Interaction with Both Domain I and III of Albumin Is Required for Optimal pH-dependent Binding to the Neonatal Fc Receptor (FcRn)*

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Background: FcRn regulates the long serum half-life of albumin.
Results: The C-terminal DIII of HSA is the principal domain for FcRn binding, whereas two loops in DI at the N terminus modulate the interaction.
Conclusion: DI of albumin contributes to optimal FcRn binding.
Significance: We highlight the importance of DI for pH-dependent binding to FcRn.

Albumin is an abundant blood protein that acts as a transporter of a plethora of small molecules like fatty acids, hormones, toxins, and drugs. In addition, it has an unusual serum half-life of humans in nearly 3 weeks, which is attributed to its interaction with the neonatal Fc receptor (FcRn). FcRn protects albumin from intracellular degradation via a pH-dependent cellular recycling mechanism. To understand how FcRn impacts the role of albumin as a distributor, it is important to unravel the structural mechanism that determines pH-dependent binding. Here, we show that although the C-terminal domain III (DIII) of human serum albumin (HSA) contains the principal binding site, the N-terminal domain I (DI) is important for optimal FcRn binding. Specifically, structural inspection of human FcRn (hFcRn) in complex with HSA revealed that two exposed loops of DI were in proximity with the receptor. To investigate to what extent these contacts affected hFcRn binding, we targeted selected amino acid residues of the loops by mutagenesis. Screening by in vitro interaction assays revealed that several of the engineered HSA variants showed decreased binding to hFcRn, which was also the case for two missense variants with mutations within these loops. In addition, four of the variants showed improved binding. Our findings demonstrate that both DI and DIII are required for optimal binding to FcRn, which has implications for our understanding of the FcRn-albumin relationship and how albumin acts as a distributor. Such knowledge may inspire development of novel HSA-based diagnostics and therapeutics.

Albumin is synthesized by hepatocytes in the liver, which secrete an impressive 13–14 g of albumin into the blood every day. In the blood albumin plays a pivotal role as a transporter of a range of small insoluble molecules such as fatty acids, metal ions, hormones, heme, and bilirubin in addition to being a carrier of small chemical drugs (1, 2). Albumin (molecular mass 66.5 kDa) is built up of three homologous domains (DI, DII, and DIII), where each is composed of two subdomains with distinct helical folding patterns connected by flexible loops (see Fig. 1A). Furthermore, albumin has a serum half-life of nearly 3 weeks in humans. This feature is of great interest to the pharmaceutical industry, as the exceptional half-life can be utilized to extend the serum persistence of therapeutics that are chemically or genetically linked to albumin (3–5). Examples are biopharmaceuticals such as cytokines, hormones, and antibody fragments. Also small albumin binding scaffolds, peptides, or fatty acids fused or conjugated directly to a drug of interest, which target or associate with albumin when injected into the bloodstream, are used to improve pharmacokinetics (3–6).

The long serum half-life is a feature that albumin shares with the IgG class of antibodies (150 kDa), whereas other serum proteins have a half-life of hours or a few days. This is solely due to their molecular size above the renal clearance threshold and binding to a cellular receptor named the neonatal Fc receptor.
Albumin DI Modulates Binding to FcRn

(FcRn)\(^8\) (7–9). Mice deficient in FcRn expression were shown to have 4–5- and 2–3-fold lower serum levels than normal mice of IgG and albumin, respectively (7, 8). A genetic link is also found in humans, as the rare disease, familial hypercatabolic hypopro- teinemia, is characterized by abnormally low levels of IgG and albumin, which correlate with a lack of FcRn expression (10). Thus, FcRn is a regulator of the serum levels and circulatory half-lives of these structurally and functionally unrelated proteins. The finding is of particular importance for our understanding of how albumin and its cargo is distributed throughout the body and how albumin can be engineered and utilized for diagnostic or therapeutic applications.

FcRn belongs to a family of major histocompatibility complex class I-related molecules and consists of a unique transmembrane heavy chain that is non-covalently associated with the common soluble β2-microglobulin (11, 12). The two ligands bind simultaneously and in a non-cooperative manner to structurally separate binding sites on FcRn (13–15). A hallmark of both interactions is the strict pH dependence, binding at acidic pH and no binding or release at physiological pH (7, 14, 16–18). This is mediated by histidine residues that become positively charged at acidic pH and are located to the elbow region of the IgG Fc, to DIII of albumin, and to the receptor itself (14, 17–19).

