Investigating the Interaction between the Neonatal Fc Receptor and Monoclonal Antibody Variants by Hydrogen/Deuterium Exchange Mass Spectrometry*§

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The recycling of immunoglobulins by the neonatal Fc receptor (FcRn) is of crucial importance in the maintenance of antibody levels in plasma and is responsible for the long half-lives of endogenous and recombinant monoclonal antibodies. From a therapeutic point of view there is great interest in understanding and modulating the IgG–FcRn interaction to optimize antibody pharmacokinetics and ultimately improve efficacy and safety. Here we studied the interaction between a full-length human IgG1 and human FcRn via hydrogen/deuterium exchange mass spectrometry and targeted electron transfer dissociation to map sites perturbed by binding on both partners of the IgG-FcRn complex. Several regions in the antibody Fc region and the FcRn were protected from exchange upon complex formation, in good agreement with previous crystallographic studies of FcRn in complex with the Fc fragment. Interestingly, we found that several regions in the IgG Fab region also showed reduced deuterium uptake. Our findings indicate the presence of hitherto unknown FcRn interaction sites in the Fab region or a possible conformational link between the IgG Fc and Fab regions upon FcRn binding. Further, we investigated the role of IgG glycosylation in the conformational response of the IgG–FcRn interaction. Removal of antibody glycans increased the flexibility of the FcRn binding site in the Fc region. Consequently, FcRn binding did not induce a similar conformational stabilization of deglycosylated IgG as observed for the wild-type glycosylated IgG. Our results provide new molecular insight into the IgG–FcRn interaction and illustrate the capability of hydrogen/deuterium exchange mass spectrometry to advance structural proteomics by providing detailed information on the conformation and dynamics of large protein complexes in solution. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.042044, 148–161, 2015.

Antibodies and variants thereof constitute the fastest growing category of therapeutic agents, and currently more than 30 immunoglobulins (Igs)1 have been approved for the treatment of cancer, immunological diseases, and infectious diseases (1). The success of therapeutic monoclonal antibodies (mAbs) is based on the ability to specifically target diverse antigens and activate immunological effector responses. An Ig is a “dimer of a dimer” consisting of light chains and heavy chains in which each light chain is linked to a heavy chain and the light–heavy dimers are connected by disulfide bridges to form the intact antibody. IgG is the most prevalent Ig isotype in plasma and is the most commonly used isotype for therapeutic antibodies because of its strong ability to induce antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (2). The IgG1 subtype is a 150 kDa Y-shaped glycoprotein. Its stem and arms are referred to as the fragment crystallizable (Fc) and fragment antigen binding (Fab) regions, respectively. The Fab region is composed of a variable (V) and constant (C) domain from both the light chain and the heavy chain (V<sub>L</sub>, C<sub>L</sub>, V<sub>H</sub>, C<sub>H</sub>1). Antigen binding is achieved through three highly variable complementary determining regions in each variable domain (V<sub>L</sub> and V<sub>H</sub>) of the Fab region. The Fc region is composed of additional constant domains of the heavy chain (C<sub>H</sub><sub>2</sub> and C<sub>H</sub>3); it mediates antibody-dependent cellular cytotoxicity through interaction with Fcγ receptors (3, 4) and activates complement-dependent cytotoxicity through interaction with C1q (5). The Fc region also interacts with the neonatal Fc receptor (FcRn), which

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1 The abbreviations used are: Ig, immunoglobulin; Ab, antibody; β<sub>m</sub>, β<sub>2</sub>-microglobulin; ETD, electron transfer dissociation; Fab, fragment antigen binding; Fc, fragment crystallizable; FcRn, neonatal Fc receptor; HDX-MS, hydrogen/deuterium exchange mass spectrometry; UPLC, ultra-high-performance liquid chromatography.
regulates the maintenance of antibody levels in plasma and thus the half-life of endogenous and recombinant monoclonal antibodies (6). The interaction between IgG and FcRn displays a characteristic pH dependence that is the basis for the function of FcRn in IgG recycling (7). FcRn rescues and recycles IgG from lysosomal degradation by binding with low micromolar affinity to internalized IgG in the slightly acidic late endosome of, for example, vascular endothelial cells (pH < 6.5). The IgG is rescued from intracellular degradation as the IgG–FcRn complex returns to the cell surface, where the IgG is released into circulation as FcRn binding is abolished in the neutral pH of plasma (6). FcRn-mediated IgG recycling contributes to the long catabolic half-life of endogenous and therapeutic antibodies of ~22 days (8).

The FcRn is a heterodimer of an MHC-class-I-like heavy chain and a β2-microglobulin (β2m) light chain. The FcRn heavy chain (α-chain) is composed of three structural domains, α1, α2, and α3, followed by a transmembrane region and a cytoplasmic domain. The three-dimensional structure of FcRn is similar to that of MHC class I molecules in which domains α1 and α2 are stacked against domain α3 and β2m (9, 10). The pH dependence of the IgG–FcRn interaction is attributed to highly conserved residues in both FcRn and IgG (10). The first crystal structures of rat FcRn and rat Fc revealed that FcRn binds to the C2 and C3 domains of the IgG Fc region—specifically, C2 residues 252–254 and 309–311, as well as C3 residues 434–436 (11, 12). Several positively charged histidines in the IgG CH2 and CH3 domains (H310, H433, H435, and H436; the latter is not found in humans) interact with acidic residues E117, E132, W133, E135, and D137 in the FcRn α2 domain, accounting for the pH-sensitive nature of the IgG–FcRn interaction. The interface is also composed of a hydrophobic core around Fc I253 that interacts with FcRn W133 and the N-terminal I1 residue of the β2m, which has been proposed to contact Fc residues 309–311. The interaction of FcRn and IgG occurs in a 2:1 stoichiometry, where two FcRn molecules bind to one IgG through binding sites on each heavy chain (12). Two distinct binding modes have been suggested in which the FcRn molecules bind in a symmetric or asymmetric fashion to the Fc. In symmetric models FcRns bind to opposite sites on the Fc, whereas in the asymmetric models two FcRn molecules form a homodimer with only one FcRn molecule binding the Fc directly (6, 11). The extracellular domains of rat and human FcRns have 68% sequence identity and are structurally similar (9, 10). The first crystal structure of human FcRn in complex with an engineered human Fc fragment (Fc–YTE) as well as human serum albumin was published recently (13) and showed a binding mode similar to that of rodent IgG–FcRn variants, with the exception of the additional interaction sites caused by substitutions in the Fc domain. To the best of our knowledge, no crystal structures of full-length human IgG and human FcRn are currently available.

