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## Abstract

Asparagine-linked carbohydrates (N-glycans) are a common modification of eukaryotic proteins that confer multiple properties including the essential stabilization of therapeutic monoclonal antibodies. Here we present a rapid and efficient strategy to identify N-glycans that contact polypeptide residues and apply the method to profile the five N-glycans attached to the human antibody receptor CD16A (Fc  $\gamma$  receptor IIIA). Human embryonic kidney 293S cells expressed CD16A with [ $^{13}\text{C}$ ]-labeled N-glycans using standard protein expression techniques and medium supplemented with 3 g/L [ $^{13}\text{C}$ ]-glucose. Anomeric resonances on the protein-linked N-acetylglucosamine residue at the reducing end of the glycan are particularly well suited to studies of multiply-glycosylated N-glycoproteins because only one reducing end and nitrogen-linked residue is present in each N-glycan. Correlations between anomeric  $^1\text{H}$ - $^{13}\text{C}$  nuclei on the reducing end residue generate crosspeaks in a conventional 2d heteronuclear single quantum coherence NMR experiment that appear in a region of the spectrum devoid of other carbohydrate peaks or background protein signals. Two N-glycan peaks corresponding to the N45 and N162 N-glycans were dispersed from the rapidly averaged peaks corresponding to the N38, N74 and N169 N-glycans. We used a combination of NMR and 1  $\mu\text{s}$  all-atom computational simulations to identify unexpected contacts between the N45 N-glycan and CD16A polypeptide residues.

## Disciplines

Biochemistry | Biophysics | Molecular Biology | Structural Biology

## Comments

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## Carbohydrate-polypeptide contacts in the antibody receptor CD16A identified through solution NMR spectroscopy

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### Abstract

Asparagine-linked carbohydrates (N-glycans) are a common modification of eukaryotic proteins that confer multiple properties including the essential stabilization of therapeutic monoclonal antibodies. Here we present a rapid and efficient strategy to identify N-glycans that contact polypeptide residues and apply the method to profile the five N-glycans attached to the human antibody receptor CD16A (Fc  $\gamma$  receptor IIIA). Human embryonic kidney 293S cells expressed CD16A with [ $^{13}\text{C}_U$ ]-labeled N-glycans using standard protein expression techniques and medium supplemented with 3 g/L [ $^{13}\text{C}_U$ ]-glucose. Anomeric resonances on the protein-linked *N*-acetylglucosamine residue at the reducing end of the glycan are particularly well suited to studies of multiply-glycosylated N-glycoproteins because only one reducing end and nitrogen-linked residue is present in each N-glycan. Correlations between anomeric  $^1\text{H}$ - $^{13}\text{C}$  nuclei on the reducing end residue generate crosspeaks in a conventional 2d heteronuclear single quantum coherence NMR experiment that appear in a region of the spectrum devoid of other carbohydrate peaks or background protein signals. Two N-glycan peaks corresponding to the N45 and N162 N-glycans were dispersed from the rapidly averaged peaks corresponding to the N38, N74 and N169 N-glycans. We used a combination of NMR and 1  $\mu\text{s}$  all-atom computational simulations to identify unexpected contacts between the N45 N-glycan and CD16A polypeptide residues.

### Graphical Abstract

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Present Addresses

n.a.

Author Contributions

n.a.

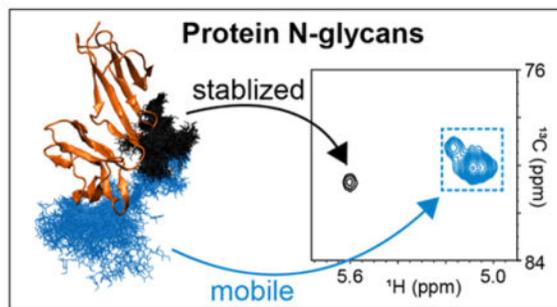
#### Notes

the authors declare no competing interests.

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website.

