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Chemical and Structural Analysis of an Antibody Folding Intermediate Trapped during Glycan Biosynthesis

Thomas A. Bowden,*,†,‡ Kavitha Baruah,‡,† Charlotte H. Coles,‡,† David J. Harvey,‡ Xiaojie Yu,‡ Byeong-Doo Song,§ David I. Stuart,†,|| A. Radu Aricescu,† Christopher N. Scanlan,§ E. Yvonne Jones,† and Max Crispin§,*

Division of Structural Biology, University of Oxford, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN, United Kingdom
Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom
Scripps Korea Antibody Institute, 192-1 Hyoja-dong, Chuncheon, Gangwon 200-701, Korea
Science Division, Diamond Light Source Ltd., Diamond House, Harwell Science and Innovation Campus, Didcot, Oxfordshire OX11 0DE, United Kingdom

Supporting Information

ABSTRACT: Human IgG Fc glycosylation modulates immunological effector functions such as antibody-dependent cellular cytotoxicity and phagocytosis. Engineering of Fc glycans therefore enables fine-tuning of the therapeutic properties of monoclonal antibodies. The N-linked glycans of Fc are typically complex-type, forming a network of noncovalent interactions along the protein surface of the Cγ2 domain. Here, we manipulate the mammalian glycan-processing pathway to trap IgG1 Fc at sequential stages of maturation, from oligomannose- to hybrid- to complex-type glycans, and show that the Fc is structurally stabilized following the transition of glycans from their hybrid- to complex-type state. X-ray crystallographic analysis of this hybrid-type intermediate reveals that N-linked glycans undergo conformational changes upon maturation, including a flip within the trimannosyl core. Our crystal structure of this intermediate reveals a molecular basis for antibody biogenesis and provides a template for the structure-guided engineering of the protein–glycan interface of therapeutic antibodies.

INTRODUCTION

Antibodies are multifunctional glycoproteins, able to bind antigens through variable Fab domains and cellular receptors via the constant Fc region. This dual functionality enables the recruitment of the cellular immune system to sites of infection by antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), and can lead to the localized activation of the complement system. Glycan and protein engineering of the Fc domain can generate therapeutic monoclonal antibodies with tailored receptor binding functionality.1,2 In contrast to chemical and chemoenzymatic methods to modulate glycans,3–9 we use glycosidase inhibitors and a cell line deficient in a glycosyltransferase to generate antibody glycoforms containing specific carbohydrate structures.

The Fc region of immunoglobulin G (IgG) is a homodimer consisting primarily of heavy chain Cγ2 and Cγ3 domains. The C-terminal Cγ3 domain protomers interact through an extended protein–protein interface, occluding over 1100 Å² of protein surface,10 and adopt rigid conformations that exhibit little structural variation.11 In contrast, the Cγ2 domain protomers have only been observed to interact via glycan–glycan contacts between opposing N-linked chains at Asn297.11–13 Glycan-mediated maintenance of the spacing between the Cγ2 domains is critical for cellular Fcy receptor (FcyR) binding, which occurs asymmetrically at the tip of the Cγ2 domains and lower hinge region.14 Deglycosylation, for example, by bacterial endoglycosidases, leads to disruption of Cγ2 spacing and significantly impairs FcyR binding.15,16

The impact of Asn297 glycosylation upon Fc structure is not limited to influencing Cγ2 spacing. IgG Fc glycosylation also stabilizes the protein through an approximately 500 Å² glycan–protein interface along the surface of the Cγ2 domain.11,13,17 These glycan–protein contacts are believed to limit both the processing by Golgi-resident glycosyltransferases and the conformational freedom of the glycan.18 This model is supported by an NMR study, which proposes that Fc glycans exist in an equilibrium with an approximately equal proportion of a “free” state, with highly mobile glycans, and a less mobile “bound” state, observable by X-ray crystallography, with