From a therapeutic point of view there is great interest in understanding and modulating the IgG–FcRn interaction to optimize the pharmacokinetics and thus ultimately the efficacy of therapeutic monoclonal antibodies. The goal of FcRn modulation is typically prolongation of the in vivo half-life in order to reduce dosing frequency and ultimately the cost of treatment. However, a shorter half-life can also be desirable, for example, for antibody–toxin conjugates or antibodies used in bioimaging (6). Several engineered therapeutic mAb variants with improved in vitro FcRn binding affinity and extended in vivo half-life have been generated via mutation of residues in the Fc domain (14–19). For example, the engineered variants of palivizumab (M252Y/S254T/T256E) (15, 16) and bevacizumab (M428L/N434S) (17) show 10- and 11-fold increases in relative FcRn affinity that result in increases of the in vivo half-life in cynomolgus monkeys of 4- and 3-fold, respectively. Mutation can also impact half-life negatively: mAb engineering can improve FcRn affinity at both pH 6 and 7.5 such that the pH-dependent release of IgGs is prohibited, leading to increased IgG clearance (16). Interestingly, post-translational modifications such as oxidation of conserved methionines in the C2 and C3 domains of IgG1 and IgG2 have been shown to affect FcRn affinity negatively. Antibody oxidation that can occur during production or storage significantly reduces FcRn binding in vitro (20, 21), which also translates to a reduced in vivo half-life in human FcRn transgenic mice models (22). The molecular origins of the effect of post-translational modifications on the IgG–FcRn interaction are, however, unclear. Further, the impact of FcRn binding on the conformational properties and dynamics of IgG in solution is currently not well understood.

In this study we investigated the interaction between human FcRn and two variants of a full-length IgG1 by means of hydrogen/deuterium exchange monitored by mass spectrometry (HDX-MS). HDX-MS has become a popular approach for studying protein dynamics and interactions (23–27), as the technique provides access to proteins at native solution conditions with modest sample requirements. Amide HDX rates in native proteins are highly influenced by higher order structure: fully solvated (non-hydrogen-bonded) amides exchange rapidly, whereas structurally protected (hydrogen-bonded) amides exchange up to 7 orders of magnitude slower (28, 29). Protein interactions can be studied and mapped via HDX-MS, as binding events can perturb HDX rates as solvation and hydrogen bonding changes directly in the binding interface or indirectly in conformationally linked regions. The structural resolution of a classic peptide-level HDX-MS experiment is dependent on the generation of overlapping peptides by acid-stable proteases, such as pepsin, typically used in HDX-MS workflows. More recently, the use of gas-phase fragmentation of deuterated peptides with ETD (30–33) has become a viable option for sublocalizing deuterium uptake to short peptide stretches or even individual amino acids, thus increasing
the spatial resolution of the classical bottom-up HDX-MS method.

Here, we used HDX-MS to probe the solution-phase interactions of human FcRn with a full-length recombinant human IgG1, and its deglycosylated variant. Our results allowed us to map antibody and FcRn regions that displayed changes in HDX upon complex formation and examine the impact of antibody glycosylation on FcRn binding. Additionally, by coupling ETD to the HDX-MS workflow in a targeted manner, we obtained high-resolution information on the HDX of individual sites that became protected upon IgG1–FcRn complex formation.

EXPERIMENTAL PROCEDURES

Proteins and Variants—Recombinant human monoclonal IgG, antibody was expressed in CHO cells and buffered in 50 mM Na phosphate, 50 mM NaCl, pH 6.5 (Roche Diagnostics, Penzberg, Germany). Recombinant human FcRn expressed in HEK293F cells was purified as described previously (34) and buffered in 50 mM Na phosphate, 50 mM NaCl, pH 6.5 (Roche Diagnostics). All other chemicals were of the highest grade commercially available.

Deglycosylation of Antibody and FcRn—The antibody and FcRn were deglycosylated by N-Glycosidase F (Roche Diagnostics) in 0.1 M sodium phosphate, pH 7.1 added to a substrate:enzyme ratio of 0.14 U/μg Ab or 0.07 U/μg FcRn. The mixture was incubated at 37 °C for 16 h. For confirmation of deglycosylation, samples were analyzed by reduced intact mass as described below.

Characterization of Antibody and FcRn by Mass Spectrometry—Reduced intact mass analysis was performed on 50 μg of Ab or FcRn reduced and denatured using 0.5 M TCEP (Perbio, Bonn, Germany) in 4 M guanidinium HCl at 37 °C for 30 min. Samples were desalted via size exclusion chromatography (Sephadex G-25, isocratic, 40% ace-tonitrile with 2% formic acid). Electrospray ionization mass spectra were recorded on a MaXis Q-TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a Triversa NanoMate (Advion, Ithaca, NY). For data evaluation, in-house-developed software was used.

