The N-linked oligosaccharide at FcyRIIIa Asn-45: an inhibitory element for high FcyRIIIa binding affinity to IgG glycoforms lacking core fucosylation

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Human leukocyte receptor IIIa (FcyRIIIa) plays an important role in mediating therapeutic antibodies’ antibody-dependent cellular cytotoxicity (ADCC), which is closely related to the clinical efficacy of anticancer processes in humans in vivo. The removal of the core fucose from oligosaccharides attached to the Fc region of antibodies improves FcyRIIIa binding, allowing the antibodies to enhance dramatically the antibody effector functions of ADCC. In this study, the contribution of FcyRIIIa oligosaccharides to the strength of the FcyRIIIa/antibody complex was analyzed using a serial set of soluble human recombinant FcyRIIIa lacking the oligosaccharides. A nonfucosylated antibody IgG1 appeared to have a significantly higher affinity to the wild-type FcyRIIIa than did the fucosylated IgG1, and this increased binding was almost abolished once all of the FcyRIIIa glycosylation was removed. Our gain-of-function analysis in the FcyRIIIa oligosaccharide at Asn-162 (N-162) confirmed that N-162 is the element required for the high binding affinity to nonfucosylated antibodies, as previously revealed by loss-of-function analyses. Interestingly, beyond our expectation, the FcyRIIIa modified by N-162 alone showed a significantly higher binding affinity to nonfucosylated IgG1 than the wild-type FcyRIIIa. Attachment of the other four oligosaccharides, especially the FcyRIIIa oligosaccharide at Asn-45 (N-45), hindered the high binding affinity of FcyRIIIa to nonfucosylated IgG1. Our data clearly demonstrated that N-45 is an inhibitory element for the high FcyRIIIa binding affinity mediated by N-162 to nonfucosylated antibodies. This information can be exploited for the structural-based functional study of FcyRIIIa.

Keywords: FcyRIIIa Asn-45/FcyRIIIa binding affinity/IgG1 lacking core fucosylation/N-linked Fc oligosaccharides/N-linked FcyRIIIa oligosaccharides

Introduction

Most therapeutic antibodies that have been licensed and developed as medical agents are of the human IgG1 isotype. Human IgG1 is a heavily fucosylated glycoprotein bearing two N-linked biantennary complex-type oligosaccharides bound to the antibody constant region (Fc) via Asn-297, and it exercises biological activities referred to as “effector functions” of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) through the interaction of the Fc with either leukocyte receptors (FcyRs) or complement components. Genetic analyses of FcyR polymorphisms of cancer patients have demonstrated that ADCC is one of the major critical mechanisms responsible for the clinical efficacy of therapeutic antibodies such as anti-CD20 rituximab (Rituxan®) and anti-Her2 trastuzumab (Herceptin®) (Cartron et al. 2002; Weng and Levy 2003; Dall’Ozzo et al. 2004; Gennari et al. 2004; Kim et al. 2006). For patients carrying the high-affinity FcyRIIIa allotype (FcyRIIIa-Val-158), in contrast to those carrying the low-affinity allotype (FcyRIIIa-Phe-158), superior clinical responses have also been demonstrated in cases such as rituximab-treated systemic lupus erythematosus (SLE) and Waldenstrom’s macroglobulinemia, Crohn’s disease treatment with anti-TNF-α infliximab (Remicade®), and pregnant women with fetal hemolytic disease treated with anti-RhD (Anolik et al. 2003; Louis et al. 2004; Miescher et al. 2004; Treon et al. 2005). Thus, the importance of ADCC for the clinical efficacy of therapeutic antibodies is now widely recognized.

