Most cells active in the immune system express receptors for antibodies which mediate a variety of defensive mechanisms. These receptors interact with the Fc portion of the antibody and are therefore collectively called Fc receptors. Here, using high-speed atomic force microscopy, we observe interactions of human, humanized, and mouse/human-chimeric immunoglobulin G1 (IgG1) antibodies and their cognate Fc receptor, FcγRIIIa. Our results demonstrate that not only Fc but also Fab positively contributes to the interaction with the receptor. Furthermore, hydrogen/deuterium exchange mass spectrometric analysis reveals that the Fab portion of IgG1 is directly involved in its interaction with FcγRIIIa, in addition to the canonical Fc-mediated interaction. By targeting the previously unidentified receptor-interaction sites in IgG-Fab, our findings could inspire therapeutic antibody engineering.

The combination of antigen recognition and expression of effector functions typified by antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity is a major function of the antibody1–4. To exert this hub function, the antibody structure is divided into Fab arms and Fc stem. The Fab portions exhibit sequence variability in the N-terminal domains V H and V L, which recognize various antigens, followed by constant domains C L and CH1. By contrast, the Fc region has a two-fold-symmetric homodimeric structure comprising two CH2 and two CH3 domains. The relative orientation of the Fab arms with respect to the Fc stem varies because of their connection through a flexible linker called a hinge1,5.

The Fc portion of immunoglobulin G (IgG) provides interaction sites for effector molecules such as complement1–4. Most of the cells working in the immune system express receptors for IgG, possessing extracellular regions comprising Ig-fold domains and interacting with the Fc portion of IgG2-11. Therefore, they are collectively termed Fcγ receptors (FcγRs). FcγRs are classified into three major isoforms: FcγRI, FcγRII, and FcγRIII, each exhibiting different binding affinities to the IgG isotypes, and distinct expression profiles on immune cells12–14. Human FcγRIII is further divided into two isoforms—transmembrane FcγRIIIα and glycosylphosphatidylinositol-linked FcγRIIIβ—that share 96% amino acid sequence identity in their extracellular regions. FcγRIIIα, expressed primarily on natural killer cells, promotes ADCC by interacting with the IgG in complex with antigen, whereas FcγRIIIβ, expressed exclusively on neutrophils, mediates the degranulation and phagocytosis of the IgG-labeled target cells15–19.

The interaction modes of human IgG1 and FcγRIII molecules have been structurally characterized by X-ray crystallography using the Fc fragments and the soluble forms of FcγRIII (sFcγRIII) molecules, comprising the D1 and D2 domains20–23. These studies have identified their primary interaction sites—namely, the hinge-proximal

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segments in the C_H2 domains of Fc and the loops in the membrane-proximal D2 domain in FcγRs—and also revealed the domain rearrangements in both proteins. Furthermore, the functional significance of intermolecular carbohydrate-carbohydrate interactions has been underscored in the interaction between human IgG1-Fc and the extracellular region of FcγRIIIa, considered to be one of the most critical factors for clinical applications of human IgG1-based therapeutic antibodies that target cancers\(^{22,23,26-28}\). However, a large gulf exists between the structural views thus obtained and the functional and therapeutic insights gained from observations under physiologically realistic conditions where IgG1 interacts with FcγRIIIa anchored on the cell surfaces through a C-terminal transmembrane segment. Here, using high-speed atomic force microscopy (HS-AFM) and human, humanized, and mouse/human-chimeric IgG1 antibodies (Supplementary Fig. 1) and their Fc fragments, along with human sFcγRIIIa immobilized through its C-terminal segment on the scanning surface, we perform real-time observation of IgG-FcγR interactions. The interaction modes of these IgGs with sFcγRIIIa have also been characterized by hydrogen-deuterium exchange mass spectrometry (HDX-MS) in solution.