For peptide mapping of Ab and FcRn (250 μg), protein denaturation and reduction of disulfide bridges was induced by adding 0.4 mM Tris, 8 M guanidinium HCl, pH 8, and 0.24 M DTT for 1 h at 37 °C. For alkylation 0.6 M iodoacetic acid in water was added and samples were incubated for 15 min at room temperature in the dark. The samples were buffer exchanged to 50 mM Tris/HCl, pH 7.5, using NAP 5 VanGuard column, Waters), and an analytical C18 column (ACQUITY UPLC BEH C18 column (Waters; 1 × 150 mm, 1.7 μm particle diameter, 300 Å pore size) was used for separation, and the solvents were 0.1% formic acid in water (A) and in acetonitrile (B) (Sigma Aldrich, Munich, Germany). A linear gradient of 60 μl/min from 1% to 40% B was run over 120 min at 50 °C. Mass analysis was performed by coupling the UPLC system to an LTQ Orbitrap XL tandem mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany) operating in positive ion mode through a Triversa NanoMate interface (Advion).

Hydrogen/Deuterium Exchange Mass Spectrometry—HDX-MS experiments were performed on glycosylated human IgG1 (Abwt), deglycosylated human IgG1, (Abdegly), human FcRn, and deglycosylated FcRn (FcRndegly) using the following samples.

Abwt and Abdegly—Abwt (61 pmol/μl) or Abdegly (50 pmol/μl) was diluted in 99.9% D2O 50 mM Na phosphate, 50 mM NaCl, pH 6.5, to a final D2O content of 90% and an Ab concentration of 1.2 pmol/μl.

Abwt ± FcRn—Abwt (73 pmol/μl) and FcRn (112 pmol/μl) were mixed and diluted in 99.9% D2O 50 mM Na phosphate, 50 mM NaCl, pH 6.5, to a final D2O content of 90% and Ab and FcRn concentrations of 1.2 pmol/μl and 8.96 pmol/μl, respectively.

Abdegly ± FcRn—Abdegly (50 pmol/μl) and FcRn (112 pmol/μl) were mixed and diluted in 99.9% D2O 50 mM Na phosphate, 50 mM NaCl, pH 6.5, to a final D2O content of 90% and Ab and FcRn concentrations of 1.2 pmol/μl and 8.5 pmol/μl, respectively.

FcRn ± Abwt or ± Abdegly—FcRn (112 pmol/μl) and Abwt (73 pmol/μl) or Abdegly (50 pmol/μl) were mixed and diluted in 99.9% D2O 50 mM Na phosphate, 50 mM NaCl, pH 6.5, to a final D2O content of 90% and FcRn, Abwt and Abdegly concentrations of 1.2 pmol/μl, 6.4 pmol/μl, and 4.4 pmol/μl, respectively.

Ustekinumab (Stelara®) ± FcRn—Ustekinumab (72 pmol/μl) and FcRn (165 pmol/μl) were mixed and diluted in 99.9% D2O 50 mM Na phosphate, 50 mM NaCl, pH 6.5, to a final D2O content of 90% and Ab and FcRn concentrations of 1.2 pmol/μl and 8.5 pmol/μl, respectively.

Abwt ± FcRn or ± FcRndegly—Abwt (61 pmol/μl) and FcRn or FcRndegly (145 pmol/μl) were mixed and diluted in 99.9% D2O 50 mM Na phosphate, 50 mM NaCl, pH 6.5, to a final D2O content of 90% and Ab and FcRn concentrations of 1.2 pmol/μl and 11.3 pmol/μl, respectively.

Theoretical calculations were based on a Kd of 0.6 μM for the IgG1–FcRn interaction and a final volume of 25 μl following dilution into D2O. Following 15-min pre-incubation of samples, deuterium labeling was initiated at room temperature for different time intervals: 0 min, 1 min, 10 min (only for Abwt and Abdegly), 1 h, 2.5 h, and 5 h. For Ustekinumab ± FcRn and for Abdegly ± FcRn or ± FcRndegly, data were acquired for samples labeled for 15 s and 1 h. At each time interval, aliquots (25 μl) of 30 pmol of target protein were removed from the labeling reaction and quenched to a final pH of 2.5 in an ice-cold mixture of 25 μl of 50 mM Na phosphate, 50 mM NaCl, pH 6.5, and 0.5% of 0.5 M TCEP, 6 M guanidinium HCl, pH 2.3, and frozen to −80 °C until LC-MS analysis. Fully deuterated samples were prepared via overnight incubation of Abwt, Abdegly, or FcRn in 6 M deuterated guanidinium HCl (final D2O content of 90%). The quenched deuterated proteins were loaded onto a refrigerated HDX-UPLC system coupled to a hybrid Q-TOF Synapt G2 mass spectrometer (Waters). The UPLC system was operated at 0 °C and equipped with an in-house-packed pepsin column with a 60 μl internal volume (IDEX, Oak Harbor, WA) containing pepsin immobilized on agarose (Thermo Scientific Pierce), a trap C18 column (ACQUITY UPLC BEH C18 1.7 μm VanGuard column, Waters), and an analytical C18 column (ACQUITY UPLC BEH C18 1.7 μm, 1 × 100 mm column, Waters). Proteins were digested in-line at 20 °C and desalted on the trap column with a flow rate of 200 μl/min mobile phase A (0.23% formic acid). Peptic peptides were eluted to the mass spectrometer by a 7 min gradient from 8% to 40% mobile phase B (acetonitrile, 0.23% formic acid) a flow rate of 40 μl/min. The electrospray ionization source was operated in positive ion mode, and the instrument was enabled for ion mobility analysis. A reference lock-spray signal of Glu-Fibrinopeptide (Sigma-Aldrich) was acquired for internal calibration. Identification of peptides was done by tandem MS/MS using a combination of data-independent acquisition methods without ion mobility (MS2 - MS elevated) and with ion mobility (HDMSe - High Definition MS2) as well as data-dependent acquisition MS/MS. Peptide identifications were made through database searching in PLGS v. 2.5, and HDX-MS data were processed in DynamX v. 2.2.1. All HDX-MS experiments were performed in triplicate, and significant changes in deuterium uptake were defined as values greater than two times the standard deviation. HDX-MS data of overlapping peptides were only used to localize deuterium uptake to smaller segments if the back-exchange of the fully deuterated antibody peptides was similar (less than 7%) (35, 36).
HYDROGEN/DEUTERIUM EXCHANGE Mass Spectrometry with ETD—

