Role of Oligosaccharide Residues of IgG1-Fc in FcyRIIb Binding

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Yusuke Mimura‡§, Peter Sondermann‡, Rodolfo Ghirlando‡, John Lund‡, Stephen P. Young‡, Margaret Goodall‡, and Roy Jefferis‡

From the 2Division of Immunity and Infection, The Medical School, University of Birmingham, B15 2TT, United Kingdom, the Max-Planck-Institut für Biochemie, Abteilung für Strukturforschung, Am Klopferspitz 18a, D-82152 Martinsried, Germany, and the Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892

Engagement of Fcγ receptors (FcyRs) with the Fc region of IgG elicits immune responses by leukocytes. The recent crystal structure of FcγRIII in complex with IgG-Fc has provided details of molecular interactions between these components (Sondermann, P., Huber, R., Oosthuizen, V., and Jacob, U. (2000) Nature 406, 267–273). One of the most intriguing issues is that glycosylation of IgG-Fc is essential for the recognition by FcyRs although the carbohydrate moieties are on the periphery of the FcγRIII-Fc interface. To better understand the role of Fc glycosylation in FcγR binding we prepared homogeneous glycoforms of IgG-Fc (Cri) and investigated the interactions with a soluble form of FcγRIIb (sFcyRIIib). A 1:1 complex stoichiometry was observed in solution at 30 °C (Kd, 0.94 μM; ΔΔG, −8.4 kcal mol−1; ΔΔH, −6.5 kcal mol−1; ΔΔS, 1.9 kcal mol−1; ΔΔCp, −160 cal mol−1 K−1). Removal of terminal galactose residues did not alter the thermodynamic parameters significantly. Outer-arm GlcNAc residues contributed significantly to thermal stability of the C1γ2 domains but only slightly to sFcyRIIb binding. Truncation of 1,3- and 1,6-mannose residues generates a linear trisaccharide core structure and resulted in a significantly decreased affinity, a less exothermic ΔΔH, and a more negative ΔΔCp for sFcyRIIib binding, which may result from a conformational change coupled to complex formation. Deglycosylation of the C1γ2 domains abrogated sFcyRIIib binding and resulted in the lowest thermal stability accompanied with noncooperative unfolding. These results suggest that truncation of the oligosaccharides of IgG-Fc causes disorder and a closed disposition of the two C1γ2 domains, impairing sFcyRIIib binding.

IgG molecules are glycosylated at Asn-297 of the C1γ2 domain within the Fc region; the complex biantennary-type oligosaccharides attached have been shown to be essential for Fc effector functions mediated through Fcγ receptors (FcyRs) and complement C1q (1, 2). Deglycosylation of IgG compromises the recognition by all three cellular FcγRs (3–5). Analysis of carbohydrates isolated from normal human serum IgG has yielded up to 30 different structures. The minimal oligosaccharide structure is a hexasaccharide (GlcNAcMan3GlcNAc) with variable sugar residues attached, which results in the generation of multiple glycoforms. The oligosaccharide can be traced in x-ray crystal electron density maps of the IgG-Fc and is sequenced within the internal space enclosed by the C1γ2 domains. There are extensive noncovalent interactions between the carbohydrate and the protein moiety, resulting in reciprocal influences on conformation (6, 7). Recently we have shown that the C1γ2 domains exhibit a progressive reduction in thermal stability and functionality with successive removal of outer-arm sugar residues (8). However, the precise manner in which the oligosaccharide influences or determines Fc structure and functions remains to be determined. Detailed information of the glycosylation influence of IgG-Fc on FcγR binding and complement activation is critical for application of IgG for therapeutic use.