Results

HS-AFM observations of IgG1-FcγRIIIa. We prepared a recombinant sFcγRIIIa glycoprotein with a C-terminal hexahistidine moiety for immobilization onto a Ni\(^{2+}\)-coated mica surface. When the IgG solution was added to the observation buffer in the sample chamber of HS-AFM, IgG molecules visited the extracellular domains of FcγRIIIa, transiently forming a 1:1 complex (Supplementary Videos 1–3). We compared interactions of a panel of IgGs by HS-AFM imaging (Fig. 1a) by quantifying dwell times of the IgG molecules and summarize them in Fig. 2. The human, humanized, and mouse/chimeric IgG1 antibodies—PMF37, trastuzumab, and rituximab, respectively—exhibited comparable dwell times of around 1 s.

We subjected the Fc fragments derived from these IgGs to HS-AFM observation (Fig. 1b, Supplementary Videos 4–6). Unexpectedly, dwell times of the Fc fragments were remarkably decreased (0.31 s–0.35 s) in comparison with those of the intact IgGs (Fig. 2). We confirmed that mock-treated control (PMF37 incubated in the
absence of papain) maintained the longer dwell time (Supplementary Fig. 2). These data indicate that the Fab portions of IgG contribute positively to its interaction with FcγRIIIa.

**HDX-MS characterization of IgG1-FcγRIIIa interaction.** The HS-AFM observation raised the possibility of direct involvement of the Fab portion of IgG1 in the interaction with sFcγRIIIa. Hence, using the human IgG1 PMF37, we attempted to identify the interaction sites of IgG1 and sFcγRIIIa by HDX-MS analysis. In this experiment, at least over 80% coverage was achieved for PMF37 and sFcγRIIIa glycoproteins, thereby enabling HDX-based detection of changes in solvent exposure of their peptide segments upon their interactions. We subjected the HDX-MS results from three independent experiments to t-test with 99% confidence and considered percent differences greater than 4% for at least two time points as significant. The average error was 0.7% or less for corrected data of three replicates at each time point. Consequently, significant changes in the deuterium uptake rate were observed for PMF37 and its interacting sFcγRIIIa (Figs 3–5 and Supplementary Figs 3–5).

With regard to PMF37, we observed significant decreases in the deuterium uptake rate for Ser65-Leu72 in VL, Thr105-Glu124 and Thr163-Tyr177 in CH1, and Phe54-Asn62 in VH, Pro127-Leu142, Asn159-Leu174, and Val186-Thr197 in CH2, Val263-Phe275, Tyr278-Thr300, and Tyr319-Glu333 in CH2, and Trp381-Leu398 in CH3. On the other hand, sFcγRIIIa showed a deuterium uptake rate reduction in Ile49-Tyr56 and Leu84-Leu92 in the membrane-distal D1 domain and Lys101-Leu118 and His119-Phe139 in the membrane-proximal D2 domain, whereas Phe11-Trp16 and Leu12-Asp23 in the D1 domain exhibited an increased uptake rate upon binding to PMF37. We map the HDX data on 3D-structural models of PMF37 and sFcγRIIIa (Fig. 6). Our results indicate protection against deuterium uptake observed not only in Fc but also in Fab, implying Fab's involvement in interactions with sFcγRIIIa.

**Figure 2.** Dwell times of three kinds of IgG1 and their Fc fragments on sFcγRIIIa.
Discussion

The interaction of FcγR with IgG is widely assumed to be mediated through the Fc region, as its name indicates. This concept was established from the 1960s through the 1970s, when FcγRs were putative molecules and their interactions with IgGs were characterized mainly by cell-based assays, such as rosette formation. These studies demonstrated their interactions to be inhibited by the Fc fragments but not the Fab fragment, and were followed by molecular cloning of FcγRs and subsequently a number of structural studies using the Fc fragments in complex with the soluble forms of their cognate receptors.

However, the earlier studies also pointed to the possible roles of Fab in modulating effector functions expressed by FcγR. For example, the Fc fragment isolated from human IgG4 was shown to have a greater ability to bind C1q than that of the intact IgG4, which was interpreted as steric obstruction of the C1q-binding site by the Fab arms. Most notably, Birshtein et al. reported that the protein ICR16,—a mouse IgG variant having the CH1 domain of IgG2b and the remaining heavy chain constant region of IgG2a—did not inhibit rosette formation between a macrophage line and IgG2a-coated sheep red blood cells. Based on this paradoxical observation, the Fab arms were thought to affect the functional capacity of the Fc region either by inducing conformational changes or by obscuring the putative FcγR-binding site in Fc. The present study unintentionally revisited this issue.