Long serum half-life is maintained via a cellular recycling mechanism that relies on the pH-dependent binding. FcRn resides predominantly within intracellular endosomes, into which IgG and albumin enter after pinocytosis and where they bind FcRn as a consequence of the low pH. The ternary complex is recycled to the cell surface where exposure to the physiological pH of the blood triggers release. Thus, FcRn rescues both its ligands from intracellular degradation, whereas proteins that do not bind the receptor are directed to lysosomes. This recycling pathway has been shown to take place in hematopoietic cells and endothelial cells lining the vascular space (8, 20, 21).

Engineering of the interactions between FcRn and its ligands has resulted in altered binding properties that favor efficient recycling and thereby extended half-life. A number of human IgG molecules with increased binding affinity at acidic pH, but not physiological pH, have been identified that show extended half-life and improved therapeutic efficacy (22–27). Regarding albumin, recent insights into the mechanism of the FcRn-albumin interaction have resulted in publication of the first examples of engineered HSA mutant variants with altered pH-dependent binding to hFcRn, which translates into decreased or increased serum half-life in both mice and rhesus monkeys (18, 28).

Furthermore, in regard to conjugation or genetic fusion of biopharmaceuticals to HSA, it is important to control how FcRn binding is affected. Importantly, we recently showed that fusion of a short peptide or an antibody-derived single-chain FcRn binding is affected. Importantly, we recently showed that recombinant form of DIII bound hFcRn but with reduced affinity compared with full-length HSA (16, 18). Another interesting finding was that a recombinant form of DIII bound hFcRn but with reduced affinity compared with full-length HSA (16, 30). Furthermore, based on a large set of mutagenesis studies, we constructed a docking model of the FcRn-HSA complex using the available crystallographic data of hFcRn and wild type (WT) HSA (16). The best model suggested that the N-terminal DI may also make direct contacts with hFcRn, which was also confirmed by two recently published co-crystal structures of hFcRn in complex with a WT HSA molecule or a HSA mutant containing four amino acid substitutions (HSA13) that binds the receptor but with reduced pH discrimination (18, 31). Notably, the co-crystal structures show a fit that differs somewhat from the docking model due to changes in conformation and orientation of the three HSA subdomains upon binding to FcRn, including alterations within DIII whereby DIIIB rotates relative to DIIIA (18, 31).

In the present study we have investigated how DI of HSA impacts binding to hFcRn. Stretches of amino acid residues in two exposed loops within DI were targeted by mutagenesis, and the mutant HSA variants were expressed and compared with WT HSA with respect to binding to hFcRn using in vitro binding assays. A total of 12 amino acid residues were replaced with alanine residues. Four mutations negatively affected binding to hFcRn, whereas four improved binding. Two of the mutants shown to bind hFcRn less well are naturally existing missense HSA variants. Thus, we demonstrate that not only DIII but also DI of albumin is important for optimal pH-dependent binding to FcRn, and we pinpoint amino acid residues within two exposed loops of DI that are important for receptor binding. Our data have implications for our understanding on how FcRn regulates the long serum half-life of albumin and transports its cargo throughout the body, and such knowledge will translate into design of novel albumin-based therapeutics with improved properties.

EXPERIMENTAL PROCEDURES

Construction and Production of Recombinant FcRn Molecules—The construction of a eukaryotic pcDNA3 vector encoding a recombinant truncated form of WT hFcRn, which contains the cDNA encoding the three extracellular domains (α1–α3) C-terminally fused to a cDNA encoding glutathione S-transferase (GST) from Schistosoma japonicum, has been described (32, 33). The vector also contains a cDNA encoding human β2-microglobulin and the Epstein-Barr virus origin of replication sequence. GST-tagged hFcRn was produced by transient transfection of human embryonic kidney 293E (HEK293E) cells using polyethyleneimine Max (Polysciences), and the receptor was purified from harvested supernatant using a GSTrap FF column as described (32, 33).