FcyRs are expressed on various hematopoietic cell types (e.g. macrophages, eosinophils, neutrophils, natural killer cells, and lymphocytes) and link cellular and humoral immunity by serving as a bridge between antibody specificity and effector cell function. Stimulation of cells through FcyRs results in a wide variety of effector functions, including Ab-dependent cell-mediated cytotoxicity (9), phagocytosis (10), oxidative burst (11), and release of inflammatory mediators (12). FcyRs constitute a subfamily of the immunoglobulin gene superfamly and have been classified into three classes (13). FcγRI binds monomeric IgG with high affinity. FcγRII and FcγRIII are low affinity IgG receptors and responsible for the clearance of immune complexes. Furthermore, FcyRs occur in various isoforms (FcγRIa, -b1, -b2, -c; FcγRIIa1–2, -b1–3, -c) and alleles (FcγRIIIa1-HR, -LR; FcγRIIIb-NA1, NA2) (14). Indirect protein engineering approaches suggest that interaction sites for the cellular FcγRs are localized to the hinge proximal region of the C1γ2 domain (4, 15–17). Crystal structures have been reported for extracellular portions of FcγRIIa (18) and FcγRIIb (19), suggesting conflicting models for the formation of IgG-Fc-FcγR complexes. Direct evidence has now been provided from the crystal complex of human IgG1-Fc with recombinant human FcγRIII (20), and NMR studies of the interaction of mouse IgG2b-Fc with mouse FcγRII (21), demonstrating a 1:1 stoichiometry as the consistent requirement for the expression of Fc effector functions without triggering permanent activation through a single IgG molecule.
To better understand how the oligosaccharide contributes to the expression of the IgG-Fc effector function, we investigated the interaction between a recombinant soluble form of FcγRIIb (sFcγRIIb) and homogeneous Fc glycoforms using isothermal titration calorimetry and surface plasmon resonance. A 1:1 stoichiometry for the interaction of sFcγRIIb with Fc in solution is demonstrated although the binding of two sFcγRIIb molecules to one IgG molecule has been proposed by a modeling approach (19). We extend this finding to examine the thermodynamic analysis of defined glycoforms of IgG-Fc on sFcγRIIb binding and show that the mannose residues exert an important influence on sFcγRIIb binding. Finally, we discuss how the oligosaccharide residues of IgG-Fc affect FcγR binding.

**EXPERIMENTAL PROCEDURES**

**Proteins**

sFcγRIIb—Recombinant soluble human FcγRIIb was expressed in *Escherichia coli*, refolded, and purified, as described previously (22). Briefly, the extracellular region of hFc-γRIIb (23) was overexpressed as inclusion bodies in *E. coli* which were purified by lysozyme treatment of the cells and subsequent sonication. The resulting suspension was centrifuged (30 min, 30,000 × g) and washed with buffer containing 0.5% (w/v) lauryldimethylamine oxide. The centrifugation step and resuspension in lauryldimethylamine oxide-containing buffer was repeated twice, and then twice more without lauryldimethylamine oxide. The inclusion bodies were dissolved in 6 M guanidine hydrochloride and the protein renatured. The dialyzed and filtered protein solution was applied to a human IgG-Sepharose column and eluted by pH jump. The concentrated neutralized fractions were subjected to size-exclusion chromatography on a Superdex-75 column (Amersham Pharmacia Biotech). Protein concentrations were determined spectrophotometrically using a calculated ε280 of 29,400 M⁻¹ cm⁻¹ (24).

**Homogeneous Glycoforms of IgG-Fc (Cri)**—The monoclonal protein IgG1 (Cri, allotype G1m(f)) was isolated from serum from a patient with multiple myeloma and the IgG1-Fc fragment generated as previously reported (25). The following protocols were used to generate truncated glycoforms of IgG1-Fc (Cri). For (NGA2F)₂ glycoform, the native IgG1-Fc (5 mg) in 50 mM citric acid, Na₂HPO₄, pH 4.8, was exposed to N-acetyl-b-galactosaminidase (0.225 unit, *D. pneumoniae*, Roche Molecular Biochemicals) at 37°C for 24 h. For deglycosylated IgG1-Fc the native IgG1-Fc (5 mg) in 40 mM KH₂PO₄, 10 mM EDTA, pH 7.4, was exposed to peptide-N-glycosidase F (30 units; *Flavobacterium meningosepticum*, Roche Molecular Biochemicals) at 37°C for 72 h. Following exposure to glycosidases all proteins were affinity purified using a Strepptococcal protein G-Sepharose 4B (Amersham Pharmacia Biotech) column, eluted with 0.1 M glycine-HCl buffer, pH 2.7. Eluates were immediately neutralized by the addition of 1 M Tris/HCl, pH 8.0, and dialyzed extensively against phosphate-buffered saline.

