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FcRn binding properties of an abnormal truncated analbuminemic albumin variant

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ABSTRACT

Background: The major histocompatibility class I-related neonatal Fc receptor, FcRn, salvages both IgG and albumin from degradation and thus contributes to maintain high serum levels of these proteins. Analbuminemia is a rare autosomal recessive disorder characterized by clinically observed allelic albumin variants that are absent or found in very low concentrations in the blood circulation. Such variants may have altered FcRn binding properties that affect their half-life, biodistribution and thereby transport ability.

Methods: We established an easy cloning, expression and purification strategy to obtain recombinant GST-tagged human serum albumin (HSA) variants for evaluation of pH dependent FcRn binding properties using an enzyme-linked immunosorbent assay (ELISA) and a real time surface plasmon resonance (SPR) biosensor system.

Results: The strategy yielded purified GST-tagged albumin variants. A recombinant truncated HSA variant similar to a clinically observed splice mutant denoted Bartin, here abrogated HSA^{Bartin}, showed no detectable pH dependent FcRn binding compared to a fully functional albumin wild type variant, HSA^{Wt}, and a truncated HSA variant consisting of only the carboxy terminal domain III (HSA^{DIII}).

Conclusions: The approach described can be used to rapidly screen clinically observed truncated or otherwise mutant or modified HSA variants regarding their pH dependent FcRn binding properties. Here, we demonstrate that a recombinant truncated HSA variant, HSA^{Bartin}, does not interact with FcRn, which gives a molecular explanation for the low serum levels. In addition, DIII of HSA alone was shown to retain its FcRn binding property.

Introduction

Albumin is a multifunctional abundant protein that constitutes 60–65% of the blood plasma protein pool with a normal concentration of 35–45 g/L [1]. It functions primarily to support the osmotic pressure and to carry a wide variety of insoluble and hydrophobic endogenous and exogenous metabolites and drugs [1]. In addition, albumin is an important antioxidant and it possesses enzymatic properties [1-3].

The serum level of albumin is maintained by its liver synthesis rates, its molecular weight that exceeds the renal clearance threshold and its interaction with the neonatal Fc receptor, FcRn [4]. This receptor is a major histocompatibility complex class I-related heterodimeric molecule that consists of a membrane bound heavy chain that is non-covalently associated with the common β2-microglobulin [5,6]. FcRn is expressed in almost every cell type and tissue of the body, and has evolved to be a versatile player in several important biological processes, as for instance transepithelial and maternofetal transport of IgG (passive immunization of the fetus), antigen presentation and phagocytosis by immune cells, and half-life regulation of the serum levels of IgG and albumin [4,7-10].

FcRn binds IgG and albumin simultaneously in a pH dependent fashion to opposite sites on the α2-domain of the FcRn heavy chain, binding at acidic pH and no or very weak binding at physiological pH [9,11]. The strict pH dependence favors the unique FcRn recycling pathway that has been suggested to take place mainly in endothelial cells that cover the bloodstream and in hematopoietic cells [8,12,13]. Ligands are continuously taken up by fluid phase pinocytosis and enter acidified endosomes where FcRn predominantly resides. The low pH herein triggers binding of IgG and albumin to FcRn. The ternary complex is transported to the cell surface where the exposure to the physiological pH of the bloodstream triggers release of ligands back into the circulation. Ligands that escape FcRn binding are destined for lysosomal degradation. This sophisticated pathway relying on pH dependent binding to FcRn explains the uniquely long half-life of 19–22 days for IgG and albumin and their direct concentration–catabolism relationships.

The importance of FcRn has unequivocally been demonstrated in mice lacking the receptor that show dramatically reduced serum levels of both ligands [12,14-16]. A human example is illustrated by the rare familial hypercatabolic hypoproteinemia condition that is characterized by very low serum levels of both albumin and IgG [17]. The molecular reason for this condition has been a matter of debate,

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but was recently shown to be caused by a genetic defect in the FcRn heavy chain associated β2-microglobulin gene causing expression of FcRn to be only 20% of normal [18].