Deuterated samples were prepared using the same procedure as in the HDX-MS experiments, except the injection amount was adjusted to 100 pmol Abwt and a 5-fold dilution into D2O buffer (80% D2O) was employed, resulting in Ab and FcRn concentrations of 4 pmol/µl and 14 pmol/µl, respectively. HDX-ETD was performed in a targeted manner on selected Abwt peptides with differential deuterium uptake between the FcRn bound and unbound states. The electrospray ionization source and source T-wave were operated at settings optimized for minimal hydrogen/deuterium scrambling as described previously (37) with the following parameters: capillary voltage, 2.8 kV; desolvation gas flow, 800 L/h; cone gas flow, 0 L/h; source temperature, 90 °C; desolvation gas temperature, 300°C; sampling cone, 20 V; extraction cone, 2 V; T-wave trap wave velocity, 300 m/s; and wave height, 0.2 V. ETD was performed in the trap T-wave using 1,4-dicyanobenzene (Sigma-Aldrich) as the ETD reagent. 1,4-dicyanobenzene was introduced into the ion source using a nitrogen makeup flow of 20 ml/min over the reagent crystals stored in a sealed container. The radical anions were generated via glow discharge with a current of 40 µA. We analyzed ETD data by determining the average mass of generated c- and z-type ETD fragment ions. We calculated the deuterium content by subtracting the deuterium content of the unlabeled product ions from those of the deuterated samples. To verify the absence of hydrogen/deuterium scrambling, we monitored the loss of ammonia from the charged reduced species in ETD spectra recorded from peptic peptides from a fully deuterated Abwt sample as described previously (38).

FcRn Affinity Chromatography of Antibodies—FcRn affinity chromatography was performed as described previously (34, 39) for Abwt, Abdegly, and ustekinumab using buffer A (20 mM MES, 140 mM NaCl, pH 5.5) and buffer B (20 mM TrisHCl, 140 mM NaCl, pH 8.8) and a linear gradient from 20% to 100% buffer B in 70 min.

FcRn Surface Plasmon Resonance Analysis—The FcRn binding properties of Abwt and Abdegly were analyzed via surface plasmon resonance using a T200 instrument as described previously (34). FcRn was immobilized onto a Biacore CM5-Biosensor chip at a concentration of 10 µg/ml and 400 RU. Solutions of Abwt and Abdegly were injected as 2-fold dilution series starting from 500 nM in PBS 0.05% Tween-20 (pH 6.0) running buffer at a flow rate of 30 µl/min at room temperature. The association time was 180 s, and the dissociation time was 600 s.

Generation of an IgG–FcRn Homology Model—A model of the Fab fragment of Abdegly was generated with an in-house script based on DiscoveryStudio Pro (Accelrys Inc., San Diego, CA, USA). Two copies of this Fab were superimposed via their C|C1 and C|C domains on the crystal structure of a complete antibody (PDB: 1H2Z) to obtain a model of the full-length Abwt antibody. The recently published crystal structure of human FcRn in complex with a human Fc fragment and human serum albumin (PDB 4n0u) (13) was then superimposed with each of the heavy chains in the antibody model via the C|C atoms of the C|C2 and C|C3 domains. We thereby generated a model comprising full-length Abwt and two copies each of the large and small subunits of FcRn.

RESULTS

Preparation and Characterization of Antibodies and FcRn—
The deglycosylated variant of the antibody (Abdegly) was prepared via enzymatic cleavage of the N-glycan structures on the conserved N305 residue (N297; Eu numbering is indicated in brackets throughout the text for reference) in the antibody heavy chain as described in “Experimental Procedures.” Successful deglycosylation was validated by mass spectrometric analysis of the reduced antibody after N-glyco-

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82% of both heavy and light chains (Fig. 1 and supplemental Fig. S6). Comparison of the HDX profiles of unbound and FcRn-bound Abwt revealed that several sites in both the Fc and Fab domains showed a reduction in deuterium uptake upon binding to FcRn (Figs. 1 and 2). These regions included heavy-chain peptides in regions 1–23, 153–182, 188–205, 243–260, 315–374, and 434–454 and light-chain peptides in region 55–83. The reduced deuterium uptake in heavy-chain peptides spanned the CH2 and CH3 domains (243–260, 315–356, and 434–454) indicates stabilization of these regions due to FcRn binding to the full-length IgG and correlates nicely with a direct interaction between IgG and FcRn as determined by ETD analysis of peptides of this region (described later). In region 434–454, analysis of overlapping peptides showed protection of residues A439 and L440. The A439–L440 stretch lies in close proximity to H433 and H435, which have been implicated in the pH-dependent binding of IgG and FcRn (11, 12). To further sublocalize reductions in HDX upon FcRn binding, we also performed ETD analysis of peptides of this region (described later).