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ordered protein−glycan interactions less accessible to enzymatic processing.19 The composition of IgG Fc glycans is largely directed by the protein.18,20 The glycosylation exhibits limited processing and consists of a predominantly biantennary complex-type framework with partial occupancy of galactose, core $\alpha1\rightarrow6$-linked fucose, low levels of "bisecting" GlcNAc, and sialic acid.18 This limited processing is in contrast to the highly sialylated complex-type glycosylation typically observed on secreted glycoproteins.18

The human FcγRs (FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa) display binding properties dependent upon the presence and composition of the Fc glycan.1 For example, afucosylated antibody glycoforms, which may find utility in anticancer treatment, are inflammatory and exhibit enhanced ADCC due to elevated binding to the activatory FcγRIIIa.21 In contrast, anti-inflammatory IgG glycoforms display increased levels of terminal sialylation and are under investigation for enhanced intravenous immunoglobulin therapy.22

Biosynthetic Fc precursors have also been investigated for therapeutic applications due to their altered FcγR-dependent effector functions.9,23–26 Monoclonal antibodies found in the early steps of carbohydrate maturation including oligomannose- or afucosylated hybrid-type glycans, for example, display increased affinity for FcγRIIIa and enhanced ADCC functionality,24,26 albeit with potentially elevated serum clearance.27,28 Here, we have generated and characterized a panel of such glycoform intermediates and present the crystal structure of the key precursor bearing hybrid-type glycosylation. In the context of the biosynthetic pathway of N-linked carbohydrates, this glycoform represents the intermediate formed between the immature oligomannose and the native, complex-type states.29

This Fc glycoform, generated by recombinant mammalian protein expression in the presence of the Golgi α-mannosidase II inhibitor, swainsonine,30,31 was crystallized and subjected to X-ray crystallographic analysis to 2.4 Å resolution. Examination of this structure reveals a novel interaction between carbohydrate and protein components. Together with thermo-stability analyses, the structure provides a model for the conformational transitions that IgG Fc undergoes upon glycoprotein maturation and provides a template for the structure-guided engineering of therapeutic antibodies.

■ RESULTS AND DISCUSSION

Expression and Purification of IgG Fc Glycoforms. A panel of IgG1 Fc glycoforms, corresponding to key stages of the
protein-directed and substantially less processed than other cells with no inhibitors present. MALDI-TOF MS analysis bearing complex-type glycans were generated using HEK 293T hydrolysis of the Man\(\alpha\)1-mannosidase II inhibitor, swainsonine. Swainosine prevents the ER, and the early, medial, and late Golgi apparatus, are generally representative of the carbohydrates appearing in hybrid-, and complex-type glycan structures. These glycoforms “\(\beta\) glycans indicating variable terminal revealed an heterogeneous spectrum of putative hybrid-type linked glycans of IgG1 Fc expressed with 10 hybrid-type glycans (\(m/z = 1743.6\); Figure 1A). Expression of the Fc region in GnT I-deficient human embryonic kidney (HEK) 293S cells resulted in a similarly homogeneous spectrum dominated by Man\(\alpha\)GlCNAC\(\beta\) glycans (\(m/z = 1257.4\); Figure 1B).

The next glycoform in the N-linked biosynthetic pathway, the hybrid-type glycan, was isolated using the Golgi \(\alpha\)-mannosidase II inhibitor, swainosine. Swainosine prevents hydrolysis of the ManA and ManB saccharides of the “6-arm” of the trimannosyl core. However, this inhibition does not impede the addition of a GlcNAc residue to the “3-arm” Man4 by GnT I or subsequent structural elaborations. Consistent with this mode of action, MALDI-TOF MS analysis of the N-linked glycans of IgG1 Fc with 10 \(\mu\)M swainosine revealed an heterogeneous spectrum of putative hybrid-type glycans indicating variable terminal \(\beta1\rightarrow4\)-linked galactose, “bisecting” \(\beta1\rightarrow4\)-linked GlcNAc, and a population of sialylated hybrid-type glycans (\(m/z = 2081.7\); Figure 1C). Finally, Fc bearing complex-type glycans were generated using HEK 293T cells with no inhibitors present. MALDI-TOF MS analysis revealed biantennary complex-type glycans with variable terminal galactose (Figure 1D). This spectrum is consistent with previous observations that Fc glycosylation is highly protein-directed and substantially less processed than other glycoproteins similarly expressed using the pHLSec expression vector in HEK 293T cells.