A spectrum of clinically observed albumin variants has been characterized over the past decades by routine plasma electrophoretic analysis and genetically by sequencing of the patient's albumin genes. A variety of albumin allelic variants have been reported in peer-reviewed papers, as recently summarized by Minchiotti and colleagues [19] and listed in the albumin mutation register [20]. Their relevance to *pathophysiology* of diseases is uncertain, but it has for instance been shown that the familial dysalbuminemic hyperthyroxinemia phenotype results from an albumin mutation that is caused by replacement of histidine by arginine at position 218 [21].

Analbuminemia is a rare recessive disorder characterized by low or no detectable circulating albumin in affected individuals that correlate with the presence of abnormal genetic albumin variants. In some cases, carboxy terminal truncated albumin products is generated [19]. These variants will partly or completely lack domain three (DIII) of albumin that notably has been shown to be crucial for FcRn binding [22].

Recently, Dolcini and co-workers described a novel analbuminemic albumin variant, denoted Bartin, that had evolved as a result of a splice mutation [23]. The resulting variant lacks 175 amino acids carboxy terminally, which includes all of DIII, except the first 25 amino acids. In addition, the 4 last amino acids are altered relative to the wild type sequence. As the truncation and alteration of DIII are rather drastic, it is tempting to speculate that the clinical manifestations observed are due to abrogation of FcRn binding [24]. Despite the fact that several other analbuminemic albumin variants with such alterations have also been reported, a molecular characterization of their FcRn binding properties has so far not been considered. Furthermore, purification of such abnormal albumin variants from blood plasma is obviously hampered as they exist in low concentrations, ranging from 2 to 30% of total albumin [19]. In this paper we describe a sophisticated, rapid and easy system to produce and purify HSA variants for fast and real time SPR screening of pH dependent binding to FcRn. The results show that a recombinant variant of Bartin is not capable of interacting with human FcRn, in sharp contrast to its wild type counterpart.

Results

Construction of recombinant HSA variants

To evaluate the impact of albumin truncation on pH dependent binding to FcRn, we established an easy cloning, expression and purification protocol to generate recombinant forms of such albumin variants. As a proof of concept we chose the recently discovered Bartin splice mutant (HSA^{Bartin}) [23] in addition to a full length wild type variant (HSA^{Wt}). The Bartin mutant is a carboxy terminal truncated HSA variant that lacks the whole of DIII except the first 25 amino acids. Illustrations of the crystallographic structure of HSA^{Wt} (amino acid 1–585) as well as a predicted structure of HSA^{Bartin} (amino acid 1–410) are shown in Fig. 1a.

The cDNA segments encoding HSA^{Wt} (1827 bp) and $HSA^{Batrtin}$ (1302 bp) that include the albumin signal peptide, were PCR-amplified from a human liver cDNA library (Fig. 1b), and cloned in frame of a cDNA encoding a carboxy terminal GST-tag on unique EcoRI and XhoI restriction sites in a previously constructed plasmid, pcDNA3-GST-oriP (Fig. 1c) [25]. The reported Bartin splice mutant encodes a truncated protein of 410 amino acids where position 406–410 are also altered compared to the HSA^{Wt} sequence [23], as highlighted in Fig. 1d. Our truncated HSA^{Bartin}-like variant has the wild type amino acids retained at these positions. The obtained cDNAs were sequenced and shown to be identical with published HSA cDNA sequences (data not shown).

Production of GST-tagged HSA^{Wt} and HSA^{Bartin}

The plasmids encoding the two albumin variants were transiently transfected into adherent HEK 293E cells, and subsequently, supernatants were harvested every second day for 2 weeks. Collected supernatants were filtrated and applied on a simple one-step glutathione affinity purification column. Eluted fractions were pooled, concentrated and evaluated by SDS–PAGE analysis where protein bands were visualized by Coomassie staining. Fig. 1e shows a representative gel where bands with molecular weights of ~90 kDa and ~70 kDa for the GST-fused HSA^{Wt} and HSA^{Bartin} variants, respectively, are indicated. In addition, some covalent aggregates are present that are created as a consequence of exposed cysteine residues on the GST-tag as previously described [26,27]. Their molecular weights are in accordance with their theoretical values. The total yield of secreted and purified HSA^{Bartin} (~600 μg/L supernatant) was approximately twice as high as for the wild type. Thus, genetic truncation of HSA does not affect secretion from HEK 293E cells.