In addition to perturbed HDX kinetics in distinct regions of the Fc, reduced deuterium uptake could also be observed in several peptides in regions outside the Fc domain, including residues in region 1–23 in the V₃ domains, regions 153–182 and 188–205 in the C₃,1 domains, and light-chain peptides in region 55–83 in the V₅ domain. In region 1–23 of the V₅ framework region 1, analysis of HDX in overlapping peptides 5–18, 5–22, and 5–23 showed that no additional protection could be observed for the C-terminal end of this region (19–23). Comparison of peptide 1–18 with other overlapping pep-
tides (4–18, 5–18) could not be performed accurately, as the difference in back-exchange of the fully deuterated sample exceeded 15% (as detailed under “Experimental Procedures”). Thus the protection from HDX upon FcRn binding can be localized to residues 3–18, as the deuterium on the first two N-terminal residues in peptides is lost prior to detection in HDX-MS experiments (26, 43). Comparison of the HDX of peptides 57–79 is included as an example of an instance when FcRn had no significant effect on Ab wt (Ab wt in the presence of FcRn is indicated with a dashed black curve). The deuterium incorporation was monitored in triplicate at 1 min, 1 h, 2.5 h, and 5 h. The full deuterium level measured in control experiments (at 90% D2O) is shown in black at the 5 h time point. In the central schematic, the HDX-MS results are mapped onto a homology model of Ab wt. Regions with no significant change in deuterium uptake upon FcRn binding are shown in gray, and the N-linked glycans are shown in blue. Colored regions indicate heavy-chain (red) or light-chain (orange) peptides with reduced deuterium uptake upon FcRn binding.

Additionally, the same level of HDX protection was observed in HDX-MS experiments of Ab wt bound to either glycosylated or deglycosylated FcRn (supplemental Fig. S2), indicating that the single N-linked glycosylation of human FcRn did not contribute to IgG binding (supplemental Fig. S8). This was further substantiated by a comparison of IgG binding kinetics of glycosylated and deglycosylated FcRn via surface plasmon resonance, in which no difference in on/off rate behavior was observed (data not shown).

To gain further insights into the HDX of IgG when bound to FcRn, we performed a targeted HDX-ETD analysis of antibody peptides displaying a difference in deuterium uptake upon FcRn binding. We verified the absence of gas-phase perturbations of the deuterium label during ETD due to hydrogen/deuterium scrambling for the individual peptic peptides analyzed by monitoring the deuterium content of the reduced species ion and its deammoniated counterpart generated in each ETD mass spectrum (38). ETD fragment spectra of three peptides in region 249–260 (249–259, 249–260, 250–260) showed that the HDX protection could be sublocalized to amides of the C-terminal half. In peptide 249–260 (FLFPPK-
PKDTLM, 478.6, MH3) we measured the deuterium levels of a full c-ion series, except the c1 ion (Fig. 3). No differences in HDX were observed in the c2, c5, c7, c8, and c9 ions, whereas both the c10 and the c11 ion showed reduced deuterium uptake in the presence of FcRn. Thus, the ETD data resolve the reduction in deuterium uptake upon FcRn binding of ~0.7 D in peptide 249–260 to a site-specific protection from HDX at residue L259 (0.8 D). The residue L259 (L261) is located in a small α-helix-loop structure at the bottom of a hydrophobic pocket in the CH2 domain (Fig. 3E). The HDX-ETD data point to stabilization of the small α-helix containing L259 upon FcRn binding. Similar protection patterns were observed for all three peptides that were subjected to ETD in this region (data not shown). Additionally, ETD of peptide 441–454...

Fig. 3. Site-specific HDX analysis of peptide 249–260 of Abwt by ETD. A, ETD fragment ions of Abwt peptide 249–260 (FLFPPKPDKTLM, 478.6, MH3+) when undeuterated (bottom) and following 1 h of HDX in the absence (middle) and presence of FcRn (top). The average mass is depicted by dashed lines for Abwt (gray) and Abwt with FcRn (red), and the mass shift in the two states is indicated by arrows for the c11 and MH+ ions. B, chemical structure and fragment ions observed for peptide 249–260. Detected fragment ions are marked in bold italic letters. C, bar chart of the deuterium uptake in the c-ions of peptide 249–260 when unbound (gray) or bound to FcRn (red). Error bars show standard deviation of three replicate measurements. D, bar chart of the deuterium content in individual residues (red fill) and for peptide segments (white fill). Error bars show standard deviation of the difference of three replicate measurements. E, ETD data mapped onto the homology model of Abwt with peptide 249–260 indicated in red and residue L259 displayed in sticks.
(HNYTQKSLSPLPG, $523.9$, $MH^{+}$) showed that the majority of the sites protected from HDX were localized to H443–T445 (supplemental Fig. S9), correlating with a contribution of H443 in the pH-dependent interaction of IgG and FcRn. ETD was also performed on several other peptides, including peptides 164–182, 188–205, and 194–205; however, this did not allow further sublocalization. As previously observed (31, 32, 44), the ETD fragmentation efficiency was optimal for triply charged precursor ions, whereas less informative fragmentation was observed for doubly charged peptides (164–182, 188–205, and 194–205).