**Cleavage of Interchain Disulfide Bridges**

The IgG1 or IgG1-Fc (Cri) was reduced and alkylated according to Fleischman et al. (26). Briefly, IgG1 or IgG1-Fc (Cri, 10 mg/ml) was dissolved in 0.5 M Tris/HCl containing 2 mM EDTA, pH 8.0. The protein was treated with 0.01 M dithiothreitol at room temperature for 1 h after being sparged with N₂ gas. Iodoacetamide was added to a final concentration of 0.02 M and incubated on ice for 30 min with gentle stirring. The protein was dialyzed extensively against phosphate-buffered saline. Cleavage of the interchain disulfide bonds was confirmed by nondenatured SDSPolyacrylamide gel electrophoresis and electrospray ionization mass spectrometry. Alkylation of Cys residues involved in inter-chain disulfide bridges (L chain, 1; H chain, 3) was confirmed by an increase in their masses by 59 and 178 Da, respectively, relative to those of reduced L and H chain (data not shown), which corresponds well to the mass of the -CH₂-CONH₂ group (58 Da) added by alkylation.

**Electrospray Ionization Mass Spectrometry**

Electrospray ionization mass spectrometry was performed using an LC-ITTM instrument and MassLynx data acquisition (Micromass, Manchester, UK). This instrument is an orthogonal acceleration time-of-flight mass spectrometer, with a Z-spray electrospray ion source. The mass spectrometer was operated in the positive-ion mode. Resolution of the mass spectrometer was 5000 at m/z 1500. The cone voltage was set to 35 V. The purified IgG1-Fc Cri proteins (1 mg/ml) were extensively dialyzed against distilled water and formic acid added to a final concentration of 1% (v/v). Ten microliters of the sample solution was injected into a Rhenodeyne valve into the mobile phase of 50% acetic acid, and 50% of 0.1% (v/v) formic acid in water. Ten to twenty spectra were averaged, baseline subtracted, and deconvoluted using MAX.ENT. software (Micromass).

**Isothermal Titration Calorimetry (ITC)**

ITC was performed at 30.0 °C using the MCS-ITC system (MicroCal, Northampton, MA). To examine the stoichiometry of the interaction between IgG-Fc and sFcγRIIb, one of the proteins was dissolved in phosphate-buffered saline, pH 7.4, to 10–13 μM and placed in the calorimeter cell (1.34 ml). The injection syringe (250 μl) was filled with a concentrated solution (0.30–0.37 mM) of the other component dissolved in the same buffer. sFcγRIIb from the syringe was titrated into

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**Fig. 1.** Stoichiometry of the interaction between IgG-Fc and sFcγRIIb. A, Fc into sFcγRIIb titration. Five μl of Fc (0.37 mM) was injected into the cell containing 10 μM sFcγRIIb at 30 °C, except for the first injection which was 1 μl. B, sFcγRIIb into Fc titration. Eight μl of sFcγRIIb (0.33 mM) was injected into the cell containing 14 μM Fc except for the first injection which was 1 μl. Top, raw heat data obtained for a series of injections. Bottom, integrated curve showing the best fit. The data were fitted to a “one-set of sites” model. The data set presented is one of two sets of independent experiments.

**TABLE I**

<table>
<thead>
<tr>
<th>Titrant</th>
<th>n (Stoichiometry)</th>
<th>K_d (μM)</th>
<th>ΔG (kcal/mol)</th>
<th>ΔH (kcal/mol)</th>
<th>ΔS (cal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1-Fc → sFcγRIIb</td>
<td>1.00 ± 0.04</td>
<td>0.98 ± 0.01</td>
<td>-8.30 ± 0.01</td>
<td>-6.20 ± 0.20</td>
<td>2.10 ± 0.20</td>
</tr>
<tr>
<td>sFcγRIIb → IgG1-Fc</td>
<td>0.91 ± 0.01</td>
<td>0.94 ± 0.06</td>
<td>-8.35 ± 0.04</td>
<td>-6.49 ± 0.27</td>
<td>1.86 ± 0.31</td>
</tr>
<tr>
<td>sFcγRIIb → intact IgG1</td>
<td>0.97 ± 0.04</td>
<td>0.95 ± 0.02</td>
<td>-8.35 ± 0.01</td>
<td>-6.35 ± 0.27</td>
<td>2.00 ± 0.28</td>
</tr>
<tr>
<td>sFcγRIIb → R/A IgG1*</td>
<td>1.06 ± 0.02</td>
<td>1.89 ± 0.40</td>
<td>-7.93 ± 0.13</td>
<td>-3.80 ± 0.06</td>
<td>4.12 ± 0.13</td>
</tr>
</tbody>
</table>

* Reduced and alkylated IgG.
The thermodynamic parameters were calculated from basic equations of the thermodynamics: \[ \Delta G = \Delta H - T \Delta S = -RT \ln K, \]
where \( K \) is the dissociation constant, \( \Delta G \) is the change in free energy, \( \Delta H \) is the enthalpy, and \( \Delta S \) is the entropy of binding, respectively. \( T \) is the temperature in Kelvin, and \( R \) is 1.98 cal mol\(^{-1}\) K\(^{-1}\).