Evaluation of FcRn binding properties

To verify the functional integrity of the HSA variants regarding pH dependent FcRn binding, two detection methods were established, namely ELISA and SPR. In the ELISA, a mouse monoclonal anti β2-microglobulin IgG2a antibody, a subclass that does not in itself bind to the human form of FcRn [28,29] was coated in MaxiSorp™ microtiter wells followed by capture of serial dilutions of soluble human FcRn (shFcRn). Production of functional shFcRn was recently reported in a cost-effective high yield bacterial expression system [29,30]. GST-tagged HSA variants were added and bound ligands visualized using an HRP-conjugated goat anti-GST antibody. Human IgG1 was also added to captured shFcRn, and detected with an HRP-conjugated goat anti mouse light chain antibody. The ELISA was performed at both pH 6.0 and pH 7.4, and as expected, IgG1 was shown to bind more strongly at pH 6.0 than at physiological pH (Fig. 2a). Similarly, HSA^{Wt} bound pH dependently while the HSA^{Bartin}-like variant did not bind at either pH (Figs. 2b, c).

Furthermore, to establish a more rapid and sensitive screening method, an SPR biosensor system was used. This allows real time measurements of specific shFcRn pH dependent binding without the need for any detection reagents. A dextran coupled CM5 sensor chip was used for covalent immobilization of high levels of GST-tagged HSA variants using amine coupling. We have previously shown that this procedure does not interfere with functional ligand binding to FcRn [11,30]. One hundred nanomolars of shFcRn was then injected over both HSA variants at pH 6.0 and pH 7.4. Fig. 2d shows representative sensorgrams of reversible and pH dependent binding to HSA^{Wt}. Importantly, the binding profile is similar to that reported for shFcRn binding to non-tagged HSA [11,30]. Furthermore, pre-formed shFcRn-human IgG1 complexes bound immobilized HSA^{Wt} (Fig. 2e). This finding is in accordance with reports indicating that the two ligands bind to distal binding sites on FcRn [22,30]. Moreover, injection of shFcRn over high levels of the immobilized HSA^{Bartin}-like mutant did not generate any detectable binding responses at acidic pH, and as expected, no binding at physiological pH (Fig. 2f). Thus, Bartin-like truncation of HSA leads to elimination of FcRn binding.

Finally, to demonstrate that the major FcRn binding site is localized within DIII, we genetically engineered an HSA variant consisting of only carboxy terminal DIII (amino acid 381–585) fused to the GST-tag. To facilitate secretion from 293E cells, the endogenous HSA signal sequence was added in front of and in frame of the DIII cDNA sequence. Production of HSA^{DIII} in 293E cells was dramatically reduced (50 μg/L supernatant) compared with carboxy terminal truncated albumin variants. To test its FcRn binding capacity, 5 μg/mL of each variant was used in the established ELISA. In contrast to DIII