Levels of both galactose and sialic acid in the hybrid-type spectrum were higher than those observed for the complex-type glycoforms. As sialic acid can alter the ionization efficiency of glycans in mass spectrometry, we also subjected the hybrid and complex-type glycans to electrospray ionization (ESI) mass spectrometry (Figure 2). No sialylated structures were detected by ESI–MS of the glycans released from the Fc glycoform bearing complex-type glycans (Figure 2B). In contrast, the spectrum of glycans deriving from Fc expressed in the presence of swainosine contained a prominent peak at \(m/z = 2035.7\) corresponding to a sialylated core-fucosylated hybrid-type glycan (Figure 2A).

Isomeric assignments of the hybrid-type structures were determined by negative-ion ESI MS/MS of the enzymatically released glycans. The fragmentation spectra of the most abundant species are presented in Figure 3. The \(m/z\) values of the “D-type ions”, as defined by Harvey, are a signature of the 6-arm. These D-type ions are annotated in the spectra and are formed by the formal loss of the 3-arm and the fucosylated di-N-acetyllactosamine core. Similarly, the D’-type ions, formed by cleavage of the 6-arm, reveal the cluster of mannose residues on the 6-arm. Bisecting GlcNAc residues are revealed by an abundant \([D – 221]^{-}\) ion at \(m/z = 629\) and the virtual absence of a D-type ion (Figure 3B). The absence of an ion at \(m/z = 306\) shows that the sialic acid residue is \(a2\rightarrow3\)-linked (Figure 3A). This linkage contrasts \(a2\rightarrow6\)-linked sialic acid observed in serum-derived antibodies.

To assess the level of sialic acid in the hybrid-type glycans, we expressed an intact IgG1 antibody hybrid-type glycoform and subjected the fluorescently derivatized glycans to normal-phase HPLC. This analysis revealed that 20% of these swainosine-induced hybrid-type glycans were sialylated (Figure 4A). Consistent with our MALDI–MS and ESI–MS data of IgG1 Fc glycans, no sialylated structures were observed in IgG produced in the absence of swainosine (Figure 4B).

Swainosine is not known to affect galactosyltransferase or sialyltransferase activity, as confirmed by the similar glycan profiles of the same glycoproteins expressed in HEK 293T cells.
Figure 3. Negative ion fragmentation spectra of the major N-linked glycans from the hybrid-type glycoform of IgG1 Fc. Neutral glycan ions are \([M + H_2PO_4]^-\), while the sialylated glycan forms a \([M - H]^-\) ion. (A) Sialylated, fucosylated hybrid glycan (Man5Gal1GlcNAc3Fuc1NeuNAc1), \(m/z\) 2035.7. (B) Bisected, fucosylated hybrid glycan (Man5GlcNAc4Fuc1), \(m/z\) 1883.6. (C) Fucosylated hybrid glycan (Man5Gal1GlcNAc3Fuc1), \(m/z\) 1842.6. (D) Agalactosylated, fucosylated hybrid glycan (Man5GlcNAc3Fuc1), \(m/z\) 1680.5. The nomenclature describing fragmentation ions follows that of Domon and Costello and is distinct from the carbohydrate residue labels (Figure 1).