Supplemental Methods and two supporting figures (PDF)



Asparagine-linked carbohydrates (N-glycans) are common eukaryotic protein modifications that contribute structural stabilization, epitopes for receptor interactions, longevity in the serum, markers for intracellular trafficking, protection from antibody recognition and proteases, as well as antigen presentation in the immune system<sup>1–8</sup>. A high percentage of secreted and cell surface human proteins are likely N-glycosylated and many contain multiple modifications though not all N-glycans contribute to structural stabilization<sup>9,10</sup>. Glycoprotein stabilization through carbohydrate/polypeptide contacts is a critical role for N-glycans and is the primary function of the essential and conserved immunoglobulin G N-glycan in therapeutic monoclonal antibodies and the human immune system<sup>1,2,11</sup>. Currently, rapid structure-based methods to identify and characterize N-glycans that contribute to glycoprotein structure and function by interacting with polypeptide residues do not exist. Furthermore, approaches to characterize N-glycans lag behind other related protein spectral methods because common recombinant protein expression systems like *Escherichia coli* do not N-glycosylate. Here we present a solution-based method to identify N-glycans that form intramolecular interactions with polypeptide residues that uses standard glycoprotein expression techniques.

N-glycans can be extensive features with dozens of sugar residues<sup>12,13</sup>, however, Kelly and coworkers demonstrated the predominant N-glycan contribution to the stabilization of CD2 came from the *N*-acetylglucosamine (GlcNAc) residue at the reducing end and point of attachment to asparagine<sup>14</sup>. Thus, we probed the 1-bond correlation between anomeric carbon (C1) and proton (H1) nuclei by solution NMR spectroscopy as a potential reporter of intramolecular interactions between N-glycan and polypeptide residues using the soluble extracellular domain of human CD16A (Fc  $\gamma$  receptor IIIa) that contains five N-glycosylation sites to identify which contribute to antibody binding. CD16A is a membrane protein with two N-terminal extracellular antibody-binding domains, a transmembrane spanning region and a short intracellular domain. CD16A is expressed mainly on natural killer cells and monocytes/macrophages and activates an immune response by binding antibody-coated particles.

CD16A expressed from HEK293S cells grown in commercially available, chemically-defined media formulations contained a high degree of glycosylation with nearly homogenous oligomannose N-glycans (complete details are available in the Supplemental Methods section)<sup>15–17</sup>. Supplementing commercial HEK293 growth medium with 3 g/L [<sup>13</sup>C<sub>U</sub>]-glucose efficiently labeled CD16A carbohydrate residues but showed minimal, if any

metabolic scrambling into amino acids in a 2d  $^1\text{H}$ - $^{13}\text{C}$  HSQC experiment which is not surprising because the medium contains high concentrations of amino acids (Fig 1). Correlations between anomeric protein and carbon nuclei provide clear signals for each sugar residue in the glycan, and are resolved from other protein and carbohydrate peaks with  $^{13}\text{C}$  resonance frequencies between 80–105 ppm. Unfortunately, this strategy provides as many as 35 signals for CD16A and it is challenging to identify signals coming from a single N-glycan. The *N*-acetylglucosamine residue at the N-glycan reducing end ((1)GlcNAc) represents the only residue of each glycan that contains a linkage to a nitrogen atom; all other residues are linked through an oxygen atom. This difference isolates peaks from the (1)GlcNAc residues on CD16A in region of the spectrum from 80–85 ppm ( $^{13}\text{C}$ ) that lacks protein or other carbohydrate resonances (*dashed* box, Fig 1). Expansion of the spectrum to cover these correlations reveals four peaks (Fig 2); two are resolved and separated and two intense peaks partially overlap in the spectrum. The peak at the far left of the spectrum is considerably weaker and broader than the other three peaks. It was interesting to note that digesting CD16A with endoglycosidase F (Fig S1), which cleaves between glycan residues 1 and 2, did not alter the spectrum to a significant extent, consistent with the report by Kelly and coworkers noted above that the (1)GlcNAc residue provided the predominant stabilizing contribution of the N-glycans <sup>14</sup>.