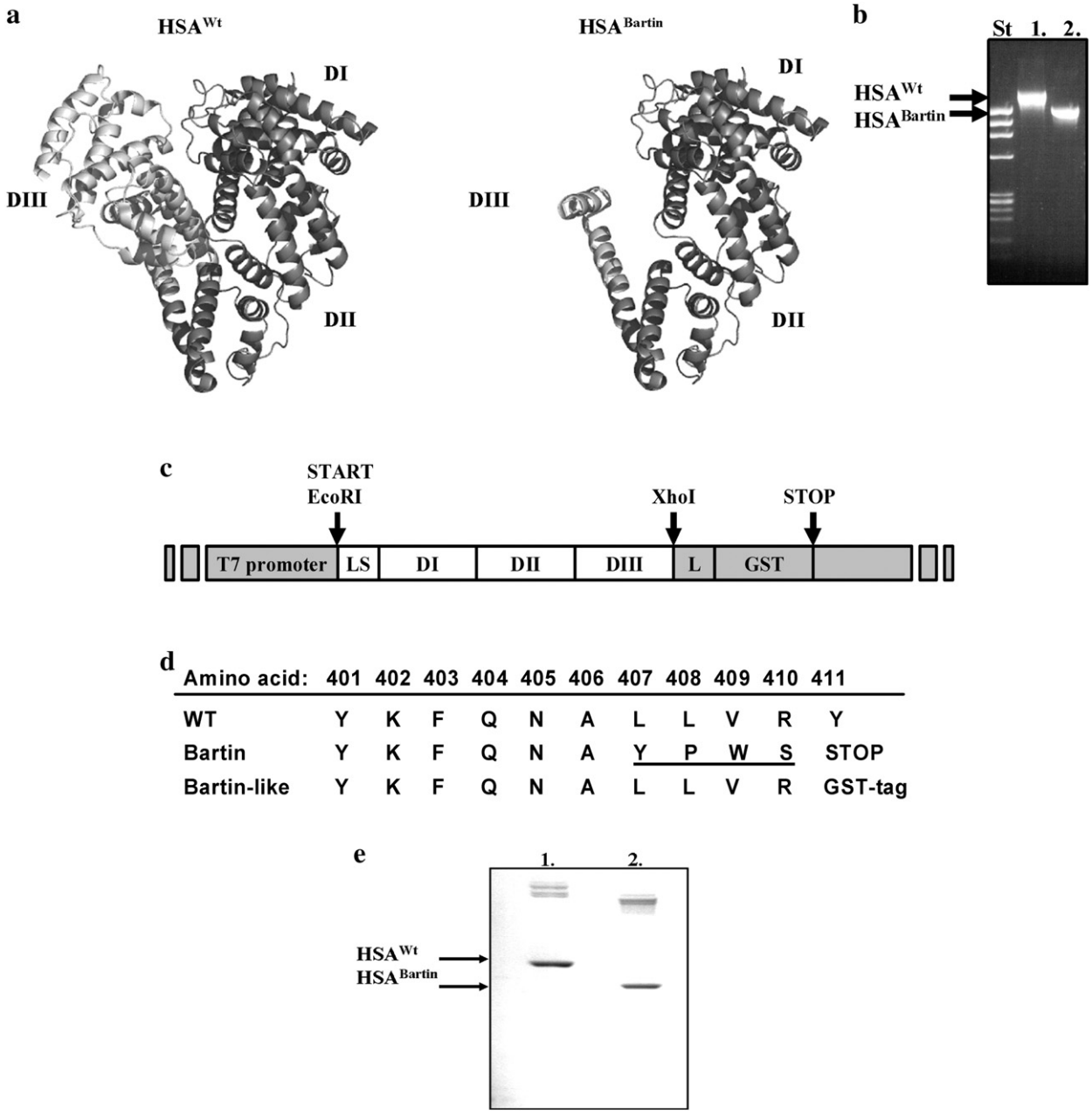

Fig. 1. Construction and production of GST-tagged HSA variants. (a) Illustrations of the crystal structure of the wild type HSA (amino acid 1–585) and a predicted crystal structure of a Bartin-like truncated HSA variant (1–410). HSA is composed of three structurally similar globular domains, DI (1–195), DII (196–383) and DIII (384–585) that encompass amino acids 1–195, 196–383 and 384–585, respectively. DI and DII are highlighted in dark gray ribbon structures and DIII in light gray ribbon structures. The figures were design using PyMOL and the crystallographic data of HSA available on the Protein Data Bank site [40]. (b) The PCR-amplified cDNA segments encoding HSA^{Wt} (1827 bp) and the Bartin-like HSA variant (1302 bp) analyses on a 1% agarose gel. (c) An overview of the cloning site of the pcDNA3-GST-oriP vector map showing directional subcloning of the HSA cDNA insert on the restriction sites EcoRI and XhoI in genetic frame of a cDNA encoding the GST-tag. LS; albumin leader sequence, DI, DII and DIII; the three albumin domains, L; linker sequence containing an enterokinase cleavage site, GST; glutathione-*S*-transferase tag from *Schistosoma japonicum*. Amino acid alignment of the HSA^{Bartin}-like variant with published HSA sequences [23,40]. The aberrant amino acids and introduction of a stop codon caused by a mutational frameshift in the Bartin variant are underlined. (e) Analyses of the protein integrity of purified GST-fused HSA variants on a 12% SDS–PAGE gel. The protein bands corresponding to monomeric HSA^{Wt} and HSA^{Bartin} are indicated by arrows.