The change in heat capacity of binding (\( \Delta C_p \)), which is assumed to be independent of temperature, was determined using the following expression: \[ \Delta C_p = \Delta H/\Delta T. \]

**Differential Scanning Microcalorimetry (DSC)**

Experiments were performed on a VP-DSC microcalorimeter (MicroCal) with IgG-Fc sample concentrations of 4 \( \mu \)g and a scan rate of 60 °C h\(^{-1}\). No concentration dependence of the thermal unfolding temperatures (\( T_u \)) and calorimetric enthalpies (\( \Delta H \)) was observed at this low concentration. Data were collected and analyzed using Origin 4.1 software (MicroCal), essentially as previously described (28). The area below each transition peak corresponded to \( \Delta H \), and the van’t Hoff enthalpy (\( \Delta H_v \)) was calculated using the formula: \[ \Delta H_v = 4.5 \Delta T/\ln(2), \]
where \( \Delta C_p \) and \( \Delta T \) were the measured heat capacity at the peak maximum, and \( R \) is the gas constant. The cooperativity of the heat transition was calculated as the ratio \( \Delta H/\Delta H_v \) (29).

**Surface Plasmon Resonance Measurements**

sFcRIIb binding was measured using Biacore 3000 and CM5 biosensor chips (BIAcore, Uppsala, Sweden). Homogeneous glycoforms of IgG-Fc were immobilized onto the chips by amide coupling following the manufacturer’s instructions. Assays were performed with sFcRIIb in a mobile phase at flow rate of 10 \( \mu \)l/min using 20 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.05% (v/v) surfactant P20, pH 7.4. The carbodiimide/ethanolamine-treated blank was included as a control. \( K_d \) values for the binding of the Fc glycoforms to sFcRIIb were estimated from the plots of the steady-state binding levels against sFc in the form of the binding isotherm:

**RESULTS**

The calorimeter cell containing the homogeneous glycoform of IgG-Fc. A series of injections was made, and the observed heat pulses integrated and fitted to a theoretical titration curve using software Origin 2.9 (MicroCal) with \( \Delta H \) (the enthalpy change in cal mol\(^{-1}\)), \( K_d \) (the equilibrium association constant in \( \mu \)M), and \( n \) (the number of binding sites per monomer) as adjustable parameters. The quantity c values (27) were between 5 and 30 in the present study.

**TABLE II**

<table>
<thead>
<tr>
<th>Glycoform</th>
<th>Molecular mass (Da)</th>
<th>Observed</th>
<th>Calculated</th>
</tr>
</thead>
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<tr>
<td>Deglycosylated</td>
<td>49,923</td>
<td>49,931</td>
<td></td>
</tr>
<tr>
<td>(MN2F)(_2)</td>
<td>51,347</td>
<td>51,358</td>
<td></td>
</tr>
<tr>
<td>(M3N2F)(_2)</td>
<td>51,993</td>
<td>52,007</td>
<td></td>
</tr>
<tr>
<td>(NGA2F)(_2)</td>
<td>52,811</td>
<td>52,820</td>
<td></td>
</tr>
<tr>
<td>NGA2F + NA1F(\alpha)</td>
<td>52,972</td>
<td>52,982</td>
<td></td>
</tr>
<tr>
<td>NGA2F + NA2F(NA1F)(_2)</td>
<td>53,135</td>
<td>53,144</td>
<td></td>
</tr>
<tr>
<td>NA1F + NA2F</td>
<td>53,293</td>
<td>53,306</td>
<td></td>
</tr>
<tr>
<td>Monosialyl NA1F + NA2F</td>
<td>53,590</td>
<td>53,598</td>
<td></td>
</tr>
</tbody>
</table>

\( \alpha \) NA1F, fucosylated and monogalactosylated glycoform.

\( \alpha \) NA2F, fucosylated and digalactosylated glycoform.

**FIG. 2.** Electrospray ionization mass spectrometry of homogeneous glycoforms of IgG1-Fc (CRI). a, native Fc; b, Fc treated with sialidase and \( \beta \)-galactosidase, and N-acetylgalcosaminidase; c, Fc treated with sialidase, \( \beta \)-galactosidase, and N-acetylgalcosaminidase; d, Fc treated with sialidase, \( \beta \)-galactosidase, N-acetylgalcosaminidase, and \( \alpha \)-mannosidase; e, peptideN-glycosidase F-treated protein. Degly refers to deglycosylated protein. Open symbols in panel a represent variable attachment of terminal galactose and sialic acid residues; The sugars are represented as follows: ●, fucose; ■, N-acetylgalcosamine; ●, mannosae; △, galactose; ○, sialic acid; p, protein.