Figure 4. HPLC analysis of fluorescently labeled N-linked glycans from recombinant IgG1 expressed in HEK 293T cells in (A) the presence and (B) the absence of the GnT I inhibitor, swainsonine. The lower panels show the spectra of glycans following digestion with Arthrobacter ureafaciens sialidase. Symbols for the structures are as described in Figure 1.
in the presence of swainsonine and in HEK 293T Lec36 cells that are devoid of Golgi α-mannosidase II activity. Similarly, there was no evidence of increased terminal processing upon disruption of Golgi α-mannosidase II activity as compared to glycoproteins expressed in HEK 293T cells. Therefore, the increased abundance of galactose and sialic acid residues in the 3-arm of the hybrid-type glycoform, as compared to the complex glycoform, may be indicative of increased steric accessibility of the Fc glycans to processing enzymes.

Thermodynamic Stability of Fc Glycoforms. One explanation for the increased 3-arm processing of the hybrid-type glycoform as compared to that of the complex-type glycan is that the accessibility of the glycans is influenced by composition and structure of the glycans–protein interface. Because of the highly processed composition of the hybrid-type glycoform, we hypothesized it would exhibit distinct glycan–protein packing interactions with altered stability. We assessed our panel of Fc glycoforms by differential scanning fluorimetry (Figure 5).

This analysis revealed that relative to the melting temperature ($T_m$) of the complex-type glycoform, the other glycoforms exhibited reduced $T_m$ values: Man$_9$GlcNAc$_2$ ($T_m = -2.8 \pm 0.7 \, ^\circ C$), hybrid-type glycoforms ($T_m = -4.0 \pm 0.7 \, ^\circ C$), and endoglycosylated-treated Fc ($-5.2 \pm 1.0 \, ^\circ C$). Therefore, in addition to reducing glycan accessibility to glycosyltransferases, the enzymatic action of Golgi α-mannosidase II, to produce complex-type glycans, is permissive for the biosynthesis of a more thermally stabilized Fc structure. We sought to investigate the structural basis for this observation by X-ray crystallography.

Structural Characterization of the Hybrid-Type Fc Glycoform. Crystallographic structures of IgG Fc have been reported for a number of glycoforms ranging from oligomannose Man$_9$GlcNAc$_2$ to homogeneous complex-type, and endoglycosidase-deactivated and aglycosylated states. However, no crystallographic information is available for the hybrid-type Fc glycoform that represents the structural basis for this observation by X-ray crystallography.

The hybrid-type glycoform crystallized in the primitive orthorhombic spacegroup, $P2_12_12_1$, with one homodimer in the asymmetric unit. The $C_{\gamma3}$ protomers were very similar in structure (0.3 Å root-mean-square deviation over 103 equivalent Ca residues) and exhibited noncrystallographic 2-fold rotational symmetry. However, the orientations of the $C_{\gamma3}$ domains may arise due to differences in stabilizing crystallographic packing interactions. This hypothesis is supported by analysis of the crystalline assembly. This analysis reveals that the $C_{\gamma3}$ domain of the more ordered chain A exhibits higher B-values where some $C_{\gamma3}$-distal loop regions and associated glycan residues were disordered and not clearly visible in the electron density (Figure 6A).

Biosynthetic Conformational Transitions. Overall Conformation. In chain A of our hybrid-type glycoform, we observe electron density for eight saccharide residues, corresponding to the entirety of the fucosylated oligomannose component of the hybrid-type glycan (Figure 6B). No electron density was observed for the processed 3-arm beyond Man$_9$.

The 3-arm is entirely solvent exposed with the nearest
component of a symmetry-related molecule located 9 Å away. While we cannot formally exclude the possibility of selective crystallization of a subset of hybrid-type glycoforms, this distance, and the large accessible volume surrounding the 3-arm, provides no evidence for such a phenomenon.

From the observation that the conformations of complex-type glycans are not influenced by the presence of fucose, we suggest that the structure of the Man$_{9}$GlcNAc$_{2}$ component of the hybrid-type glycan reported herein is likely to resemble the Man$_{9}$GlcNAc$_{2}$ glycoform that occurs in the preceding stage of the pathway (Figure 1). This assertion, combined with analysis of the previously reported Man$_{9}$GlcNAc$_{2}$ Fc structure and the series of complex-type structures by Krapp et al., now enables us to propose a model of glycan maturation during antibody biogenesis.