deleted HSA^{Bartin}, HSA^{DIII} was shown to bind in a similar fashion as HSA^{Wt} (Fig. 2g). The binding signal is slightly higher for HSA^{DIII} than for the wild type as explained by the fact that the molecular weight of the HSA^{DIII} variant is approximately half that of HSA^{Wt}.

Discussion

Minor or major structural alterations caused by genetic changes or chemical post-translational modifications may have an impact on half-life and biodistribution of circulating proteins such as IgG and albumin. This has been extensively demonstrated in several preclinical studies using chemically or genetically modified albumin variants [31-34], although a molecular explanation for the altered pharmacokinetics is not necessarily given. The discovery of putative albumin receptors and elucidation of how they bind and transport albumin variants, with or without bound ligands, may bring new insights. Similarly, the characterization of a set of distinct IgG binding receptors, such as FcRn and the Fcγ receptors [9,35], has contributed to the understanding of how IgG is transported and functions in immunity. Surprisingly, FcRn was subsequently shown to bind albumin, and rescue both IgG and albumin from intracellular degradation [4,12]. The capacity of the FcRn salvaging pathway is

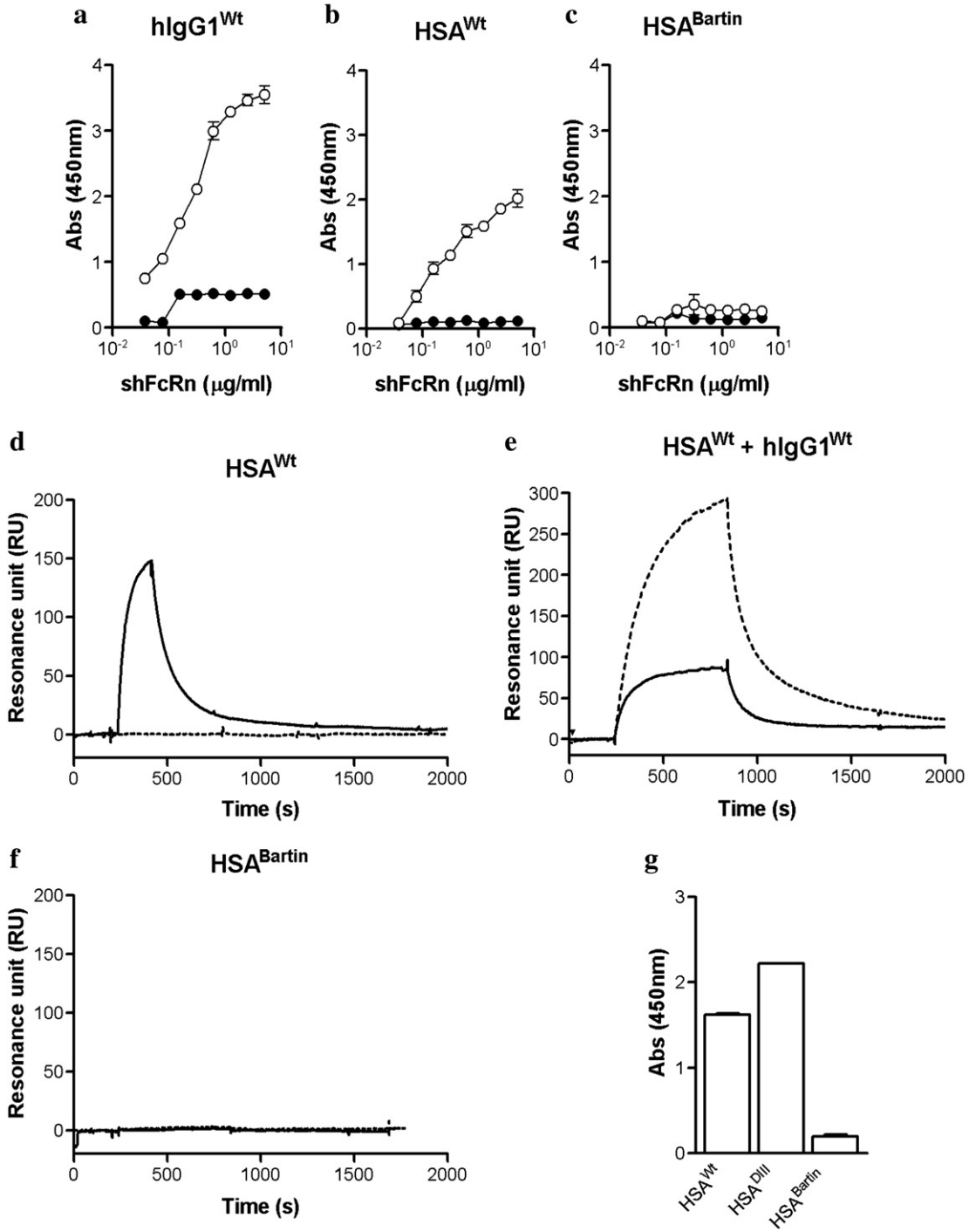

Fig. 2. FcRn binding properties of human IgG1 and HSA variants. Serial dilutions of shFcRn (5–0.04 μg/mL) were captured on an monoclonal mouse IgG2a anti-β2-microglobulin antibody (2 μg/mL) absorbed to MaxiSorp™ microtiter wells followed by binding of (a) human IgG1 wilt type (2 μg/mL, $hIgG1^{Wt}$) at pH 6.0 (o) and pH 7.4 (●), (b) HSA^{Wt} (5 μg/mL) at pH 6.0 (o) and pH 7.4 (●), and (c) the HSA^{Bartin}-like variant (5 μg/mL) at pH 6.0 (o) and pH 7.4 (●). (d) Binding of 100 nM shFcRn to immobilized HSA^{Wt} at pH 6.0 (—) and pH 7.4 (---), (e) binding of 100 nM shFcRn (—) and preformed shFcRn-human IgG1 complexes (---) to immobilized HSA^{Wt} at pH 6.0, and (f) binding of 100 nM of shFcRn to HSA^{Bartin} at pH 6.0 (—) and pH 7.4 (---). (g) Comparison of the FcRn binding capacity of HSA^{DIII} with HSA^{Wt} and HSA^{Bartin} (5 μg/mL of each variant) at pH 6.0. The ELISA data shown represent means ± SD of triplicates, and the SPR sensorgrams are representative for serial injections.