Intramolecular interactions between glycan and polypeptide residues were destroyed by trypsin digest to generate unstructured peptide and glycopeptide fragments. A spectrum of proteolyzed CD16A showed the dispersed spectrum collapsed into two intense and partially overlapped peaks that indicate the two peaks identified in the intact CD16A spectra shifted from the intense overlapped peaks due to glycan/polypeptide interactions and not amino acid sequence differences near the N-X-S/T sequon (Fig 2). This result also provides a clear expectation for resonance frequencies of (1)GlcNAc  $^1\text{H}$ - $^{13}\text{C}$  correlations from N-glycans in minimally or unstructured regions.

Mutating asparagine residues located in the three residue N-X-S/T N-glycan consensus sequons to glutamine prevented N-glycan attachment during protein expression and provided N-glycan specific assignments for CD16A. Proteins with a single N-glycan site expressed poorly and proved unstable. Combinations of single or triple mutations identified the asparagine residue position for each N-glycan peak. The two intense and partially overlapped peaks contained resonances from N-glycans at N38, N74 and N169 (Fig 2). These N-glycans do not appear to interact with the CD16A polypeptide based on peak intensity and a position that is coincident with N-glycans from proteolyzed CD16A that suggest rapid motional averaging of multiple conformations. The peak located just to the upper left of these resonances, shifted by 0.1 ppm  $^1\text{H}$ -1.0 ppm  $^{13}\text{C}$  relative to the N38/N74/N169 peaks, belongs to the N162 N-glycan. The peak in the lower left, shifted by 0.5 ppm  $^1\text{H}$ /0.5 ppm  $^{13}\text{C}$ , belongs to the N-glycan attached to N45.

We investigated the relative motion of the five CD16A N-glycans with molecular dynamics simulations to provide more insight into possible motions experienced by each N-glycan. An 850 ns simulation of the extracellular domain of CD16A revealed that the N45 N-glycan experienced less motion than the N-glycans at N38, N74, N162 or N169 at each residue position based on analysis of a root mean square fluctuation value (RMSF; Fig S2). The

N38, N74 and N169 N-glycans neither stabilize CD16A (Fig 2) nor contribute much to antibody binding<sup>18–20</sup> and therefore investigation of CD16A with only the N45 and N162 N-glycans is justified. A 1  $\mu$ s simulation of CD16A lacking the three most mobile N-glycans (N38/N74/N169) showed a similar difference in N45 and N162 mobility as in simulations of the wild-type CD16A (Fig 3). The N45 N-glycan residues showed low RMSF values that were  $\sim 1/3$  the values for corresponding N162 N-glycan residues and RMSF clearly separates N162 and N45 N-glycans at the (1)GlcNAc residue, corresponding to the NMR measurements (Fig 2).

A detailed analysis of intramolecular interactions involving the N45 N-glycan revealed a mode of stabilization that is centered around interactions with the (1)GlcNAc residue. The (1)GlcNAc residue on the N45 N-glycan forms two important interactions with E68 and D64 that are maintained for the duration of the 1  $\mu$ s simulations and are poised to restrain residues 53 to 66. The importance of these contacts was verified in a 1  $\mu$ s all-atom simulation of aglycosylated CD16A. Residues 40–55 and 60–70 showed much greater disorder in a simulation of aglycosylated CD16A than the corresponding regions of glycosylated CD16A (Fig S3). Furthermore, the N45-stabilized loop corresponding to residues 60–70 occupied a stabilized structure that completely unfolds in the simulation of aglycosylated CD16A. Shields and coworkers identified the importance of these residues and connected secondary structural elements that include W90 and W113 that form a cage around P329 of IgG1 Fc<sup>21</sup>. W90 and W113 reside between N45 and N162 and are poised to be affected by local stabilization mediated by N45. The role of stabilizing N45 N-glycan contacts is further supported by our observations that N45 enhances recombinant CD16A expression. A CD16A mutant with only the two N45 and N162 N-glycans expressed at a high level, but the CD16A form with one N-glycan at N162 expressed with very low yield (data not shown).