The folded Man$_{9}$GlcNAc$_{2}$ glycoform is generated following the hydrolysis of the glucose cap and consequent release from the calnexin/calreticulin folding check-point. All available structures of Fc glycoforms show a conserved mode of interaction of the saccharide residues proximal to the protein attachment site. The Man$_{9}$/4GlcNAc$_{2}$/1→4GlcNAc core and the observable 3-arm residues exhibit highly similar conformations and glycan–protein packing interactions. In contrast, the 6-arm residues exhibit divergent conformations reflecting the different chemical compositions and/or surrounding environments of the glycan residues (Figure 7).

Transition from Man$_{9}$GlcNAc$_{2}$ to Man$_{5}$GlcNAc$_{2}$. The structural rearrangements that occur within the oligosaccharide when carbohydrates are modified are illustrated by the hydrolysis of terminal α1→2 mannose residues of the Man$_{5}$GlcNAc$_{2}$ glycoform. The ManD3 residue in the Man$_{5}$GlcNAc$_{2}$ structure anchors the oligosaccharide chain to the protein surface at the junction of the C$_{2}$/3 and C$_{3}$/3 domains (Figure 7A). As the solvent accessible ManD1 and ManD2 residues are disordered in the crystal structure, hydrolysis of ManD3 is likely to induce the observed rearrangement of the 6-arm and cause the 6 Å shift of ManB (Figure 7A,B). This cleavage also results in the associated relaxation of the Man$_{4}$4GlcNAc$_{2}$/1→6ManB linkage (from $\varphi = 69^\circ$, $\psi = -178^\circ$, $\omega = 62^\circ$ to $\varphi = 77^\circ$, $\psi = 101^\circ$, $\omega = 30^\circ$; Figure 7A,B). Despite this movement, the Man4' residue in the Man$_{9}$GlcNAc$_{2}$ glycoform is orientated in a direction opposite to that of complex-type structures (Figure 7C). This orientation is maintained by the presence of ManA and ManB residues of the 6-arm that sterically prevent further rotation of the Man$_{4}$4GlcNAc$_{2}$/1→6Man3 linkage (Figure 7B).

Transition from Man$_{9}$GlcNAc$_{2}$ to Hybrid-Type. The action of GnT I on Man$_{9}$GlcNAc$_{2}$ catalyzes the formation of hybrid-type glycans and allows downstream carbohydrate processing (Figure 1). GnT I transfers β1→2-linked GlcNAc to the Man4 residue of the 3-arm to form the GlcNAc$^5$/β1→2Man4 linkage (Figure 7B,C). As for many structures of complex-type glycoforms, we do not observe interpretable electron density for the solvated and mobile residues on this arm. Additionally, GnT I catalysis also renders the core GlcNAc residue (GlcNAc1) of the hybrid-type glycan susceptible to α1→6-fucosylation. We observe electron density for the fucose and note the conformation closely resembles that of the complex-type glycoform (Figure 7B,C). This supports our assertion that Man$_{5}$GlcNAc$_{2}$ glycans are not affected structurally by fucosylation.

Formation of Complex-type Glycans. Golgi α-mannosidase II hydrolyses the α1→3-linked ManA and α1→6-linked ManB residues from the 6-arm and is dependent upon the prior activity of GnT I (Figure 1). Elimination of these residues relieves steric restraints around the 6-arm and allows the reorientation of the Man$_{4}$/α1→6Man3 linkage (from $\varphi = 77^\circ$, $\psi = 101^\circ$, $\omega = 30^\circ$ to $\varphi = 62^\circ$, $\psi = 171^\circ$, $\omega = -175^\circ$; Figure 7B,C), causing close alignment of the glycan to the protein surface.