The HS-AFM data visualized the dwell times of IgG1 molecules on clustering FcγRIIIa to be significantly longer than those of their Fc fragments, indicating that their Fab portions stabilized the complexes formed with the receptor. Furthermore, the HDX-MS data detected a reduction in deuterium uptake not only in the canonical interaction sites, i.e., the hinge-proximal segments of the Fc Cβ2 domains and the protruding loops of the FcγRIIIa D2 domain—but also in the Fab region, along with the FcγRIIIa D1 domain (Fig. 6). Based on these observations, we conclude that the Fab arm is directly involved in interactions with FcγRIIIa.

Sequence comparison of these putative FcγRIIIa-binding sites in the Fab region of IgG1 highlights the CL and CH1 segments conserved among the three IgG1 antibodies—PMF37, trastuzumab, and rituximab—suggesting the secondary interaction is mediated most likely through the CH1 and CL domains of IgG1 and the D1 domain of FcγRIIIa. The additional IgG1-binding segment in the D1 domain of FcγRIIIa—that is, Ile49-Tyr56—is conserved in FcγRIIIb (Supplementary Fig. 1c), suggesting that the Fab-binding property is commonly shared by these FcγRIII receptors. However, contradictory HDX-MS data have been reported regarding sFcγRIIIa-induced microenvironmental change in the Cβ2 segments around the position 160 (Asn159-Leu174 in this study): Houde et al. reported that 157–164 (Val156-Leu163 in our numbering system) was protected upon sFcγRIIIa binding, consistent with our results, whereas, in contrast, Shi et al. did not shed light on such
In these two reports, no significant difference in deuterium uptake has been described for the remaining parts in the Fab region. The apparent discrepancy among the results might be attributed to differences in HDX-MS experimental conditions, including protein concentrations, deuterium incubation time, pH, and temperature besides variations in glycoforms of the IgG1 and sFcγRIIIa samples. Intriguingly, Shi et al. showed that the CH1 segment Val150-Tyr162 (Val146-Trp158 in our numbering system) was exposed upon binding to sFcγRIIIa by a fast photochemical oxidation experiment, which enables sensitively probing fast dynamics in protein conformational change. Their explanation of this result is that Fab and Fc regions are in close contact conformationally in its prebound state and Fab is released from the interaction with Fc upon binding to the receptor. A possible integrative interpretation of these data along with our data is that binding of the FcγRIIIa D2 domain to Fc causes transient exposure of the CH1 segment in either or both Fab arms, which is followed by association of one Fab arm with the D1 domain.

Our HDX-MS experiments also detected allosteric conformational changes in both IgG1 and sFcγRIIIa. We observed protection against deuterium uptake for the segments located at the interface between the CH2 and CH3 domains of IgG1-Fc—that is, Leu242-Leu251 and Trp381-Leu398, which are distal from the FcγRIIIa-binding sites. This is consistent with the structural data showing quaternary-structure deformation of Fc induced by binding to sFcγRIIIa. Furthermore, we observed the enhanced solvent exposure for the Phe11-Trp16 and Leu12-Asp23 segments, which are located at the interface between the D1 and D2 domain of FcγRIIIa. This is also consistent with the crystal structures of sFcγRIIb showing a domain rearrangement upon binding to IgG1-Fc. We noted the putative FcγRIIIa-binding site in Fab to partially overlap with the area involved in interaction with protein G, which has been reported to be dependent on antigen binding. This suggests the intriguing possibility that antigen binding has potential impacts on the conformations of these sites, thereby allosterically affecting the Fab-FcγRIIIa interaction.

FcγRIIIa, expressed on NK cells, mediates ADCC, and is therefore considered to be a critical target of therapeutic antibodies for cancer treatments. One of the promising approaches in attempting to improve therapeutic efficacy of IgG drugs is to enhance their affinities to FcγRIIIa. So far, such undertakings have been focused on the Fc portion of IgG by amino acid substitutions at the previously identified FcγRIIIa-binding site, and also by engineering of the N-glycans attached to Fc, best exemplified by the removal of the core fucose residue, causing ADCC enhancement. Our findings in the present study offer a novel strategy for developing therapeutic
IgG antibodies with higher affinities for FcγRIIIa by rational and evolutionary engineering targeting the previously unknown—but commonly shared—interaction sites in their Fab portions.