Position-specific N-glycan assignments and all-atom molecular dynamics simulations provided a new window to CD16A structure and function. It is not surprising that the N162 peak was not resolved in a spectrum of the CD16A N45Q mutant. The stabilization provided by the N45 N-glycan appears to impact the conformation near N162 because the N45 N-glycan appears to form the strongest interactions with the CD16A polypeptide based on the degree of peak shift when compared to the spectrum of proteolyzed CD16A and the simulations of glycosylated and aglycosylated CD16A. It was surprising, however, that the N45 N-glycan showed the greatest interaction of the five N-glycans. Perhaps the unique location of N45 at the end of a loop formed by residue 42–50 provides more potential N-glycan/polypeptide interactions. This location is comparable to the N297 N-glycan on IgG1 Fc that forms a distinct intramolecular interface with polypeptide residues and unlike the four other CD16A N-glycans<sup>1</sup>. Previous work indicated that primarily the N45 and N162 N-glycans contributed to antibody binding<sup>18, 19</sup>. N-glycans at N38, N74 and N169 likely do not contribute and were removed for crystallization of the CD16A/IgG1 Fc complex<sup>19, 20</sup>. The N162 N-glycan is involved in IgG1 Fc binding and the N45 N-glycan is located on a domain that does not contact IgG1 Fc directly.

This glycoprotein labeling and NMR technique provides an accessible, rapid and efficient method to identify N-glycans that contact polypeptide residues and are poised to contribute

to glycoprotein stability and function. This technique uses standard protein expression and isotope labeling methods that are accessible to most laboratories. N-glycan resonances from the anomeric carbon at the reducing end of the N-glycan provide a limited number of well resolved signals that are observable using standard 2d NMR experiments available on standard spectrometers with two channel capability. These methods are likely applicable to most glycoproteins and isolated domains with one or more N-glycans, as demonstrated here.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