Interestingly, the Fc oligosaccharide structures of therapeutic antibodies greatly influence FcyRIIIa binding, and the removal of the core fucose from Fc oligosaccharides dramatically enhances the effector functions of ADCC via improved FcyRIIIa binding both in vitro and in vivo (Shields et al. 2002; Shinkawa et al. 2003; Niwa, Hatanaka, et al. 2004; Niwa, Shoji-Hosaka, et al. 2004; Yamane-Ohnuki et al. 2004; Niwa et al. 2005; Iida et al. 2006; Kanda et al. 2006; Satoh et al. 2006; Suzuki et al. 2007). Although fucose depletion from the Fc oligosaccharides of antibodies is found to improve binding affinity to FcyRIIIa via an enthalpy-driven and association-rate-assisted mechanism (Okazaki et al. 2004), the precise, structurally based mechanisms of the affinity enhancement remain to be elucidated. In the FcyRIIIa/IgG complexes, the interaction sites on the Fc for binding to FcyRIIIa form protein portions in the hinge and C12 regions only (Morgan et al. 1995; Clark 1997). The generation of the essential Fc tertiary conformation for binding to FcyRIIIa depends on the presence of the Fc oligosaccharides attached to the C12 domains, and the antibody effector functions mediated via FcyRIIIa are severely abrogated in aglycosylated forms of antibodies (Tao and Morrison 1989; Krapp et al. 2003). The crystal
structure analysis of human IgG1 has revealed that the antibody oligosaccharides linked to the Fc are integral to the protein portion of the Fc and form multiple noncovalent interactions with the C12 domains (Huber et al. 1976; Harris et al. 1998; Radaev et al. 2001). Thus, multiple noncovalent interactions between the oligosaccharides and the protein exert a reciprocal influence of each on the conformation of the other, and these complexities of human IgG1, along with the core fucose heterogeneity of the Fc oligosaccharides, delicately affect the binding affinities with FcγRIIIa proteins. Schematic structures of the hexa-His-tagged soluble human recombinant FcγRIIIa (shFcγRIIIa-His) lacking the N-linked oligosaccharides we expressed in this study are illustrated. Arrows indicate the N-glycosylation sites of each shFcγRIIIa-His, and Qs represent altering asparagine of N-linked glycosylation sites into glutamine to delete the glycosylation.

Human FcγRIIIa is also a glycoprotein bearing five N-linked oligosaccharides bound to the asparagine residues at positions 38, 45, 74, 162, and 169 (Ravetch and Perussia 1989). Recently, based on the crystal structure analysis, the ADCC enhancement by IgG1 lacking core fucosylation was attributed to a subtle conformational change in a limited region of the Fc of IgG1 (Matsumiya et al. 2007), and the high affinity of nonfucosylated antibodies for FcγRIIIa was attributed to a subtle conformational change in a limited region of the Fc of IgG1 (Ravetch and Perussia 1989). Recently, based on the crystal structure analysis of human IgG1 has revealed that the antibody oligosaccharides linked to the Fc are integral to the protein portion of the Fc and form multiple noncovalent interactions with the C12 domains (Huber et al. 1976; Harris et al. 1998; Radaev et al. 2001). Thus, multiple noncovalent interactions between the oligosaccharides and the protein exert a reciprocal influence of each on the conformation of the other, and these complexities of human IgG1, along with the core fucose heterogeneity of the Fc oligosaccharides, delicately affect the binding affinities with FcγRIIIa.

Purification of N-linked oligosaccharide-depleted FcγRIIIa
A serial set of the hexa-His-tagged soluble human recombinant FcγRIIIa (shFcγRIIIa-His) lacking the N-linked oligosaccharides was generated by altering asparagine of the N-glycosylation sites into glutamine using the wild-type FcγRIIIa cDNA. The mammalian expression vector carrying each cDNA for the wild-type and mutants expression of original FcγRIIIa was introduced into Chinese hamster ovary (CHO) cell line CHO/DG44, and the expressed products were purified from the culture medium by Ni-NTA chromatography and subjected to nonreducing (A) and reducing (B) 5–20% SDS–PAGE analyses. Lane 1: the wild-type shFcγRIIIa-His (1 μg), lane 2: N162-shFcγRIIIa-His (1 μg).