Following this rearrangement, GnT II catalyzes the transfer of β1→2-linked GlcNAc to Man4', allowing the formation of hydrophobic stacking interactions between GlcNAc$^5'$ and Phe243 (Figure 7C). The formation of these canonical glycan–protein interactions is consistent with the increased stability (Figure 5 and Supporting Information Figure S1) and decreased enzymatic processing of the mature complex-type glycoform relative to the artificially trapped hybrid-type glycoform (Figures 1, 2, and 4). Limited downstream compositional heterogeneity of the complex-type glycoform subsequently arises from the partial transfer of galactose to terminal GlcNAc5 and GlcNAc5' residues and leads to little change with respect to carbohydrate conformation or thermal stability.

Evidence for the suppression of galactosylation and sialylation by the interaction between GlcNAc5' and Phe243 is provided by the analysis of IgG from a patient with a homozygous mutation in the Mge2 gene. Mge2 encodes GnT II that catalyzes the transfer of the 6-arm GlcNAc5' to Man4'. The IgG Fc glycans isolated from the patient lacked 6-arm GlcNAc5' but exhibited significantly elevated 3-arm
processing as compared to wild-type structures with the majority of glycans containing the NeuNAc\(^{7}\)\(\alpha\)\(2\)\(\rightarrow\)6Gal\(^{6}\)\(\beta\)1\(\rightarrow\)4GlcNAc\(^{5}\) motif.\(^{50}\) Together with our structural observations, we suggest that the action of Golgi \(\alpha\)-mannosidase II and GnT II enhance the glycan–protein interface and limit glycosyltransferase accessibility to the 3-arm.

### SUMMARY AND CONCLUSIONS

The use of glycosidase inhibitors and cell-lines with genetically modified glycan processing enzymes offers a powerful route to the isolation of glycoproteins with defined glycan structures.\(^{34,51}\) These methods offer an attractive alternative to direct chemical synthesis and can be readily combined with chemoenzymatic methodologies.\(^{3,7,52,53}\)

Analysis of isolated biosynthetic intermediates of IgG1 Fc revealed distinct differences in the susceptibility of discrete glycan states to glycosyltransferases. Specifically, we have shown by mass spectrometry that the trapped hybrid-type glycans are more readily accessible to galactosyl and sialyltransferases than are complex-type structures. The generation of hybrid-type glycoforms with increased Fc sialylation is of note given the enhanced anti-inflammatory functionality exhibited by sialylated Fc in, for example, intravenous immunoglobulin therapy.\(^{22}\)

The biosynthetic intermediates also exhibited reduced stability, an important parameter in the development of antibody therapeutics.\(^{54}\) Through our X-ray crystallographic analysis, we correlate this stability to structural transitions that occur during antibody biogenesis. We offer a molecular-level explanation for how stability arises from rearrangements of the glycan–protein interface. We deduce that glycan-dependent stabilization occurs during Golgi \(\alpha\)-mannosidase II and GnT II processing, which respectively cause the relaxation of the 6-arm toward the protein surface and the formation of hydrophobic glycan–protein interfaces.

Given the promising portfolio of effector functions exhibited by IgG bearing oligomannose and hybrid-type glycans,\(^{15,24,25,27,28}\) knowledge of their three-dimensional structure and defined molecular transitions provides a template to support structure-guided stabilization and optimization for the clinic.