### Funding Sources

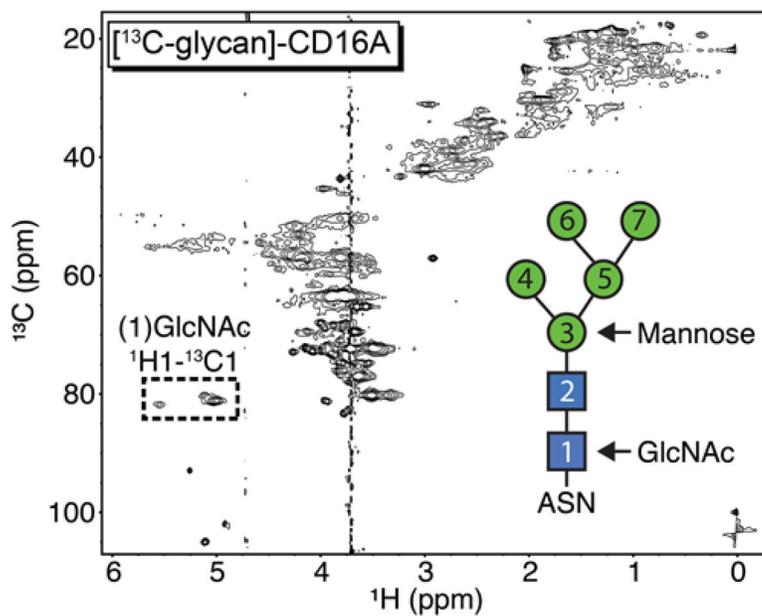
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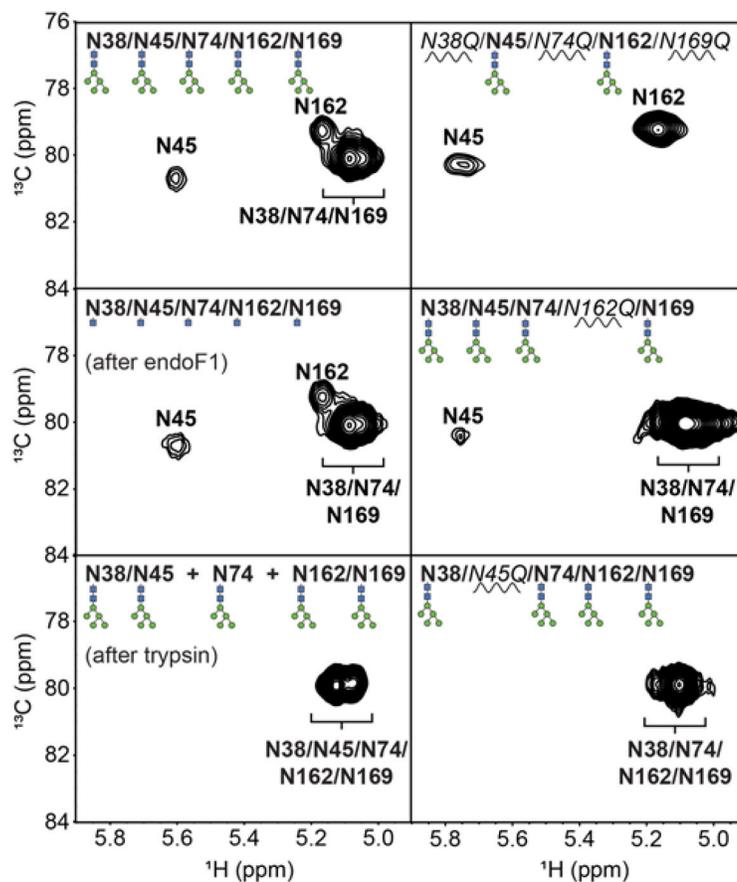
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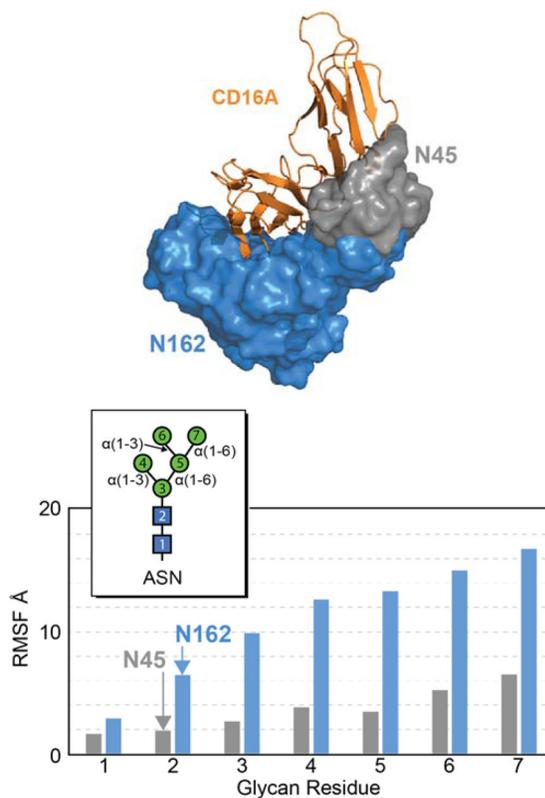


**Figure 1.**  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectrum of  $^{13}\text{C}$ -glycan-CD16A. The region corresponding to the peaks from the anomeric correlations of asparagine-linked *N*-acetylglucosamine residues are highlighted with a *dashed* box. Recombinant CD16A contains exclusively oligomannose (Man5) N-glycans as shown by the cartoon in the lower right.



**Figure 2.**

A region from  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra of [ $^{13}\text{C}$ -glycan]-CD16A show anomeric correlations originating from the asparagine-linked *N*-acetylglucosamine residue. Glycosidase or trypsin digestions of wild type CD16A with 5 *N*-glycans are shown in the *left* column. CD16A mutated to prevent one or more *N*-glycosylation modifications revealed peak assignments (*right* column).



**Figure 3.** All-atom molecular dynamics simulation (1  $\mu$ s) of N-glycosylated CD16A reveals large differences in N-glycan mobility. The N162 N-glycan samples a large space (*blue cloud, left figure*) unlike the N45 N-glycan with restricted mobility (*grey cloud*). RMSF values for N-glycan residues in these simulations likewise reflect the relative mobility of each N-glycan.