The thermodynamic parameters were obtained irrespective of whether IgG-Fc was titrated into sFcRIIb (Fig. 1A) or sFcRIIib into IgG1-Fc (Fig. 1B) (Table I). Both binding isotherms were best fitted to a 1:1 association model. If the stoichiometry were different from 1:1, the values of \( \Delta H \) and \( n \) would differ depending on which component is in the calorimeter cell and which is being titrated as shown for the interac-
tion between IgG and neonatal FcR (33). Thus, the results verify that the stoichiometry for the interaction is 1:1 in solution, suggesting that sFcRIIb binds to the hinge-proximal region of the C1q2 domains of Fc in a manner similar to that observed for the structure of Fc in complex with sFcRIIIB (20). The thermodynamic parameters for sFcRIIib binding to intact IgG molecules were almost the same as those for IgG-Fc (Table I), indicating that the Fab regions of IgG do not contribute significantly to binding of FcRIIib. We disrupted the hinge inter-chain disulfide bridges of IgG by mild reduction and alkylation to examine its effect on the stoichiometry of the interaction. The stoichiometry for sFcRIIib interaction with reduced and alkylated IgG remained the same as that for sFcRIIib-intact IgG interaction (1:1) although the affinity was slightly and marked decrease in affinity, respectively (Fig. 4; Table III). Removal of terminal GlcNAc residues to generate a less favorable entropic contribution (Fig. 3, b and c; Table III). The interactions between sFcRIIib and homogeneous glycoforms of IgG-Fc (Cri) were investigated by ITC (Fig. 3). The thermodynamic parameters were characterized at 30 °C (Table III). The native Fc and its nongalactosylated glycoform, (NGA2F), showed very similar thermodynamic parameters (Fig. 3, a and b; Table III). Removal of terminal GlcNAc residues to generate showed very similar thermodynamic parameters (Fig. 3, a and b; Table III).

### Table III

<table>
<thead>
<tr>
<th>Glycoform</th>
<th>Stoichiometry</th>
<th>$K_a$</th>
<th>$ΔG$</th>
<th>$ΔH$</th>
<th>$ΔS$</th>
<th>$ΔC_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.91 ± 0.01</td>
<td>0.94 ± 0.06</td>
<td>-8.35 ± 0.04</td>
<td>-6.49 ± 0.27</td>
<td>1.86 ± 0.31</td>
<td>-160 ± 10°</td>
</tr>
<tr>
<td>(NGA2F)</td>
<td>0.93 ± 0.01</td>
<td>0.99 ± 0.01</td>
<td>-8.32 ± 0.01</td>
<td>-6.47 ± 0.06</td>
<td>1.85 ± 0.06</td>
<td>ND*</td>
</tr>
<tr>
<td>(M3N2F)</td>
<td>0.96 ± 0.07</td>
<td>1.19 ± 0.04</td>
<td>-8.21 ± 0.02</td>
<td>-6.58 ± 0.21</td>
<td>1.63 ± 0.14</td>
<td>ND*</td>
</tr>
<tr>
<td>(MN2F)</td>
<td>0.90 ± 0.02</td>
<td>3.03 ± 0.55</td>
<td>-7.63 ± 0.12</td>
<td>-5.89 ± 0.15</td>
<td>1.74 ± 0.27</td>
<td>-250 ± 40°</td>
</tr>
</tbody>
</table>

* The errors represent the estimated standard error obtained from the data fitting.

**Surface plasmon resonance** was employed for thermodynamic analysis of the interactions of sFcRIIib with homogeneous glycoforms of Fc immobilized on sensor chips at six different temperatures 5, 10, 15, 20, 25, and 30 °C. A representative binding profile is shown at 25 °C in Fig. 4A. Although the dissociation constants obtained by surface plasmon resonance were generally greater by about 3-fold (Table IV), relative to those obtained by ITC (Table III), both data sets show the same trends in that trimming of terminal GlcNAc and mannose residues of the oligosaccharide chains of IgG-Fc results in a slight and marked decrease in affinity, respectively (Fig. 4B). In addition, it was shown that the binding affinity to sFcRIIib was decreased with increasing the temperature for all glycoforms (Fig. 4B). The plots of $ΔG$ values against temperature were fitted to a nonlinear van’t Hoff equation (Fig. 4C). Apparently, the thermodynamics were almost the same between native Fc and the (NGA2F), glycoform because their plots over-
lapped closely (Fig. 4C). Interestingly, ΔC_p of the (M3N2F)_2 glycoform was similar to those of native Fc and the (NGA2F)_2 glycoform, however, the value for the (MN2F)_2 glycoform was more negative (Table IV).