Monomeric His-tagged hFcRn was produced using a BaculoVirus expression vector system (34). A viral stock encoding

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\(^8\)The abbreviations used are: FcRn, neonatal Fc receptor; hFcRn, human FcRn; HSA, human serum albumin; DI, DII, DIII, domains I, II, and III; SPR, surface plasmon resonance; RU, resonance units.
His-tagged hFcRn was a kind gift from Dr. Sally Ward (University of Texas, Southwestern Medical Center, Dallas, TX). Briefly, hFcRn was purified using a HisTrap HP column supplied with Ni\(^2+\) ions (GE Healthcare). The column was pre-equilibrated with \(1 \times \text{PBS with 0.05\% sodium azide, and the pH of the supernatant was adjusted with } 1 \times \text{PBS, 0.05\% sodium azide (pH 10.9)} \) to pH 7.2 before being applied to the HisTrap HP column with a flow rate of 5 ml/min. The column was washed using 200 ml of \(1 \times \text{PBS followed by 50 ml of 25 mM imidazole, } 1 \times \text{PBS (pH 7.3), and hFcRn was eluted with 250 mM imidazole, } 1 \times \text{PBS (pH 7.4). The collected protein was buffer-exchanged to } 1 \times \text{PBS using Amicon Ultra-10 filter units (Millipore) followed by isolation of the monomeric fraction before the protein was concentrated using Amicon Ultra columns (Millipore) and stored at 4°C.}

Construction and Production of HSA Variants—Recombinant WT HSA, HSA-K500A, and the fragments DI-DII, DIII, and DIII-K500A were constructed, produced in yeast, and purified essentially as previously described (16).

GST-tagged HSA variants were constructed by subcloning of cDNA fragments (GenScript) into a pCDNA3 vector that contains a cDNA encoding WT HSA in-frame of a cDNA segment encoding GST (30). cDNA fragments encoding DI substitutions (R81A, E82A, E82K, T83A, Y84A, D108A, N109A, P110A, N111A, L112A, P113A, and R114G) were ordered from GenScript and subcloned into the same vector. Vectors were transiently transfected into human embryonic kidney 293E cells using polyethyleneimine Max (Polysciences), and the HSA variants were purified from harvested supernatant using a GSTrap protein was concentrated using Amicon Ultra columns (Millipore) and stored at 4°C.

ELISA—Microtiter wells (Nunc) were coated with 100 μl of non-fused WT HSA (100 μg/ml) and incubated overnight at 4°C. Then the wells were washed 3 times with PBS, 0.005% Tween 20 (PBS/T) (pH 6.0) before blocking with 4% skimmed milk (Acumedia) for 1 h at room temperature. After washing as above, GST-tagged hFcRn (0.5 μg/ml) were diluted in PBS/T (pH 6.0) with 4% skimmed milk alone or in the presence of titrated amounts of WT HSA, HSA-K500A (16), DIII, or DI-DII (16). After incubation for 1 h at room temperature the wells were washed as above. Bound receptor was detected using a horseradish peroxidase-conjugated goat anti-GST antibody (GE Healthcare) that was added for 1 h at room temperature followed by washing with PBS/T (pH 6.0). Visualization was done using tetramethylbenzidine substrate (Calbiochem), and the absorbance was measured at 450 nm after adding 100 μl of 1 M HCl using the Sunrise spectrophotometer (TECAN).

Screening of GST-fused HSA variants were carried out by coating a human IgG1 mutant variant (M252Y/S254T/T256E/H433K/N434F) with specificity for 4-hydroxy-3-ido-5-nitrophenylacetic acid (10 μg/ml) in microtiter wells (Nunc). The plates were incubated overnight at 4°C before the wells were blocked with PBS, 4% skimmed milk for 1 h at room temperature followed by washing 4 times in PBS/T (pH 6.0). A constant amount of His-tagged hFcRn (20 μg/ml) was diluted in PBS/T, 4% skimmed milk (pH 6.0), added to the wells, and incubated for 2 h at room temperature before the wells were washed as above. Subsequently, 5 μg/ml GST-tagged WT HSA and the mutants were diluted in PBS/T, 4% skimmed milk (pH 6.0) and added to the wells for 2 h at room temperature. After washing as above, a horseradish peroxidase-conjugated anti-GST antibody (GE Healthcare) diluted (1:3000) in PBS/T, 4% skimmed milk (pH 6.0), was then added and incubated for 1 h. After washing, bound HSA variants were detected using tetramethylbenzidine substrate (Calbiochem). The absorbance was measured at 620 nm using the Sunrise spectrophotometer (TECAN).