Results
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Fig. 2. SDS-PAGE of the shFcγRIIIa-His purified by Ni-NTA chromatography. The expressed products of shFcγRIIIa-His were purified from the culture medium by Ni-NTA chromatography and subjected to nonreducing (A) and reducing (B) 5–20% SDS–PAGE analyses. Lane 1: the wild-type shFcγRIIIa-His (1 μg), lane 2: N162-shFcγRIIIa-His (1 μg).
Degradation of N162-shFcγRIIIa-His. The expressed products of N162-shFcγRIIIa-His (10 µg) purified from the culture medium by Ni-NTA chromatography were subjected to reducing 12% SDS–PAGE (A). The N-terminal amino acid sequences of the three detected bands indicated as arrows were analyzed by the Edman degradation method (B). The cleavage sites are shown in the schematic model of N162-shFcγRIIIa-His (C).

and middle bands was Glu3-Asp-Leu-Lys-Pro-Lys-Ala-V al-V al-Phe-Leu13 (Figure 3). This degradation was not inhibited even though the culture medium was prepared in the presence of protease inhibitors including 0.5 mM PMSF, 3.6 µM pepstatin A, 0.3 µM aprotinin, 16.1 µM bestatin, 5.6 µM E-64, and 4.6 µM leupeptin. Comparable degraded products were also observed in No-oligo-shFcγRIIIa-His, although no such degraded product was observed among the other three shFcγRIIIa-His recombinants (data not shown). Subsequent gel filtration chromatography excluded the degraded products to yield almost homogeneously purified (over 95%) products in SDS–PAGE analysis under the nonreducing condition (Figure 4A). All purified shFcγRIIIa-His products migrated as bands with almost the same sizes as we had expected in SDS–PAGE (Figure 4).

Characterization of N-linked oligosaccharide-depleted FcγRIIIa

SDS–PAGE analysis under the reducing condition revealed that two purified samples, that of No-oligo-shFcγRIIIa-His and that of N162-shFcγRIIIa-His, each still contained a band with a lower molecular weight than the expected products, which was observed in the products purified by Ni-NTA chromatography (Figure 4B). Interestingly, IgG affinity chromatography analysis showed that the band retained the ability to bind IgG1 (Figure 5). The majority of the attached oligosaccharides of N162-shFcγRIIIa-His were of the sialylated biantennary complex-type oligosaccharides containing two galactoses with a fucosylated core structure, and the nonsialylated neutral oligosaccharide form was a minor component of the oligosaccharides attached to N162-shFcγRIIIa-His (Figure 6). The oligosaccharide structure of the wild type and other shFcγRIIIa-His mutants produced by CHO/DG44 was confirmed to be of the complex type (data not shown).

IgG1 binding activity

The IgG1 binding activity of the wild-type and of each purified mutant shFcγRIIIa lacking the N-linked oligosaccharides
was estimated by FcγRIIa-binding ELISA and by surface plasmon resonance measurement using shFcγRIIa-His. Compared with the binding to fucosylated IgG1, the wild-type FcγRIIa showed superior binding affinity to nonfucosylated IgG1. This phenomenon was also observed in the three FcγRIIa mutants lacking the N-linked oligosaccharides except for the FcγRIIa mutant lacking all five N-linked oligosaccharides of No-oligo-shFcγRIIa-His (Figure 7). No-oligo-shFcγRIIa-His showed mostly equivalent binding to IgG1 irrespective of core fucosylation and weaker binding than the other glycosylated FcγRIIas to nonfucosylated IgG1. A binding kinetics analysis using a BIAcore™ biosensor system T100 (BIAcore, Uppsala, Sweden) confirmed the differences observed in FcγRIIa-binding ELISA (Figure 8). The sensorgrams clearly showed that N162-shFcγRIIa-His carrying only one N-linked oligosaccharide at Asn-162 had the strongest binding affinity to nonfucosylated IgG1 rather than the wild-type shFcγRIIa-His (Figure 8B and F) and that the additional attachment of N-linked oligosaccharide at Asn-45 decreased the high binding affinity (Figure 8F and G). The deletion of the N-linked oligosaccharide at Asn-45 in the wild-type shFcγRIIa-His also exhibited its negative effect on the binding affinity to nonfucosylated IgG1 to increase the binding affinity of No-N45-shFcγRIIa-His (Figure 8B and H). N45-N162-shFcγRIIa-His carrying two N-linked oligosaccharides at Asn-45 and Asn-162 showed higher binding affinity to nonfucosylated IgG1 than the wild-type shFcγRIIa-His having three more N-linked oligosaccharides (Figure 8B and G). The FcγRIIa mutant lacking all five N-linked oligosaccharides of No-oligo-shFcγRIIa-His had the weakest binding affinity to nonfucosylated IgG1 among all tested shFcγRIIa-His (Figure 8D). The interaction of fucosylated IgG1 with FcγRIIa was not as strong as that observed between nonfucosylated IgG1 and FcγRIIa as a whole. The affinity of N162-shFcγRIIa-His to fucosylated IgG1 was slightly weaker than that of No-oligo-shFcγRIIa-His, and N45-N162-shFcγRIIa-His showed the weakest affinity to fucosylated IgG1.