**Thermal Stability of the Homogeneous Glycoforms of IgG1-Fc (Cri)—**The thermal stability of homogeneous glycoforms of IgG1-Fc (Cri) was examined by DSC (Fig. 5). The first transition peak has been assigned to the unfolding of the CH2 domain of the Fc. An experiment at 25 °C is shown as a representative. B, plots of mid-injection peak data against sFcγRIIb concentrations. The data were fitted to a single binding site model for receptor binding to homogeneous glycoforms of IgG-Fc. K_d values shown are the average of two independent experiments. C, temperature dependence of ΔG of sFcγRIIb binding to each IgG-Fc glycoform. The plots were fitted to a nonlinear van't Hoff equation (see "Experimental Procedures").
IgG-Fc. The data were baseline corrected. The thermograms presented are one of two similar experiments. 

resulted in noncooperative melting of the CH2 domains, such independently of each other. Interestingly, removal of the oligosaccharide and reduced and alkylated Fc, suggesting that both samples unfold similarly, i.e. that there is at least one intermediate between the folded and unfolded states (Fig. 5, A, iv, and B, ii).

DISCUSSION

Recent crystal structures of the sFcyRIIb-Fc complex have revealed the detailed molecular interactions between these components (20, 35). However, while in one structure a primary GlcNAc residue participates directly in binding to the receptor, in the other there is no direct interaction of the oligosaccharide with the receptor. Thus, it is unclear as to why the oligosaccharide at Asn-297 of IgG-Fc on the periphery of the receptor interaction site is critical for FcγRI binding. We attempted to probe the roles of the N-linked oligosaccharide chains of IgG-Fc in binding to sFcyRIIb and IgG-Fc bearing truncated oligosaccharides. We found that removal of the mannose residues on the 1,6- and 1,3-arms of the oligosaccharide of IgG-Fc has a significant influence on the receptor binding. The cause of the inability of deglycosylated IgG to bind FcγRI is also discussed.

Stoichiometry—We established a 1:1 stoichiometry for the interaction between sFcyRIIb and IgG-Fc in solution. The molecular basis for the 1:1 stoichiometry has been detailed for the sFcyRIIb-IgG-Fc complex in the crystalline state (20). sFcyRIIb molecules bind asymmetrically at a 1:1 ratio to the lower hinge region of both C1b domains, a primary GlcNAc sugar residue and residues in the vicinity of the carbohydrate. The decreased binding of reduced and alkylated IgG to sFcyRIIb provides an insight into the role of inter-chain disulfide bridges in receptor binding (Table I). This result supports the involvement of both C1b domains of IgG in sFcyRIIb binding, suggesting that the disulfide bridges are not essential for a 1:1 association but contribute to appropriate positioning of both C1b domains to
optimize FcγR binding. Using truncated Fc mutants we recently showed that hinge disulfide bonds are not necessarily required for recognition by FcγRI of IgG1 or single-chain Fv fusion protein with Fc (36). However, the mutants lacking C13 domains (IgG lacking a C13 domain pair; single-chain Fv fusion protein with a hinge-C12 domain) fail to be recognized by FcγRI following mild reduction and alkylation. Therefore it seems likely that the C13-C13 and C12-C12 contacts, 1090 Å2 and 389 Å2, respectively (6), maintain the close apposition of the two C12 domains even in the absence of hinge disulfide bonds. Taken together, two C12 domains are required for recognition of IgG by FcγRs, either paired covalently at the N-terminal end by hinge disulfide bonds or noncovalently at the C-terminal end by the C13 domain pair. Interestingly, the oligosaccharide-C12 contact (522 Å2) is an additional requirement for optimal FcγR binding as shown in Fig. 3e. The contribution of individual sugar residues of the oligosaccharides to sFcγRIIb binding is discussed below.