SPR—SPR analyses were performed on a BIACore 3000 instrument (GE Healthcare), and CM5 chips (GE Healthcare) were immobilized with GST-tagged hFcRn or HSA variants. The proteins (2 μg/ml) were injected in 10 mM sodium acetate buffer (pH 5.0) (GE Healthcare), and unreacted moieties on the CM5 surface were blocked with 1 M ethanolamine following the manufacturer’s procedure. For all experiments, phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl, 0.005% Tween 20) at pH 6.0 or 7.4 was used as running buffer or dilution buffer. Kinetic measurements were performed by injecting serial dilutions of WT HSA (1.0 μM to 0.03 μM) or a recombinant form of DIII HSA (200.0 μM to 0.3 μM) (16) over immobilized hFcRn (1000 RU). Alternatively, soluble monomeric hFcRn (1.0 μM to 0.03 μM) was injected over immobilized GST-fused HSA variants (500 relative units) at pH 6.0. For all experiments, buffer pH 6.0 was used with a flow rate of 50 μl/min at 25°C. The flow cells were regenerated using PBS/T (pH 7.4). Nonspecific binding and bulk buffer effects were corrected by subtracting responses obtained from the control CM5 surfaces and blank injections. Kinetic rate constants were estimated using a simple Langmuir 1:1 ligand binding model or a steady state affinity model provided by the BIAevaluation 4.1 software. The closeness of the fit, described by the statistical value χ2, which represents the mean square, was lower than 4.0 in all affinity estimations.

Stability Analysis of HSA Variants in Human Serum—Before use in experiments, human serum was depleted for IgG and albumin. Briefly, IgG was removed using a HiTrap™ HP column supplied with Protein G-Sepharose (GE Healthcare). The column was pre-equilibrated with 20 mM sodium phosphate buffer, 0.15 M NaCl, 0.005% Tween 20) at pH 6.0 or 7.4. After incubation for 1 h at room temperature the wells were washed as above. Bound receptor was detected using a horseradish peroxidase-conjugated goat anti-GST antibody (GE Healthcare) that was added for 1 h at room temperature followed by washing with PBS/T (pH 6.0). Visualization was done using tetramethylbenzidine substrate (Calbiochem), and the absorbance was measured at 450 nm after adding 100 μl of 1 M HCl using the Sunrise spectrophotometer (TECAN).

Circular dichroism (CD) spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco International) calibrated with ammonium d-camphor-10-sulfonate (Ictayama Chemicals). Measurements were performed with
RESULTS

HSA-GST variants (0.15 mg/ml) in 10 mM PBS (pH 6.0) without NaCl added at 25 °C, 50 °C, and 70 °C using a quartz cuvette (Starna) with a path length of 0.1 cm. Each sample was scanned 3 times at 50 nm/min (bandwidth of 1 nm, response time of 4 s) with the wavelength range set to 190–260 nm. The data were averaged, and the spectrum of a sample-free control was subtracted. Secondary structural elements were calculated using the neural network program CDNN Version 2.1 and the supplied neural network based on the 33-member basis set (35).

Structural Analysis—Coordinates of the crystal structures of hFcRn in complex with WT HSA (PDB ID 4N0F) (31) or a HSA variant (HSA13) containing four mutations (PDB ID 4K71) (18) were retrieved from the Protein Data Bank. The structures were inspected using PyMOL (Schrodinger Inc.).

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RESULTS

DIII Competes More Weakly than Full-length HSA for Binding to hFcRn—Albumin has a heart-shaped structure that is made up of three subdomains; DI, DII, and DIII (Fig. 1A). We have previously demonstrated that the C-terminal DIII is the principal domain that is engaged in pH-dependent binding to hFcRn (16, 30). Here we used a competitive ELISA to investigate how recombinant forms of HSA DIII and DI-DII competed for binding to hFcRn compared with full-length HSA. The results showed that nearly 20-fold more DIII than complete HSA was needed to give 50% reduction in hFcRn binding. However, DIII competed more efficiently than HSA K500A, a full-length variant with a mutation within DIII that causes a 30-fold reduction in hFcRn affinity (16). The DI-DII construct inhibited binding only at concentrations above 100 μM (Fig. 1B).