Discussion

Recently, ADCC enhancement technology has been expected to play key roles in improving the efficacy of current therapeutic antibodies, especially anticancer antibodies. Enhancement of the binding of therapeutic antibodies for FcγRIIa has received considerable attention for the development of next-generation therapeutic antibodies with the improved clinical efficacy of ADCC. Indeed, several clinical trials using such therapeutics are ongoing. To understand the physiological functions and to examine in detail the efficacy of these new types of therapies in vivo, it is very important to understand the interactions between the therapeutics and the target molecule for the effector functions. Thus, in this study, we focused on the interaction between FcγRIIa and therapeutic antibodies, especially on the effects of the FcγRIIa oligosaccharides on the high binding affinity of FcγRIIa to IgG1 lacking core fucosylation.

FcγRIIa of mammalian origin is well known as a highly glycosylated protein with five N-linked glycosylation sites. However, only a few observations are available on the influences of the FcγRIIa oligosaccharides on the functions of FcγRIIa, namely, monomeric fucosylated IgG binds to FcγRIIa lacking the N-linked oligosaccharide at Asn-162 with higher affinity than to the wild-type FcγRIIa (Ferrara et al. 2006). This finding has also been reported in the loss-of-function analysis of FcγRIIib, the highly homologous FcγR having the oligosaccharide at the same position as FcγRIIa, by means of prokaryotic expression, glycosylation site mutation, and tunicamycin-treatment glycosylation depletion in mammalian cell expression (Galon et al. 1997; Drescher et al. 2003). In this study, first we found that it is hard to stably express aglycosylated FcγRIIa without any degradation by mammalian CHO/DG44 cells as host cells (Figure 2). This degradation seemed to be caused by some intracellular event(s) because the exogenous addition of protease inhibitors, including PMSF, pepstatin A, aprotinin, bestatin, E-64, and leupeptin, into culture medium did not affect the phenomenon. FcγRIIa carrying just one N-linked oligosaccharide at Asn-162 (N-162) was also degraded when expressed in mammalian CHO/DG44 cells, as was aglycosylated FcγRIIa. The cleavage sites were located in a lysine and arginine cluster region of the FcγRIIa D2 domain, and the cathepsin-like proteases appeared to be responsible for the cleavages (Figure 3). Interestingly, this degradation was not
observed in the FcyRIIIa carrying N-162 and one more N-linked oligosaccharide at Asn-45 (N-45), which means that the attachment of N-45 to the FcyRIIIa D1 domain affects the D2 domain protease sensitivity despite the different domain location. The N-45 attachment in the D1 domain might cause a conformational change of FcyRIIIa to further stabilize the D2 domain structure.

We succeed in removing the degraded products from the samples by gel filtration chromatography, and the purified samples migrated as a homogeneous band with over 95% purity when subjected to nonreducing SDS–PAGE analysis (Figure 4A). However, the two purified samples of aglycosylated FcyRIIIa and FcyRIIIa carrying one N-162 still contained a band with a relatively lower molecular weight than the intact products we had expected (Figure 4B). This unexpected band seemed to be FcyRIIIa with a nick or a small fragment deletion in the D2 domain because the band, which was also observed before gel filtration purification, had a comparable molecular weight of the intact product under nonreducing condition with the intact FcyRIIIa amino acid sequence (Figure 3). Interestingly, both the intact and derivative FcyRIIIa products showed a capability to bind IgG1 (Figure 5). The cleavage of the FcyRIIIa D2 domain at the lysine and arginine cluster region might not necessarily abolish the binding affinity to IgG antibodies.