### EXPERIMENTAL PROCEDURES

**Protein Expression and Purification.** The Fc region of human IgG1 (residues 120–329; GenBank accession no. J00228) was cloned into the pHLSec vector\(^{55}\) and transiently expressed in HEK 293T cells (ATCC number CRL-1573), GnT I-deficient HEK 293S cells,\(^{35}\) and in the presence of mannosidase inhibitors to isolate glycoforms of distinct composition.\(^{34}\) Transfections were performed using 2 mg of DNA per liter cell culture medium as previously described.\(^{55}\) Fc bearing Man\(_4\)GlcNAc\(_2\) and hybrid-type glycosylation were obtained by expression in the presence of 20 and 10 \(\mu\)M of the inhibitors, kifunensine\(^{55}\) and swainsone,\(^{51}\) respectively (Toronto Research

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**Figure 7.** Structural transitions of IgG1 Fc glycans between (A) oligomannose-type (PDB accession code 2WAH), (B) hybrid-type, and (C) complex-type glycosylation (PDB accession code 3AVE), as observed by X-ray crystallography. The left-hand column displays the glycan packing against the C\(\gamma\)2 domain. The central column shows a view with the 6-arm in the foreground. The right-hand column is a close-up and shows the conformational changes occurring within the 6-arm; an asterix indicates the location of the C6 carbon of the Man\(^4\) residue. The protein surface is colored gray, the F243 side-chain is colored pink, and the glycan is colored as in Figure 1 except in the close-up where the oxygen atoms of mannose residues are shown in red.
Thermally induced unfolding of purified Fc in the presence of Sypro Orange (Invitrogen, Paisley, UK), in triplicate, by measuring Absorbance at 610 nm at 1.5 °C.

HEK 293T cells in the presence or absence of 10 μM TriM pH 5.5 yielded 20 mg of purified IgG Fc per liter cell culture.

Plasmids encoding IgG B12 light and heavy chains were kindly provided by Professor Dennis Burton (The Scripps Research Institute, CA). The heavy and light chains were transiently cotransfected in HEK 293T cells in the presence of 10 μM swainsonine.31 IgG B12 was purified by incubation for 2 h with Protein A Sepharose (GE Healthcare, UK) at room temperature. The beads were washed with phosphate buffered saline (PBS) before elution using 0.1 M citric acid pH 3.4 followed by neutralization and size exclusion chromatography. Yields were typically 8 mg of purified IgG B12 per liter cell culture.

Thermodynamic Stability of IgG Fc Glycoforms. The thermal stability of different Fc glycoforms was assayed by differential scanning fluorimetry using a Stratagene RT PCR 305 instrument. Thermally induced unfolding of purified Fc glycoforms was monitored, in triplicate, by measuring Absorbance at 610 nm at 1.5 °C increments in the presence of Sypro Orange (Invitrogen, Paisley, UK), a fluorescent stain sensitive to hydrophobic environments.65

Glycan Analysis. Oligosaccharides were released from target glycoproteins with peptide-N-glycosidase F (New Englands Biolabs) from Coomassie blue-stained NuPAGE gels.57 Excised bands were washed five times alternatively with acetonitrile and deionized water, and rehydrated with a 3000 units/mL of aqueous PNGase F solution. After 12 h incubation at 37 °C, the enzymatically released N-linked glycans were eluted with water. Samples were analyzed by MALDI-TOF MS with a Shimadzu AXIMA TOF MALDI-TOF/TOF mass spectrometer (Kratos Analytical, Manchester, UK) fitted with delayed extraction and a nitrogen laser (337 nm). Samples were cleaned on a Nafion 117 membrane (Aldrich), and then prepared for MALDI-TOF/TOF MS by adding 0.5 μL of an aqueous solution of the glycans to the matrix solution (0.3 μL of a solution of 2,5-dihydroxybenzoic acid in acetonitrile:water; 1:1, v:v) on the stainless steel target plate and allowing it to dry at room temperature. The sample/matrix mixture was then recrystallized from ethanol.