**Methods**

**Materials.** We purchased rituximab, an anti-CD20 mouse/human-chimeric IgG1 (G1m17,1; Km3) [44], from Chugai Pharmaceutical Co., LTD. Trastuzumab, anti-HER2 humanized IgG1(G1m17,1; Km3) [45], and PMF37, human anti-hepatitis A virus IgG1(G1m3; λ2) [46], were expressed by the CHO-HcD6 and Baby Hamster Kidney (BHK) cell lines, respectively, according to previously described methods [46,47]. We cultivated the CHO-HcD6 cells in BalanCD® CHO Growth A medium (Irvine Scientific) supplemented with 2 mM L-glutamine, 1% Penicillin-Streptomycin (Thermo fisher scientific), and 7.5 μg/ml puromycin (Nacalai tesque). We cultivated the BHK cells in OptiPRO ™ SFM medium (Thermo fisher scientific) supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin (Thermo fisher scientific), and 100 μg/ml hygromycin B (Wako). Following the growth of the cells, we applied the supernatant of medium to an nProtein A Sepharose Fast Flow column (GE Healthcare) and further purified IgG1 by gel filtration using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) with a 50 mM Tris-HCl, pH 8.0, buffer containing 150 mM NaCl.

For preparation of Fc fragments, we incubated IgG1 dissolved at a final concentration of 10 mg/ml in 75 mM phosphate buffer, pH 6.0, containing 75 mM NaCl, and 2 mM ethylenediaminetetraacetic acid, in the presence of papain (Merck) with an enzyme/substrate ratio of 2% at 37 °C for 12 h. We then terminated the reaction by adding 33 mM N-ethylmaleimide. We applied the reaction mixture to an nProtein A Sepharose Fast Flow (GE Healthcare) for isolation of Fc fragments. We further purified the Fc fragment by gel filtration using a Superdex 200 Increase 10/300 GL column (GE Healthcare). We checked purity of each of the Fc preparations by SDS-PAGE (Supplementary Fig. 6). For mock-treated controls, we incubated IgG1 under the same conditions except for the absence of papain.

**Figure 5.** HDX-MS analysis of sFcγRIIIa. (a) Differential plots of deuterium uptake degrees of peptides, showing time courses; 30 s (purple), 60 s (blue), 600 s (green), 3,600 s (yellow), and 14,400 s (red), along with their summational results (gray bar). The criterion of significance of deuterium uptake difference between free and bound states is the same as that in Fig. 3. (b) Deuterium uptake curves for the representative peptides showing significant differences between free (red) and bound (blue) states.
Figure 6. Mapping of HDX-MS data on 3D-structural models of IgG1 and sFcγRIIIa. We show the peptide segments exhibiting decreases in deuterium uptake rate upon interaction in red, magenta, or pink, and the peptide showing increased deuterium uptake in cyan, on a crystal structure of sFcγRIIIa extracted from Fc-sFcγRIIIa complex (PDB code:5XJE), and a homology model of PMF37 (shown as a half molecule composed of one light chain and one heavy chain). In the protected segments of PMF37, we classify residues into three types: the residues constituting the canonical sFcγRIIIa-binding sites in Fc (magenta), the remaining conserved residues among PMF37, trastuzumab, and rituximab (red), and the unconserved residues (pink). We classify the protected segments of sFcγRIIIa into two types: residues constituting the canonical Fc-binding sites (magenta) and the remaining conserved residues between FcγRIIIa and FcγRIIIb (red). The canonical binding sites are based on the crystal structure of Fc-sFcγRIIIa complex. We built the homology model based on crystal structures of the human anti-human immunodeficiency virus-1 gp120 IgG1 (PDB code: IHZH54) using SWISS-MODEL Workspace. We prepared the molecular graphics using PyMOL (http://www.pymol.org/).