In this study, we prepared a set of FcyRIIIa recombinants produced by CHO/DG44 cells, in which all of the attached oligosaccharides were of the complex type. Firstly, we confirmed that nonfucosylated antibody IgG1 shows significantly higher binding affinity to the fully glycosylated wild-type FcyRIIIa than does the fucosylated IgG1 and that this increased binding is almost abolished once all of the FcyRIIIa glycosylation is removed (Ferrara et al. 2006). Moreover, our gain-of-function analysis in the FcyRIIIa oligosaccharides also confirmed that N-162 is the element required for the high binding affinity to nonfucosylated antibodies and slightly reduces the affinity to fucosylated IgG1, as previously revealed by loss-of-function analyses (Galone et al. 1997; Drescher et al. 2003; Ferrara et al. 2006). It is worth noting that, beyond our expectation, the glycosylation at Asn-162 in FcyRIIIa significantly enhanced binding affinity to nonfucosylated IgG1 compared to the wild-type FcyRIIIa, and attachment of the other four oligosaccharides, especially N-45, hinders the high binding affinity to nonfucosylated IgG1. The crystal structure of aglycosylated FcyRIIIa, in complex with the Fc fragment of human IgG1, indicates that an oligosaccharide moiety at Asn-162 of FcyRIIIa could point into the central cavity within the Fc fragment (Sondermann et al. 2000), where the rigid core oligosaccharides attached to the Fc of IgG at Asn-297 are also located (Huber et al. 1976). The high affinity of antibodies lacking core fucosylation to FcyRIIIa is partially mediated by interactions formed between N-162 and regions of the Fc that are accessible only when the Fc oligosaccharide is nonfucosylated (Ferrara et al. 2006). Our data clearly demonstrated that the glycosylation at Asn-45 in FcyRIIIa reduces its high binding affinity mediated by N-162 to antibody IgG1 lacking core fucosylation. The conformational change of the D2 domain and the hinge region known to directly contact IgG (Sondermann et al. 2000) might be caused by N-45 modification of the FcyRIIIa D1 domain.
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Fig. 8. Surface plasmon resonance analysis of shFcγRIIIa-His binding to IgG1. Fucosylated (Fu(+)) and nonfucosylated (Fu(−)) anti-CD20 IgG1s were injected over shFcγRIIIa-His (the wild-type shFcγRIIIa-His (A and B), No-oligo-shFcγRIIIa-His (C and D), N162-shFcγRIIIa-His (E and F), No-N45-shFcγRIIIa-His (G and H), N45-N162-shFcγRIIIa-His (I and G)) capture sensor chip at six different concentrations (ranging from 4.17 to 133.3 nM). In a control experiment, the buffer solution without IgG1 was injected over the receptor-capture sensor chip. The sensorgram obtained from the control experiment was subtracted from the sensorgrams obtained by the IgG1 injection to yield the curves presented in the figure. The dissociation constant (KD: 10^{-7} M) for the shFcγRIIIa-His calculated by steady-state analysis is shown on the right side on each sensorgram in the figure. The maximum value of the longitudinal axis was fitted to each predicted R_{max} value (maximum response).

Material and methods

Cell line
The CHO/DG44 cell line, in which the dihydrofolate reductase (DHFR) gene locus is deleted, was obtained from Drs. Lawrence Chasin and Gail Urlaub Chasin, Columbia University, New York (Urlaub et al. 1980). The CHO cell line was cultured in an IMDM medium (Invitrogen, Carlsbad, CA) containing 10% (v/v) dialyzed fetal bovine serum (dFBS; Invitrogen), 0.1 mM hypoxanthine, and 16 µM thymidine using a tissue culture flask (Greiner, Frickenhausen, Germany).