Negative ion ESI–MS was performed with a Waters Synapt G2 traveling wave ion mobility mass spectrometer (Waters MS-Technologies, Manchester, UK).58 Samples were dissolved in a solution of methanol:water (1:1, v:v) containing 0.5 M ammonium phosphate and introduced with a 20 kV thin-wall nanospray capillaries. The ESI capillary voltage was 1.2 kV, the cone voltage was 20–180 V, and the ion source temperature was 120 °C. The T-wave velocity and peak height voltages were 450 mV and 40 V, respectively. The T-wave mobility cell contained nitrogen and was operated at a pressure of 0.55 mbar and was used to provide an additional selection stage for the fragmentation experiments. Fragmentation was performed after mobility separation in the transfer cell with a 3 mass unit selection window and with argon as the collision gas. The instrument was externally calibrated with sialylated N-glycan standards. Data acquisition and processing were carried out using Waters Driftscope (version 2.1) software and MassLynx (version 4.1).

Fluorescent labeling of glycans with 2-aminoethoxysulfonic acid (2-AA) and subsequent HPLC analysis was performed as previously described.59 Briefly, a buffered glycan solution was mixed with 2-AA and sodium cyanoborohydride. Labeling was achieved by incubation for 1 h at 80 °C. Excess 2-AA was removed using a SpeedVac 2-column (Systematic Instruments, UK). HPLC was carried out at room temperature in a 20 mM ammonium hydroxide solution (pH 3.9) with a linear gradient of acetonitrile and water. Exoglycosidase digestions were performed as previously described.60

Crystallization and Structure Determination. Crystals of IgG Fc bearing hybrid-type glycans were grown by sitting-drop vapor diffusion using 100 nL of protein solution (11.4 mg mL⁻¹) plus 100 nL of precipitant using the previously described semiautomated robotics of the Oxford Protein Production Facility.61 Crystals of IgG Fc bearing hybrid-type glycans grew at 20 °C in 20% w/v PEG 3350 pH 5.5 with 0.2 M sodium/potassium phosphate after 4 days. Crystals were flash frozen by immersion in a cryoprotectant containing 25% ethylene glycol and transferred to a gaseous nitrogen stream at 100 K. Data were collected at beamline BM14 at the European Synchrotron Radiation Facility, Grenoble, France (Table 1). Diffraction data were integrated and scaled using the programs DENZO and SCALEPACK,62 and the structure was solved by molecular replacement using the program Phaser with the protein chain from a natively glycosylated IgG Fc63 (PDB accession code 3AVE) as a search model. Five percent of reflections was randomly set aside to calculate the Rmerge. Generally, the hybrid Fc structure was refined using Refmac5 in the CCP4 suite and included iterative restrained refinement with translation libration screw parametrization and incorporation of automatically generated local noncrystallographic symmetry restraints.65,66 The molecular graphics program Coot was used for manual rebuilding and MolProbity was used to validate the model.49 Data processing, refinement, and structure validation statistics are presented in Table 1.

Nomenclature. Throughout this work, we have adopted the system of Vliegenthart et al.68 for labeling residues within oligomannose- and biantennary-type oligosaccharides with the additional modification of 7′α for sialic acid, 1′ for α1→6-linked core fucose (Figure 1). These residue labels are in bold-face throughout this Article. The symbolic representation of glycans follows that of Harvey et al.33 with residues in both the schematic diagrams and the molecular graphics following the color scheme of the Centre for Functional Glycomics. Carbohydrate fragmentation ions are labeled using the Donom and Costello nomenclature65 with an extension by Harvey.70

Dihedral angles were defined using the “x→y” system for crystallography: ϕ = C5′–C1–O–C(x) and ψ = C1–O–C(x)–C(x−1). For Man1–2Man and Man1–3Man, where x = 2 or 3, respectively; ϕ = C5–C1–O–C6′, ψ = C1–O–C6′–C5′, and ω = O–C6′–C5′–C4′ for Man1–6Man linkages.71

ASSOCIATED CONTENT

Supporting Information

Atomic coordinates and crystallographic structure factors of IgG Fc bearing hybrid-type glycans have been deposited in the Protein Data Bank (PDB) with accession code 4B71. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

thomas.bowden@strubi.ox.ac.uk; max.crispin@bioch.ox.ac.uk

Author Contributions

These authors contributed equally.

Notes

The authors declare no competing financial interest.

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