According to the previous study, we generated a construct of human sFcγRIIIa as recombinant glycoprotein with a C-terminal hexahistidine tag and two N-glycosylation sites at Asn45 and Asn162, while substituting the remaining three N-glycosylation sites—namely, Asn38, Asn74, and Asn169—with glutamine. Here, we refer to this recombinant sFcγRIIIa glycoprotein simply as sFcγRIIIa. We purchased the synthesized gene for sFcγRIIIa with an IgG signal sequence from FASMAC and subcloned it into a pEHX1.2 vector (Toyobo), which we then used for protein production by dihydrofolate reductase (dhFr)-mediated gene amplification. We transfected the expression vector into the dhFr-deficient CHO cell line, CHO/dhFr−/− (ATCC® CRL-9096). After 48 h of transfection, we plated the transfected cells into 6-well plates for methotrexate (MTX) pressure selection. During the multi-round selection process, we gradually increased the MTX concentration up to 500 mM. We subjected the MTX-resistant cells to monoclonal screening by limited dilution to select clones with higher expression. We selected the high expression clones by ELISA using anti-His antibody (GE healthcare).

We cultured the high expression CHO cells in Dulbecco’s Modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 500 mM MTX. After a 3-week cell culture, we applied the supernatant of the medium to a HiLoad 16/60 Superdex 75 pg column with a 50 mM Tris-HCl, pH 8.0, buffer containing 150 mM NaCl. For desialylation, we incubated sFcγRIIIa in 50 mM sodium acetate (pH 5.5) and 150 mM NaCl at 37°C for 12 h in the presence of one unit neuraminidase from Arthrobacter ureafaciens (Nacalai tesque) per 5 mg of sFcγRIIIa.

HS-AFM observation and analysis. For the HS-AFM experiments, we used a laboratory-built high-speed atomic force microscope in tapping mode at room temperature. We used a small cantilever oscillating with a resonant frequency of ~0.6 MHz (in water), a spring constant of ~0.2 N m⁻¹, and a quality factor of ~2 at the resonant frequency and thereby detected the variation of the oscillation amplitude by a two-element segmented photodiode. We fabricated an AFM tip on the cantilever using the electron beam deposition (EBD) method. The length of the EBD tip was ~500 nm, and the tip apex radius was approximately 4 nm. We set the free oscillation amplitude of the cantilever at ~1 nm. We defined a set-point of amplitude for feedback control at approximately 90% of the free amplitude to prevent an unwanted disturbance of interactions of sFcγRIIIa with IgG1 or its Fc fragment. To immobilize sFcγRIIIa through the C-terminal hexahistidine tag, we treated the freshly cleaved mica surface with a droplet of 10 mM NiCl₂. After treatment, we placed and incubated a droplet of sFcγRIIIa in 50 mM Tris-HCl, pH 8.0, buffer containing 150 mM NaCl. After washing, we immersed the sample stage of the AFM in a chamber with approximately 70 μl of 50 mM Tris-HCl, pH 8.0, buffer containing 150 mM NaCl.

We measured the bound-state dwell time using successive HS-AFM images to estimate the binding time of IgG1 (or its Fc fragment) on sFcγRIIIa by monitoring their interactions as appearance or disappearance of bright spots in the HS-AFM images. We conducted all analysis using a custom software program based on IgorPro 6 (WaveMetrics, Inc., Lake Oswego, Ore., USA).

HDX-MS analysis. As we performed previously, optimal concentrations of proteins, estimated based on their dissociation constant, were employed for HDX-MS experiments. We performed HDX-MS analysis with
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Author Contributions

R.Y., Y.Y., S.Y, S.U. and K.K. conceived and designed the study; R.Y., H.Y., M.N., M.O., T.O. and S.Y. established the protein expression systems and carried out sample preparation; R.Y., Y.Y., T.S., M.S., T.T., S.W., D.H., T.M. and S.U. performed HDX-MS experiments and analyses; H.W. and T.U. performed HS-AFM experiments and analyses; R.Y., Y.Y., T.T., S.Y., S.U. and K.K. mainly drafted the manuscript.

Additional Information

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