Antibodies
Mouse/human chimeric nonfucosylated and fucosylated anti-human CD20 IgG1s were generated as described previously (Yamane-Ohnuki et al. 2004; Iida et al. 2006). Rituximab (Rituxan®), purchased from Genentech, Inc. (South San Francisco, CA), was used for the controls. Table I shows the oligosaccharide structures of the FeC of each prepared IgG1, characterized by modified high-performance anion exchange chromatography (HPAEC) and matrix-assisted laser

Table I. Oligosaccharide analysis of the mouse/human chimeric anti-CD20 IgG1s

| Antibody                  | G0  | G1  | G2  | G0F | G1F | G2F | Fu(−) |
|---------------------------|-----|-----|-----|-----|-----|-----|-------|
| Nonfucosylated anti-CD20 | 64.0| 33.0| 3.0 | n.d.| n.d.| n.d.| 100   |
| Fucosylated anti-CD20    | n.d.| n.d.| n.d.| 41.9| 51.1| 7.0 | n.d.  |
| Rituximab                | n.d.| n.d.| n.d.| 55.1| 40.8| 4.1 | n.d.  |

*Each composition value reflects the relative amount in the total complex-type oligosaccharides detected.

*Relative amount of nonfucosylated oligosaccharides.

*n.d.: not detected (less than 2.0%).
desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in positive-ion mode. Anti-human CD20s have an amino acid sequence equivalent to that of rituximab, which is widely used for the treatment of non-Hodgkin’s lymphoma, and thus they exhibit identical binding activity to the specific antigen on antigen-binding ELISA and flow cytometric analyses, regardless of the Fc oligosaccharide structure (Iida et al. 2006). Antibodies were stored in a 0.01 M citrate buffer, pH 6.0, with 0.15 M NaCl. The concentration of purified antibodies was measured by absorbance at 280 nm.

Wild-type and oligosaccharide-depleted FcyRIIIa mutants
The hexa-His-tagged soluble human recombinant FcyRIIIa (shFcyRIIIa-His) was prepared as described previously (Niwa, Hatanaka, et al. 2004). Briefly, the cDNA encoding FcyRIIIa was isolated by reverse transcription-PCR (Superscript Preamplification System, Invitrogen) of oligo(dt)-primed RNA from human leukocyte 5′-stretch plus cDNA library (Clontech, Palo Alto, CA) using specific primers that generated the fragment encoding an extracellular domain, and the transmembrane and intracellular domains were replaced by DNA encoding a hexa-His-tag. Using PCR with specific primers, 5′-CGGAA TTCGCCCTCCTCAAAATGAACCTCGGGCTCATTTGATT TTCTCCTGCCCTCATTTTAAAGGTGTCCAGTGTGAAGA AAGGCTG-3′ and 5′-TCACTATTGACAGGATCC CG-3′, the N-terminal amino acid of the shFcyRIIIa-His was unified to Gln3 (residue numbers excluding the signal peptide) by directly connecting to the signal peptide to avoid the N-terminal amino acid heterogeneity observed in the expression of original FcyRIIIa cDNA. Thus, the expected protein is composed of the extracellular domain of FcyRIIIa-Val-158 (a high-affinity FcyRIIIa allotype) linking hexa-His-tag at its C-terminus Gly-175. To mutate asparagine of the FcyRIIIa N-glycosylation sites into glutamine, a site-directed PCR mutagenesis kit (Stratagene, La Jolla, CA) was employed. The constructed cDNA fragments were inserted into a mammalian cell expression vector, pKANTEX93 (Nakamura et al. 2000), using the EcoRI and BamHI sites. The resultant vectors were transfected into CHO/DG44 by electroporation, and transfectants were selected in the IMDM medium containing 150 µg/mL of G418 without hypoxanthine or thymidine. The resultant transfectants were cultured in an Ex-Cell 301 medium (0.15 M NaCl, 0.05 M NaH2PO4, pH 7.4) by a buffer exchange, and the expressed proteins were purified from the culture supernatant by Ni-NTA gel: SDS–PAGE (Atto, Tokyo, Japan)). The purity of the products was estimated by SDS–PAGE analysis using a GS-800 calibrated densitometer (BioRad Laboratories, Hercules, CA).

N-Terminal amino acid sequence analysis
Samples were subjected to 12% SDS–PAGE analysis, followed by electroblootting onto a polyvinylidene difluoride (PVDF) membrane using a semidry blotting system (model AE-6675; Atto). Each band on the PVDF membrane was excised by a cutter and subjected to N-terminal amino acid sequencing via the Edman degradation method using a PPSQ-10 sequencer (Shimazu Co., Kyoto, Japan).