Next, we used SPR and injected equimolar amounts of WT HSA, DIII, and DIII containing the K500A substitution over immobilized hFcRn at pH 6.0. The resulting sensograms showed that DIII has a much faster kinetic profile than complete HSA was needed to give 50% reduction in hFcRn binding. However, DIII competed more efficiently than HSA K500A, a full-length variant with a mutation within DIII that causes a 30-fold reduction in hFcRn affinity (16). The DI-DII construct inhibited binding only at concentrations above 100 μM (Fig. 1B).

We then used an ELISA where serial dilutions of the HSA variants were added to hFcRn captured on a human IgG1 variant engineered to bind hFcRn strongly. Binding of WT HSA and the mutant variants was detected using an HRP-conjugated anti-GST antibody, and the results obtained showed that WT HSA and the mutant variants bound hFcRn at pH 6.0 (Fig. 3, B and C), which is in agreement with the fact that both ligands can bind FcRn simultaneously (13, 14,17). For the loop I variants, R81A, E82A, and Y84A gave rise to reduced binding responses, with the most negative effect detected for R81A, whereas T83A showed slightly enhanced binding compared with the WT (Fig. 3B). Furthermore, screening of the loop II variants showed that mutation of Asp-108 had the greatest negative effect followed by P110A, whereas T83A showed slightly enhanced binding compared with the WT (Fig. 3B).
SPR-based Determination of Kinetics—To determine the binding kinetics, we used SPR where titrated amounts of monomeric hFcRn were injected over immobilized HSA variants at pH 6.0. The obtained sensorgrams fitted well to a 1:1 Langmuir binding model where the estimated kinetic constants showed that the loop I mutant variants E82A, T83A, and Y84A had no major impact on the binding kinetics, whereas R81A gave a nearly 2-fold reduced affinity compared with WT HSA (Fig. 4, Table 1). Of the loop II variants, D108A showed the largest negative impact, with a 4-fold reduced binding affinity, whereas P110A did not alter the kinetics (Fig. 4, Table 1). Interestingly, alanine substitution at positions Asn-109, Asn-111, Leu-112, and Pro-113 improved binding by 1.5–2.4-fold, where L112A gave the most pronounced effect (Fig. 4, Table 1). The kinetic data were in agreement with the trend of binding observed in ELISA with the exception of P113A that showed reduced binding in ELISA compared with the WT (Fig. 3C; data not shown). Using SPR, their binding kinetics were determined, and both showed an increase in $K_D$; E82K 2-fold (Fig. 5C, Table 1) and R114G 1.8-fold (Fig. 5D, Table 1).

Protein Structure and Stability Analysis—To verify whether the introduced point mutations affected secondary structure and stability of the HSA-GST variants, we performed CD analysis of the WT and four of the mutants for which binding kinetics differed the most from the WT; D108A and E82K (decreased FcRn affinity) and L112A and P113A (improved FcRn affinity). CD was performed at 25 °C, 50 °C, and 70 °C, and we found the dominating structural element for all variants to be α-helix,
FIGURE 4. SPR binding kinetics of HSA DI variants to hFcRn. Representative SPR sensorgrams show hFcRn binding of WT HSA (A), R81A (B), E82A (C), T83A (D), Y84A (E), D108A (F), N109A (G), P110A (H), N111A (I), L112A (J), and P113A (K) at pH 6.0. Injections were performed at 25 °C, and the flow rate was 50 μL/min. RU, resonance units.
To explain our binding data, we inspected the co-crystal structure of the WT HSA-hFcRn pair (Fig. 7, A–D). For the stretch of amino acids corresponding to loop I, we found that Arg-81 forms an intramolecular salt bridge with Asp89 (Fig. 7B). Disruption of the intramolecular interaction by mutation to alanine could possibly affect the positioning of the loop. Additionally Arg-81 forms hydrogen bonds with Thr-153 of hFcRn (Fig. 7B). Thus, the weaker binding detected for R81A is likely due to a combination of these two factors. The side chain of Glu-82 is not involved in any direct interactions, although the backbone carboxyl group forms a weak hydrogen bond with His-161 of hFcRn. Furthermore, mutations affecting the position of loop I have the potential to affect the intermolecular salt bridge between Glu-86 in HSA and Lys-150 in FcRn. For the loop II mutant variants, substitution of Asp-108 with an alanine had the largest negative effect. As for Glu-82, the Asp-108 side chain does not form any direct interaction with FcRn but instead forms intramolecular hydrogen bonds with His-105 and Lys-466 within DI and DIII of HSA, respectively (Fig. 7C). The removal of these interactions may destabilize the intramolecular interaction between the HSA domains and affect the backbone confirmation, disrupting the hydrogen bond between the backbone carboxyl of Asp-108 and the Lys-63 side chain of FcRn.