**Significance of Galactose Residues**—We obtained no evidence for the positive contribution of terminal galactose residues to sFcγRIIb binding or the thermal stability of the C12 domains (Figs. 3b, 4, and 5A, i; Tables III, IV, and V). The report that in the absence of the 1,6-arm galactose residue the oligosaccharides of IgG-Fc becomes freely mobile (37) does not correspond to our findings because we could not detect deglycosylated IgG-like behavior either in the binding isotherm (Fig. 3b) or thermogram (Fig. 5A, i). These results are consistent with our previous mutational studies, which showed that replacement of Lys-249 and Glu-258 that make contacts with 1,6-arm galactose does not affect the recognition of IgG by FcγRII (38). It is logical to conclude therefore, that even in the absence of galactose residues, the equilibrium for the oligosaccharide-C12 domain interaction inclines toward association. On the other hand, it has been reported that terminal GlcNAc residues of agalactosyl IgG-Fc can be recognized by the mannose receptor on macrophages and dendritic cells (40). These findings may be relevant to the pathogenesis of rheumatoid arthritis since it has been shown that serum IgG is hypogalactosylated in this disease (39).

**Significance of GlcNAc Residues**—Removal of the terminal GlcNAc residues results in a significant decrease in the thermal stability of the C12 domains (Fig. 5A, ii; and Table V) but in only a minor decrease of affinity for sFcγRIIb (Fig. 3c; Table III). This result supports our previous work on site-directed mutagenesis in which Asp-249 and Lys-246, making contacts with the terminal galactose and GlcNAc residues, are replaced with Ala and showed minimal influences on FcγRI and FcγRII binding (38). Therefore, it seems likely that loss of the GlcNAc-C12 domain interactions cause local disorder in the area of the C12 domain distant from the lower hinge and hence does not markedly influence binding of the receptor. The minor decrease in affinity of the (M3N2F)glycoform for sFcγRIIb results solely from changes in the entropic contribution (Table III). Such complex formation involves the intramolecular conversion of a partially unstructured C12 polypeptide chain into a more ordered structure. This reduction in the conformational entropy is more pronounced for the (M3N2F)glycoform, reflecting a more complexed free state.

**Significance of Mannose Residues**—A marked reduction in binding affinity of the (M2N2F)glycoform for sFcγRIIb (Figs. 3d and 4; Tables III and IV) demonstrated that the 1,3- and 1,6-arm mannose residues are among the key sugar residues which influence sFcγRIIb binding. This finding is in good agreement with our recent results obtained by superoxide production assay in which recognition of the (M2N2)glycoform of L243-IgG1 by FcγRI is significantly impaired, relative to the native IgG glycoform (8). As deglycosylated Fc did not show measurable affinity for sFcγRIIb by ITC (Fig. 3e), the (M2N2F)glycoform, which shows intermediate affinity, may provide insight into the glycoform requirements of IgG-Fc for FcγR binding. The 1,6-arm mannose residue makes contacts with Phe-241 and Phe-243 of the C12 domains (7). On the other hand, the 1,3-arm mannose makes contacts with the counterpart mannose residue of the other oligosaccharide chain, which may support a minimum separation between the C12 domains (20). Although it seems likely that removal of the 1,3-arm mannose residues abolishes the contact between the two C12 domains, the DSC results strongly suggest that C12-C12 contact is maintained without the mannose residues because the ratio ΔH/ΔHV1 of the C12 domains for (M2N2F)glycoform was similar to the value for the native protein (Table V). If the C12 domains of (M2N2F)glycoform did not contact each other and hence were unfolded independently by the temperature rise, then the ratio ΔH/ΔHV1 would be close to 2 as observed for reduced and alkylated Fc. Therefore, it is very likely that removal of the mannose residues results in a decrease in the distance between the two lower hinge regions because the oligosaccharides are no longer biantenary structures. According to the crystal structure of the sFcγRIIbIgG-Fc complex (20), the horseshoe-shaped Fc opens up on complex formation, with the distance between the Pro-329 residues located on the N-terminal tip of both C12 domains increased by 7 Å. Taken together, the decrease in the distance between the two C12 domains of uncomplexed Fc because of removal of the mannose residues may have a negative influence on sFcγRIIb binding. ΔH and ΔCp for the interaction between the (M2N2F)glycoform and sFcγRIIb (Tables III and IV) suggest a conformational change coupled to complex formation due to the folding/rearrangement of IgG-Fc/sFcγRIIb. The excess entropy change (Table IV), which gives an indication of the number of amino acid residues involved in an induced fit or conformational change
upon binding (41), was estimated from entropy change from the hydroscopic effect (ΔS_H2O) at T_cubed, subtracted by the entropy contribution due to the loss of rotational and translational degrees of freedom (ΔS_rot). The excess entropy for (MN2F)2 was notably increased, relative to the negligible values for native Fc, (NGA2F)2b, and (M3N2F)2. This result suggests that folding is coupled to the complex formation of (MN2F)2 with sFCyRIIb where the estimated value of ΔS_H2O (T_cubed) is much larger than the magnitude of ΔS_H2O (T_cubed) for rigid-body associations and ΔS_rot. We interpreted this result as an increased C_p hinge proximal domain for the uncomplexed (MN2F)2 glycoform: complex formation with sFCyRIIb now requires a conformational change within the C_p2 domains, leading to a more negative ΔC_p value. The gradual decrease in CD charides. However, deglycosylated CH2 domains do not exhibit drophobic areas that were originally covered with the oligosaccharides (Fig. 5B, iv), adding sialic acid (Lec 2) or galactose (Lec 3) to the oligosaccharides of IgG-Fc retains oligosaccharide/CH2 domain interaction with sFCyRIIb (Fig. 5A, iv) is totally different from that of the CH3 domains with respect to cooperativity as well as T_m value. The amino acid residues of the CH2 domains analogous to those in the CH3 domains involved in C3-C3p3 contact make contacts with the oligosaccharides (6). Therefore, it is likely that deglycosylation results in C1-2-C1-2 contacts through hydrophobic areas that were originally covered with the oligosaccharides. However, deglycosylated CH2 domains do not exhibit cooperativity in contrast to the CH3 domains even when released from the constraints imposed by the interchain disulfide bridges (Fig. 5B, ii). Radaev and Sun (43) have proposed that deglycosylation of Fc leads to a change in the relative orientation of the two C1-2 domains such that there is a transition from an open to a closed conformation. The gradual decrease in affinity of the IgG glycoforms for sFCyRIIb on truncation of the oligosaccharides (Fig. 3, Table II) supports this proposal. In addition to such conformational change, we emphasize that impaired FcR binding by deglycosylation could be due, in part, to destabilization of the CH2 domains (Fig. 5, Table V).

