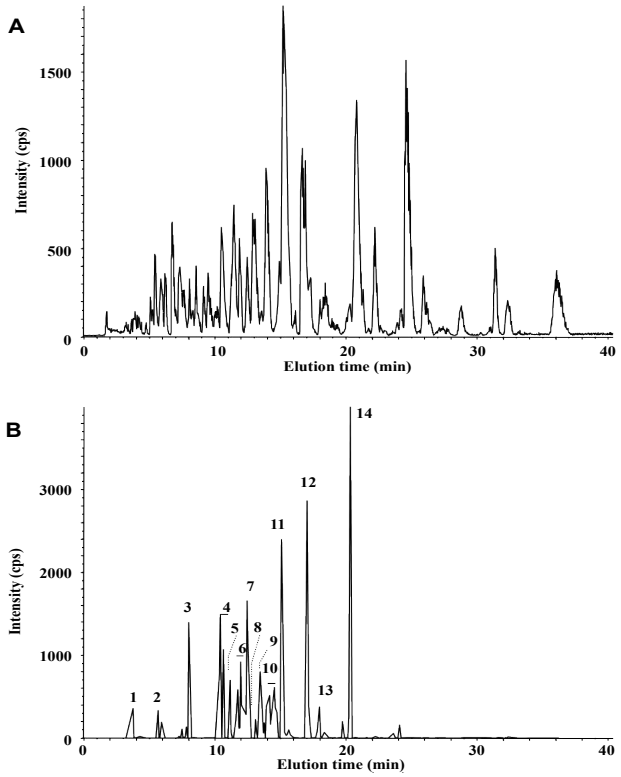

Figure 3: Detection of glycopeptides in the chromatogram of a chymotrypsin digest of recombinant human sFcγRIIIb (A) Total ion current chromatogram. (B) Mass chromatogram of product ion (*m/z* 204)

FcγRIIIb from NA1 donors was only partially observed, suggesting that the Asn45 and Asn64 sites are occupied with Con A-binding glycans typified by high-mannose-type oligosaccharides. These findings raise the possibility that the CR3 reactivities of these two alleles are different.

Site-specific *N*-glycosylation profiles have been reported for human sFcγRIIIa protein expressed in CHO and HEK293 cells [24]. In CHO-derived sFcγRIIIa, Asn162 and Asn74 are exclusively modified with fucosylated complex-type oligosaccharides, as in the case of BHK-derived sFcγRIIIb, whereas the majority (70%) of *N*-glycans at Asn45 is of the hybrid type, while the remaining are complex-type oligosaccharides. Information about the *N*-glycosylation at Asn38 and Asn169 was not available, probably owing to the lower occupancy of *N*-glycans at these sites in CHO-derived sFcγRIIIa. The *N*-glycosylation profile of sFcγRIIIa depends on the production vehicle, as exemplified by the existence of Lewis-X-containing *N*-glycans in CHO-derived sFcγRIIIa [24] and galabiose-containing *N*-glycans in NS0-expressed sFcγRIIIa and sFcγRIIIb [29]. Despite such variability, we underscore a common tendency in the major glycoforms at Asn74 and Asn162 (fucosylated complex-type *N*-glycans; Figure 4 and Supplementary Figure 2) between sFcγRIIIa and sFcγRIIIb produced in different cell types. In particular, the Asn162 glycan of FcγRIIIa is critically involved in its interaction with IgG [9,10]. Therefore, the design and development of therapeutic antibodies should be undertaken with consideration of the oligosaccharide structures displayed at this specific site, which is predominantly occupied by fucosylated complex-type oligosaccharides, at least in recombinant sFcγRIIIa and sFcγRIIIb glycoproteins, irrespective of their production vehicle. Site-specific glycosylation information about the endogenous forms of these receptors will be necessary to confirm this tendency and enable the design of therapeutic antibodies with improved efficacy.

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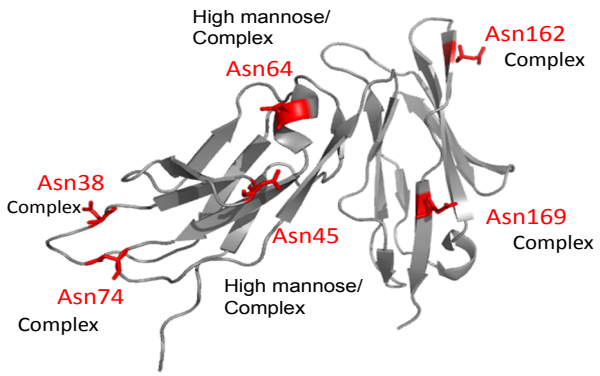

Figure 4: *N*-glycosylation sites of FcγRIIIb Crystal structure of sFcγRIIIb (PDB code: 1FNL) is presented showing the N-glycosylation sites (red).

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