To further investigate their stability, the HSA-GST variants were incubated in human serum for 0 h, 12 h, 24 h, and 48 h at 37 °C, and subsequently the effect on FcRn binding was examined in ELISA. All variants showed reduced binding to hFcRn as a function of incubation time (Fig. 6D). After 48 h 40% reduction was observed for WT HSA, whereas three of the mutants (E82K, L112A, and P113A) showed only 20–25% reduced binding to hFcRn. The D108A mutant gave rise to as much as 60% loss of activity after 48 h, which may indicate that this mutation has the most pronounced negative impact on protein stability.

\[ \text{HSA}^{\alpha} \]
\[ \begin{array}{cccc}
\text{HSA-GST}^{\beta} & \\
\text{WT} & 4.3 \pm 0.1 & 5.4 \pm 0.1 & 125.6 & 1.9 \\
\text{R81A} & 3.9 \pm 0.1 & 9.3 \pm 0.2 & 238.5 & 2.1 \\
\text{E82K} & 4.7 \pm 0.2 & 6.4 \pm 0.2 & 136.2 & 1.9 \\
\text{ERK Vibo Valentia} & 3.3 \pm 0.1 & 11.1 \pm 0.1 & 333.3 & 3.1 \\
\text{T83A} & 4.5 \pm 0.0 & 6.5 \pm 0.1 & 144.4 & 1.8 \\
\text{Y84A} & 3.5 \pm 0.1 & 4.0 \pm 0.1 & 114.3 & 2.1 \\
\text{D108A} & 3.3 \pm 0.1 & 20.5 \pm 0.5 & 621.2 & 1.0 \\
\text{N109A} & 5.2 \pm 0.2 & 5.0 \pm 0.1 & 96.2 & 2.9 \\
\text{P110A} & 4.4 \pm 0.1 & 6.5 \pm 0.2 & 147.0 & 2.1 \\
\text{N111A} & 5.9 \pm 0.2 & 4.9 \pm 0.1 & 83.1 & 2.8 \\
\text{L112A} & 5.0 \pm 0.2 & 2.5 \pm 0.1 & 50.0 & 2.4 \\
\text{P113A} & 6.6 \pm 0.3 & 0.4 \pm 0.0 & 60.1 & 3.5 \\
\text{R114G Yanomama-2} & 5.3 \pm 0.1 & 1.4 \pm 0.2 & 264.1 & 3.1 \\
\end{array} \]

\(^{\alpha}\) The HSA variants were immobilized (~500 RU) on chips, and serial dilutions of hFcRn were injected.

\(^{\beta}\) The kinetic rate constants were obtained using a simple first-order (1:1) bimolecular interaction model. The kinetic values represent the average of triplicates.

\(^{c}\) Values resulting from curve fitting using the first-order (1:1) bimolecular interaction model. \(\chi^2\) is a measure of the average squared residual (the difference between the experimental data and the fitted curve).

**Table 1**

**Binding kinetics of HSA DI mutants toward hFcRn**

- **HSA variants**: WT, R81A, E82K, ERK Vibo Valentia, T83A, Y84A, D108A, N109A, P110A, N111A, L112A, P113A, R114G Yanomama-2

- **Kinetic rate constants**:
  - \(k_a\) (in \(10^{3} / \text{s}\)), \(k_d\) (in \(10^{-5} / \text{s}\)), \(K_D\) (in \(\text{nM}\)), \(\chi^2\)

- **WT**
  - \(k_a = 4.3 \pm 0.1\) s\(^{-1}\)
  - \(k_d = 5.4 \pm 0.1\) s\(^{-1}\)
  - \(K_D = 125.6\) nM
  - \(\chi^2 = 1.9\)