IgG affinity chromatography
Nonfucosylated anti-CD20 IgG1 (0.95 mg) was immobilized onto 0.4 mL of NHS-activated Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer’s instructions. The IgG1-immobilized Sepharose was washed and equilibrated with 4 mL of running buffer, and 133 µg of purified N162-shFcyRIIIa-His (270 µL in running buffer) was loaded onto the column. After incubation at 4°C for 1 h, 400 µL of flowthrough fraction was collected. The adsorbed materials were eluted with a citrate-NaOH buffer, pH 4.0, after washing the column with 4 mL of the equilibration buffer. A sample (2 µg) of each fraction was subjected to SDS–PAGE analysis.

FcyRIIIa-derived N-linked oligosaccharide analysis
N-Linked oligosaccharides were released by digestion of the purified N162-shFcyRIIIa-His (60 µg) with two units of recombinant peptide-N-glycosidase F (PNGase F; Sigma-Aldrich) for 16 h at 37°C in 0.01 M Tris–acetate buffer, pH 8.3. The released oligosaccharides were recovered after precipitation of the protein with 75% ethanol. After the recovered supernatant was dried, the oligosaccharides were dissolved in 13 mM acetic acid and incubated at room temperature for 2 h. The acid-treated samples were desalted with cation-exchange resin (AG50W-X8, hydrogen form; BioRad Laboratories) and dried in vacuum. The dried samples were dissolved in deionized water and mixed with a matrix to be characterized by a MALDI-TOF MS spectrometer Reflex III (Bruker Daltonik GmbH, Bremen, Germany) equipped with delayed extraction. The released carbohydrates were analyzed both in a positive-ion mode using the super-DHB solution (Bruker Daltonik) as a matrix and in a negative-ion mode using 2,4,6′-trihydroxyacetophenon (THAP) as a matrix as described previously (Papac et al. 1996; Kanda et al. 2006).

IgG1-binding assay
The binding affinity of anti-CD20 IgG1 to each purified FcyRIIIa was measured by an FcyRIIIa-binding ELISA assay using plates coated with each shFcyRIIIa-His via anti-tetra-His antibodies (Qiagen) as described previously (Niwa, Hatanaka, et al. 2004). The binding kinetics of IgG1 to each of the purified FcyRIIIa-His was measured using a T100 biosensor system (BIAcore) as follows. Anti-tetra-His antibodies were immobilized onto the BIAcore sensor chip CMS using an amine-coupling kit (BIAcore) according to the manufacturer’s instructions. Each shFcyRIIIa-His was captured by the immobilized anti-tetra-His antibodies by the injection of shFcyRIIIa-His in a HBS-EP buffer (0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, 0.01 M HEPES, pH 7.4) at a flow rate of 5 µL/min.
The HBS-EP buffer lacking shFcyRIIIa-His was injected over the receptor-capture sensor surface; samples treated in this manner were used as a reference. The anti-CD20 IgG1 was diluted in the HBS-EP buffer at six different concentrations (ranging from 4.17 to 133.3 nM). Each diluted IgG1 was injected over the receptor-capture sensor surface at a flow rate of 30 µL/min. The experiments were performed at 25°C with the HBS-EP buffer as the running buffer. To obtain a blank control, the buffer solution lacking IgG1 was injected over the receptor-capture sensor surface. Prior to analysis, the data obtained by the injection of IgG1 were corrected for the reference and blank control. The dissociation constant (K_d) for each FcyRIIIa was calculated by steady-state analysis using the T100 biosensor system evaluation software version 1.0 (BIAcore). To repeat experiments, shFcyRIIIa-His and IgG1 were removed from the sensor tips by injection of 0.01 M Glycine–HCl, pH 1.5, at a flow rate of 60 µL/min for 1 min.

Conflict of interest statement
None declared.

Abbreviations
ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; ELISA, enzyme-linked immunosorbent assay; HPAEC, high-performance anion exchange chromatography; IgG1, immunoglobulin G1; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; NK, natural killer; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PNGase F, peptide-N-glycosidase F; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; THAP, 2′,4′,6′-trihydroxyacetophenone.

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