**HDX of Deglycosylated IgG1 Bound to FcRn**—To evaluate the effect of antibody deglycosylation on FcRn binding, we performed HDX-MS of the deglycosylated Ab variant in the absence and presence of FcRn. The HDX-MS analysis of $Ab_{degly}$ showed reduced deuterium uptake upon FcRn binding in the same regions as detected for the glycosylated antibody (Fig. 4, supplemental Fig. S10, and supplemental Fig. S11). However, differences in the level of HDX protection of the deglycosylated and glycosylated antibody upon FcRn binding were detected in two regions: (i) region 243–260 (illustrated in peptide 249–260), and (ii) the region containing the N-glycosylated residue N305 (N297, illustrated in peptide 286–308) (Fig. 4 and supplemental Fig. S11). As the FcRn-binding experiments of $Ab_{wt}$ and $Ab_{degly}$ were performed independently, the deuterium uptake in the antibodies was compared by determining the percentile deuterium uptake relative to the 90% control in each experiment. Thus, the relative deuterium content of peptide 249–260 at 1 h was $80\% \pm 4\%$ and $58\% \pm 1\%$ for $Ab_{wt}$ without and with FcRn, respectively, whereas for $Ab_{degly}$ it was $86\% \pm 2\%$ and $77\% \pm 1\%$ without and with FcRn, respectively. Evidently, FcRn binding reduced the HDX of the native glycosylated antibody by $22\% \pm 4\%$, whereas it was reduced by only $9\% \pm 2\%$ for the deglycosylated antibody. For glycopeptide 286–308, FcRn binding led to a maximum decrease in deuterium uptake of $13\% \pm 3\%$ (at 1 min) in $Ab_{degly}$ and only $3\% \pm 2\%$ (at 2.5 h) in $Ab_{wt}$. In addition to the HDX-MS experiments, we investigated the impact of antibody deglycosylation on FcRn binding via surface plasmon resonance and FcRn affinity chromatography (supplemental Fig. S12). A reduced level of FcRn binding was observed upon removal of the antibody glycosylation, reflected in an increased dissociation rate of $Ab_{degly}$ in comparison to $Ab_{wt}$ with dissociation half-lives of 13 s and 19 s, respectively. Likewise, the deglycosylated antibody showed less retention on the FcRn column when the antibodies were eluted by increasing the pH.

**HDX of FcRn Bound to IgG1**—To map regions on human FcRn involved in antibody binding, we performed HDX-MS on FcRn in the absence and presence of either $Ab_{wt}$ or $Ab_{degly}$. HDX-MS analysis was performed for 55 peptides spanning 71% and 97% of the FcRn $\alpha$-chain and $\beta_{\text{2m}}$, respectively (supplemental Figs. S13 and S14). We identified one N-glycosylation site (N102) on the human FcRn $\alpha$-chain that showed high diversity in the glycan structures, with the most intense glycans representing about 15% of the 10 different detected glycoforms (supplemental Fig. S2). No sequence coverage of this region was obtained during HDX-MS analysis, possibly because the combination of a high number of glycans and the diverse peptide population generated by pepsin resulted in a multiplicity of low-abundance peptic glycopeptides. Comparative HDX-MS analyses of unbound FcRn and IgG-bound FcRn revealed reduced deuterium uptake in several overlapping peptides in FcRn $\alpha$-chain regions 117–135 and 135–156 and $\beta_{\text{2m}}$ region 1–9 (Fig. 5). The reduced deuterium uptake in several overlapping $\alpha$-chain peptides from 117–135 (FMNFDLKKGTWFFWDPEARL) and $\beta_{\text{2m}}$ 1–9 (IQRTPKIQV) correlates nicely with prior crystallographic studies of sites on FcRn involved in binding (11–13, 45). Thus, the solution-phase HDX-MS data both confirm and extend such prior studies, as several overlapping peptides in region 135–156 adjacent to the consensus binding region described in the crystal structures (11–13, 45) are also protected from HDX upon antibody binding. No significant differences in HDX were observed between $Ab_{wt}$-bound FcRn and $Ab_{degly}$-bound FcRn (supplemental Fig. S14).

**DISCUSSION**

**Impact of Antibody Deglycosylation on Conformation and Dynamics**—The conserved antibody glycosylation in the Fc domain is important for the structural integrity of antibodies, and partial or full deglycosylation has been shown to influence conformation, storage stability, and solubility (46, 47). The impact of antibody glycosylations on HDX-MS was examined previously (40, 48), and in our study on $Ab_{wt}$ and $Ab_{degly}$ we observed effects upon antibody deglycosylation similar to those seen in prior studies performed on another IgG antibody. We observed increased deuterium uptake distant in sequence (243–260) to the N305 (N297) glycosylation site, which emphasizes the influence of the glycostructures on the overall conformation in the $C_{\text{2}}$ domain in the Fc region. The HDX increase indicates that this region becomes more flexible or accessible upon deglycosylation and could account for the instability associated with antibody deglycosylation. Interestingly, deglycosylation resulted in decreased HDX in the glycopeptide (286–308). This might indicate that the glycan maintains the glycopeptide in a more exchange-competent conformation. However, direct comparison of the deuterium uptake of glycopeptides and the corresponding deglycosylated variants is complicated by the fact that the presence of glycosylations might affect the intrinsic exchange rates of the neighboring amide hydrogens, as is observed for amino acid side chains (43). Furthermore, the acetamido groups of the N-glycan HexNAc moieties and the modified asparagine side chain can retain deuterium during an HDX-MS experiment (49). No difference in deuterium uptake was seen for the three glycopeptides G0F, G1F, and G2F, indicating that the addition of either one or two terminal gal-
actoses in G1F and G2F does not impact local IgG confor-
mation at the site of glycosylation.

The Interaction of Full-length IgG₁ and Human FcRn—In
this study we probed the conformation and dynamics of both
binding partners in a complex of a full-length IgG₁ and human
FcRn by HDX-MS. Our results show that FcRn binding to IgG
led to HDX protection of backbone amide hydrogens in sev-
eral regions in the Fc region. Specifically, with HDX-ETD we
confirmed the involvement of residue L259 in FcRn interaction
for wild-type IgG, which was also implicated in FcRn binding
in the crystal structure of the complex of FcRn and the Fc-
engineered Fc-YTE variant. Furthermore, protection from HDX
was detected in the Fab regions of the heavy and light chains.