- **Additional comments**
  - WT HSA-GST was found to have 82% absorption minima at 208 nm and 222 nm, in agreement with published crystal structures showing that the absorption minima at 190 nm and two complementary maxima at 208 and 222 nm are reflected by a peak of absorption at wavelength 190 nm and two absorption minima at 208 nm and 222 nm (Fig. 6, A–C). This is in agreement with published crystal structures showing that the dominating structure element for both HSA and GST is \(\alpha\)-helices (40, 41). WT HSA-GST was found to have 82% \(\alpha\)-helix and the mutants 74–82% (Table 2). L112A and P113A showed similar content as the WT (82–80%), whereas the two weak binders D108A and E82K had slightly lower \(\alpha\)-helical content, 75 and 74%, respectively. The same patterns were observed at 50 °C, where the \(\alpha\)-helix content was only slightly reduced for all variants, reflecting retained secondary structure at this temperature. At 70 °C, the spectra correspond to unfolded proteins, which is in agreement with reports showing that both DI and DII are irreversibly denatured at this temperature (42).

To further investigate their stability, the HSA-GST variants were incubated in human serum for 0 h, 12 h, 24 h, and 48 h at 37 °C, and subsequently the effect on FcRn binding was examined in ELISA. All variants showed reduced binding to hFcRn as a function of incubation time (Fig. 6D). After 48 h 40% reduction was observed for WT HSA, whereas three of the mutants (E82K, L112A, and P113A) showed only 20–25% reduced binding to hFcRn. The DI08A mutant gave rise to as much as 60% loss of activity after 48 h, which may indicate that this mutation has the most pronounced negative impact on protein stability.

**Structural Explanations of the Impact of DI Amino Acid Substitutions**—After the publishing of our docking model of the hFcRn-HSA complex (16), two studies recently reported on co-crystal structures of hFcRn in complex with HSA (18, 31), one with WT HSA and one with an engineered HSA variant (HSA13). The latter HSA variant binds with reduced pH dependence and with a 300-fold enhanced affinity at acidic pH. The two experimental structures confirm that DI makes contacts with hFcRn via the same two loops that were predicted from the docking model, but no interaction analysis was presented in these studies to investigate the impact of the DI interactions.
has evolved to secure constitutive receptor-mediated recycling and thus rescue from intracellular degradation. The process is efficient as exemplified by studies revealing that the receptor salvages one and one-half times the amount of IgG produced by plasma cells daily and half the amount of albumin produced by the hepatocytes (43, 44). A complete understanding of the molecular interplay between FcRn and its ligands is necessary so as to unravel how the receptor acts in homeostatic regulation and how the ligands are transported and distributed throughout the body.

Although IgG is the main antibody of the blood that is involved in fighting infections, albumin serves as a molecular taxi that transports a plethora of cargo in the bloodstream for delivery to their destination. These uncoupled functions are maintained by keeping the concentrations in blood stable and high at all times by balancing their production rates with the level of FcRn expression.

In this study we show that two of three HSA domains take direct part in binding to hFcRn. Compared with full-length HSA, we show that DIII on its own has a 12-fold weaker binding affinity toward hFcRn. This prompted us to investigate the contribution of DI.

Inspection of a previously published docking model of the FcRn-HSA complex (16) led us to suggest that two exposed loops within the N-terminal DI may make direct contact with hFcRn. Since then two co-crystal structures of hFcRn in complex with HSA have been published, one with WT HSA and one with HSA13, a mutant HSA molecule containing four substitutions (18, 31). The latter binds hFcRn with a 300-fold improved affinity at pH 6.0 but with loss of pH dependence as it also binds with a significant affinity at pH 7.4. Thus, HSA13 has a reduced serum half-life compared with WT HSA in hFcRn transgenic mice (18). Both co-crystal structures show that hFcRn makes contacts with DI via the same two loops as predicted from the docking model. Variation of the orientation of loop II and the associated interaction network between the WT HSA and HSA13 complexes suggests that loop II is a structural element with some flexibility.

In this study we targeted a selection of the amino acids that are positioned within the two DI loops by mutagenesis, which...
revealed that replacement of several residues with a neutral alanine modulated hFcRn binding either by gaining or loosing binding affinity at acidic pH, whereas none of the mutants bound at pH 7.4. Thus, our findings show that although the C-terminal DIII contains the principal binding site, which is very important for pH-dependent binding to FcRn, residues in DI also play a substantial role in binding.