Implications of IgG Glycosylation for Therapeutic Applications—The modulated activity of the MN2F2 glycoform of IgG may have applications for activation or deactivation of effector cells via FcγRs. Remodeling of the oligosaccharide profile of IgG constitutes an alternative approach to modulate the biological activity of therapeutic IgGs in addition to protein engineering. Therapeutic antibodies are currently approved or under clinical trial for the treatment of some types of cancer, infectious diseases, and autoimmune disorders (44). The mouse- and hamster-derived cell lines such as NS0 and Chinese hamster ovary cells can produce human IgG antibodies that express "wild-type" biological activities, however, "mutant" Chinese hamster ovary cells can emerge with defined defects in carbohydrate biosynthesis. The mutant Chinese hamster ovary cells incapable of processing the high-mannose intermediate (Lec 1), adding sialic acid (Lec 2) or galactose (Lec 3), have been reported (45, 46). The resulting IgG bearing distinct oligosaccharides exhibit different functional properties with respect to complement and FcγRI activation and in vivo half-life. Chinese hamster ovary cells do not have a capacity for the addition of bisecting GlcNAc; cell engineering with the introduction of β1-4-N-acetylglucosaminyltransferase III gene has been shown to yield antibody products that promoted killing target cells at ~10–20-fold lower concentrations than the parent molecules lacking bisecting GlcNAc (47, 48). On the other hand, aglycosylated anti-CD3 monoclonal antibody has been shown to be a promising candidate for immunosuppressive therapy due to its inability to bind to FcγRs or activate complement (49). Fcγ fragments are also proven immunosuppressants for the treatment of immune thrombocytopenic purpura capable of blocking the Fcy receptors (50) while peptide inhibitors for the IgG-FcγRs interaction have been developed for modulation of immune response (43, 51). Recently we have shown that truncation of the oligosaccharides of humanized anti-major histocompatibility complex class II (L243-IgG1) results in progressive reduction in stimulation of superoxide production through FcγRI in a monococyte-like cell line (8). The thermodynamic data presented here provide a rationale for the modulation of antibody effector functions through manipulation of glycosylation.

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REFERENCES

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Yusuke Mimura, Peter Sondermann, Rodolfo Ghirlando, John Lund, Stephen P. Young, Margaret Goodall and Roy Jefferis


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