Notably, the reduced HDX observed upon FcRn binding in

![HDX-MS raw data of peptide 249–260 showing differential HDX between the FcRn-bound states of Abwt and Abdegly.](image)

Fig. 4. Comparison of the HDX of Abwt and Abdegly in the absence and presence of FcRn. A, HDX-MS raw data of peptide 249–260 showing differential HDX between the FcRn-bound states of Abwt and Abdegly. B, left-hand and middle panels show HDX plots of Abwt peptide 249–260 (FLFPPPKDTLM) in the absence (gray) and presence (red) of FcRn and Abdegly in the absence (black) and presence (blue) of FcRn. The full deuterium level measured in control experiments (at 90% D₂O) is shown in black at the 5 h time point. The deuterium incorporation was monitored in triplicate at 1 min, 1 h, 2.5 h, and 5 h. The left-hand panel shows a bar chart of the relative deuterium incorporation as a percentage (relative to the 90% control) for peptide 249–260 at the 1 h HDX time point. C, HDX plots and bar chart of peptide 286–308 (YVDGEVHNAKTDPREEQYN/DSTY) from Abwt (G0F glycoform) and Abdegly, using the same coloring as in B.
regions remote from the Fc—namely, in the VH domain (3–18), the CH1 domain (169–182, 190–205), and VL domain (57–71)—could represent either hitherto unknown direct FcRn interaction sites or a conformational link between these regions and the FcRn binding region in the CH2 and CH3 domains of the Fc. The interaction between FcRn and antibodies has commonly been assumed to involve only Fc residues, but studies of several antibodies in recent years suggest that the antibody Fab domains might contribute to FcRn binding (34, 50, 51). The molecular origins of such Fab contributions to FcRn binding have, however, remained elusive. Previously, the IgG Fab regions were implicated in the interaction with other Fc binding proteins such as FcγRIIa (48) and Staphylococcus aureus Protein A (52–54). In particular, an earlier HDX-MS study on IgG glycovariants and FcγRIIa showed reduced deuterium uptake in peptides located in the CH1 domain as either a direct or an indirect consequence of receptor binding (48). Our solution-phase HDX measurements demonstrate that several regions in the variable and constant domains in the Fab of a full-length IgG undergo protection from HDX upon FcRn binding. The effects in the Abwt Fab upon FcRn binding were reproducible and were observed in replicate HDX-MS experiments (n = 3). This result provides strong experimental evidence of a conformational origin in line with earlier biochemical studies that indicate a role of IgG Fab in FcRn binding (34, 50, 51). The sequence of the antibody used in this study (Abwt) has standard human constant regions (hIgHG1 and light chain), and a comparison of V region sequences of Abwt with the closest human germlines showed that the V and J elements has 90% identity with the closest human germline (data not shown). To assess whether Abwt has unusual FcRn binding characteristics, we performed FcRn affinity chromatography analysis (34, 39) of Abwt and another IgG1 antibody, ustekinumab. Ustekinumab has typical FcRn binding properties and in vivo half-life (55).2 Importantly, both molecules showed similar FcRn binding characteristics with very similar elution profiles (supplemental Fig. S7). Finally, we also performed an HDX-MS analysis of the interaction between ustekinumab and FcRn and observed a comparable level of protection from HDX upon FcRn binding in the Fab (supplemental Fig. S7) and Fc region (data not shown) between Abwt and ustekinumab. Thus, through comparison to ustekinumab, we demonstrate that the mechanism for FcRn binding and the presence of changes in HDX in the Fab upon FcRn binding are not unique to the Abwt antibody and were also observed in an unrelated IgG1. The accumulated evidence listed above demonstrates a conformational role for the Fab in FcRn binding and strongly indicates that this phenomenon represents a general feature of interactions between IgG1 and FcRn. Whether our findings on two IgG1 variants are also pertinent to the FcRn binding mode of other

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2 A. Schoch, H. Kettenberger, G. Winter, J. Engert, J. Heinrich, and T. Emrich, submitted for publication.
HDX-MS Analysis of the IgG1–FcRn Complex

**Fig. 6.** Homology model of a complex between human FcRn and a full-length IgG. The model was generated via superposition of a homology model of Abwt (IgG1) with the crystal structure of a human Fc–FcRn complex (PDB: 4n0u). The IgG heavy chains and FcRn are shown in gray, the IgG light chains are in white, and the N-linked glycosylations are in blue. The FcRn N-linked glycosylation site Asn102 is shown in black. Colored regions indicate regions in IgG heavy chain (red), light chain (orange), FcRn α-chain (green), and FcRn βm (yellow) that displayed reduced deuterium uptake upon complex formation.

IgG subclasses (IgG2–IgG4) remains to be shown. The HDX-MS detection of conformational stabilization in distinct regions in the Fab upon FcRn binding can be reconciled by either a direct or an indirect structural mechanism. Antibody molecules are inherently very flexible in terms of the orientations of the Fabs and Fc, and studies of antigen–antibody complexes (57, 58) suggest that the Fabs and Fc can be conformationally linked. However, existing crystal structures of human or rat FcRn bound to Fc fragments do not provide information on the role of the Fab region in receptor binding (11–13). We have therefore generated a three-dimensional homology model of the full-length IgG1 in complex with FcRn (Fig. 6). Molecular dynamics simulation based on the homology model supports the feasibility of transient contacts between the Fab and FcRn (data not shown) at the regions protected from HDX. Thus, based on (a) experimental and *in silico* data generated in this study on the human full-length IgG–FcRn complex, (b) a consideration of the relatively close spatial arrangement of FcRn and Fabs, and (c) the inherent flexibility of the Fabs, a direct transient interaction between the Fabs and FcRn appears plausible. Notably, the interaction of FcRn with the IgG Fab region during complex formation does not appear to be the major contributor to the binding strength of full-length antibodies and FcRn, as only minor/no retention of Fab fragments is observed via analytical FcRn affinity chromatography (34). In the future it would be desirable to obtain crystal structures of full-length IgG bound to FcRn in order to substantiate the structural role of the Fab domain in interactions with FcRn.