None of the mutations hampered the secretion of the recombinant HSA-GST variants from HEK293E cells as they were produced in similar amounts, and SDS-PAGE analysis demonstrated that the mutants migrated as distinct bands similar to that of the WT. Furthermore, measurements of their \(\alpha\)-helical content by CD spectroscopy showed that the two weakest binders (D108A and E82K) have 7–8% less \(\alpha\)-helices than the WT fusion. In addition, serum stability was measured by incubation of the HSA GST variants in serum followed by binding to hFcRn in ELISA, which showed that D108A was the least stable of the mutants.

By inspecting the co-crystal structures, the observed effects of the mutations could be explained for some but not for all variants. Our analysis of mutant HSA variants suggests that substitution to alanine may impact local conformation rather than affect direct interactions, which in some instances can have a positive impact on the hFcRn-HSA interaction. The conformational flexibility of the subdomain connector loops in HSA has previously been noted (45).

Furthermore, we were intrigued by the fact that two rare naturally occurring HSA variants exist with single amino acid substitutions within the DI loops (38, 39). We expressed these as recombinant molecules and demonstrated that they bind with reduced FcRn affinity. If found in a heterozygote individual, the HSA variants may thus have decreased half-lives as they will compete with WT HSA for binding to hFcRn. Interestingly, homozygotes for the Yanomama-2 variant (R114G), found among the Brazilian tribe with the same name, have been shown to bind bilirubin poorly. This raises an important question on how structural alterations of HSA and the binding of ligands affect FcRn binding and subsequent biodistribution, a topic that should be addressed in future studies.

For determination of binding kinetics of full-length HSA variants, GST-fusions were immobilized on CM5 chips by amine coupling, and for the WT HSA-hFcRn pair the derived \(K_D\) value was shown to be almost 7-fold higher than when the receptor was immobilized and monomeric HSA injected. The difference in binding strength, which depends on the SPR setup, is in line with previous studies (16, 17, 29). The reason for this phenomenon is unknown, but it may be due to an effect of the amine coupling procedure where free amine groups of exposed amino acids are targeted. Similar but more drastic is the two ways of immobilization for measurements of the IgG-FcRn interaction, where nearly 300-fold differences are detected (13, 36, 46, 47). Thus, caution must be employed when comparing kinetic values across different experimental set-ups.

Most therapeutic molecules have a molecular weight below the renal clearance threshold and thus are rapidly eliminated from the circulation, a feature that limits their therapeutic

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**TABLE 2**
The \(\alpha\)-helix content of HSA-GST variants

| HSA-GST variants | \(\alpha\)-Helix at 25 °C | \(\alpha\)-Helix at 50 °C | \(\alpha\)-Helix at 70 °C |
|------------------|--------------------------|--------------------------|--------------------------|
| WT               | 82.3                     | 76.5                     | 20.5                     |
| E82K             | 74.7                     | 68.4                     | 43.1                     |
| D108A            | 75.3                     | 71.9                     | 18.6                     |
| L112A            | 82.6                     | 78.5                     | 21.6                     |
| P113A            | 80.4                     | 78.5                     | 15.9                     |
potential. This bottleneck may be overcome by the use of HSA as a carrier to increase the circulatory half-life and bioavailability of such molecules. Strategies to achieve this may be by association, chemical conjugation, or genetic fusion to HSA (3–5). In this regard our growing understanding of the pivotal role of FcRn in maintaining the circulatory half-life of albumin may guide the development of HSA-based therapeutics with improved properties and pharmacokinetics.

Although we have shown that the C-terminal end of DIII is very important for binding to hFcRn, genetic fusion of a peptide or an antibody-derived single-chain variable fragment to the C-terminal end slightly affects binding negatively, whereas when fused to the N terminus, binding was more or less unaffected (29). Reduced receptor binding upon fusion may be compensated by introducing amino acid substitutions in HSA that improve pH-dependent binding to hFcRn. One such example is a HSA variant with a single point mutation within DIII (K573P) that shows 12-fold improved binding toward hFcRn, which translates into 3.4 days longer half-life in rhesus monkeys (28). Our data presented here showing the requirement of DI for optimal binding to FcRn may pave the way for a next generation of engineered HSA variants that includes DI, such as the DI mutations with increased binding affinity described in this study.

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