Considering the IgG–FcRn interaction from the FcRn point of view, the HDX-MS analysis of FcRn upon antibody binding revealed protection of regions in the α2 domain and a weak protection of the βm N-terminal peptides. The α2 peptides in 117–135 contain several residues (F117, G129, W131, T132, E133) that are known to directly interact with IgG residues in the Cγ2 and Cγ3 regions (13). In the FcRn–Fc-YTE crystal structure, the residue Y88 is also involved in the interaction. No differences in deuterium uptake were observed in the peptide covering Y88 (79–97) in HDX-MS analyses, correlating with the fact that Y88 interacts with residue T254, which is unique to the engineered Fc-YTE variant (S254T) (13). βm is known to interact with the IgG via I1 (12), which cannot be measured via HDX-MS, as the deuterium on the first two N-terminal residues in peptides is lost prior to MS detection (26, 43). However, we observed weak protection in the N-terminal peptides of βm, which indicates that residues 3–9 are directly or indirectly protected from HDX when an antibody binds FcRn. Furthermore, we observed protection in several overlapping peptides in region 135–156 of the FcRn α-chain, which has not been implicated as a part of the Fc binding interface in the available crystal structures (11–13). The reduced deuterium uptake could be caused by direct stabilization upon IgG binding or an indirect stabilizing effect due to a binding event in adjacent region 117–135. A direct interaction site for the IgG Fab in this region does not seem plausible, as it is facing toward the Cγ3 domain and the direction opposite the Fab arms (Fig. 6). Alternatively, the regions in the α2 domain could be implicated in the FcRn dimerization that has been observed in rodent, but not human, FcRn–Fc crystal structures (9–11). However, dimerization of rodent FcRn was mediated by residues in the α2 domain and βm that did not show reduced HDX in human FcRn upon antibody binding in our current solution-phase studies.

**Impact of Antibody Deglycosylation on FcRn Interaction—** Our comparative HDX-MS analysis of Abwt and Abdegly showed that antibody glycosylations on N305 (N297) in the Fc region influenced the conformation and dynamics in parts of the FcRn binding interface (243–260) and possibly in the glycopeptide (286–308). Further, HDX-MS analysis of FcRn binding to either Abdegly or Abwt demonstrated that the FcRn–IgG interaction is sensitive to antibody deglycosylation. The deglycosylated peptide 286–308 was significantly protected upon FcRn binding relative to the glycosylated variant. This could be the result of an alternative binding mode or destructuring in this region upon deglycosylation that might render this region more sensitive to indirect stabilization upon FcRn binding (Fig. 4, *supplemental Fig. S4*). In contrast, the reduction in HDX in region 243–260 due to FcRn binding was lower for Abdegly than for Abwt, indicating that FcRn binding did not induce the same pronounced level of stabilization in the deglycosylated variant as in the wild type. This correlates with surface plasmon resonance and analytical FcRn affinity chromatography data showing a small decrease in FcRn binding upon removal of the glycans (*supplemental Fig. S12*). Notably, the difference in FcRn-induced protection of native versus
deglycosylated IgG was not simply caused by decreased occupancy of the binding site of FcRn on IgG, as the current HDX-MS analyses of the IgG–FcRn interaction were performed using an excess of FcRn (~7-fold). The effect of antibody deglycosylation is even more pronounced in FcγRIIia binding (48), where removal of the glycans almost completely disrupts receptor binding. FcγRIIia binding and changes in glycosylation have previously been shown to influence the conformation and dynamics of region 243–260. Our results suggest that this region is similarly implicated in differential FcRn binding upon deglycosylation. Thus, alternative IgG glycoforms could also affect FcRn binding, although this could not be resolved in the current experiments because a mixture of N-linked glycans was present on AbmH (supplemental Fig. S1). Furthermore, the 243–260 region is highly susceptible to conformational rearrangement upon antibody oxidation (M252, M428). Earlier HDX-MS studies of oxidized IgG variants showed increased deuterium uptake in region 249–260 relative to the native IgG (48, 59), similar to our data on the deglycosylated variant. This increase in structural flexibility or accessibility in region 249–260 of the oxidized variant could provide a rationale for the reduced FcRn binding affinity and in vivo half-life observed for oxidized antibodies (20–22). It appears from the current and earlier work that region 243–260 comprises a conformational environment that is highly sensitive to perturbations to the Fc region, including receptor binding (FcγRIIia and FcRn) and post-translational modifications (glycosylation and oxidation). We note that the conformational changes observed upon antibody deglycosylation do not seem to translate to changes in in vivo pharmacokinetics, as it has been shown that a deglycosylated antibody has unchanged pharmacokinetics relative to the wild-type IgG in cynomolgus monkeys (56, 60). The present HDX-MS results therefore suggest that IgG deglycosylation perturbs the conformation of local regions of the Fc in a manner that attenuates the conformational response of the IgG to FcRn binding, but due to the 1:2 binding avidity of the IgG–FcRn interaction, no net difference in the in vivo IgG clearance rate appears to occur. No such avidity effects should occur with the FcγRIIia interaction, where only one receptor binds to the IgG.

In conclusion, this study provides new molecular insights into the IgG–FcRn interaction and specifically links IgG sites in both Fc and Fab regions to FcRn binding. Furthermore, we show that IgG deglycosylation limited the conformational impact of FcRn binding in the Fc region of an IgG. From an analytical perspective, our results demonstrate the capability of HDX-MS to detect and localize changes in conformation to short stretches or even single amino acids using ETD in full-length IgG in response to FcRn binding. Thus, in the further pursuit of novel antibody variants with altered FcRn binding characteristics, HDX-MS appears to be a sensitive and useful approach for monitoring changes in the intricate conformational dynamics that govern IgG–FcRn complex formation.

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