dramatic, and without the receptor, one would have needed a great increase in the production rates of IgG and albumin from the plasma cells and liver, respectively, to maintain normal serum concentrations [36,37]. In line with this, genetic differences and chemical modifications of the FcRn ligands may affect their affinity for the receptor and thus their pharmacokinetics. For instance, methionine oxidation of human IgG subclasses was very recently shown to have an impact on FcRn binding [38,39], and site-directed mutagenesis in the Fc part of IgG can abolish shFcRn binding completely [30]. In relation to albumin variations, no studies have so far reported a direct correlation between altered *in vivo* behavior and FcRn binding properties.

In this report we describe an easy cloning and production strategy to generate HSA and genetically modified HSA variants, such as reported from clinical studies. The system allows for screening for FcRn binding properties. We chose the recently described analbuminemic

Bartin variant, which lacks ~90% of the carboxy terminal DIII, to demonstrate the feasibility of the approach. Both the HSA^{Bartin}-like variant as well as HSA^{Wt} were cloned, and the genes introduced in the human kidney cell line HEK 293E. Both were secreted in amounts sufficient for FcRn interaction analyses. Notably, truncation of DIII did not hamper the secretion rate. This may indicate that the synthesis rate of endogenously produced Bartin is not affected, although the impact of the four aberrant amino acids found in the carboxy terminal end of this variant is unknown.

Using a sensitive SPR-based assay for fast screening of FcRn binding, the Bartin-like variant bound neither at pH 6.0 nor pH 7.4. This is the first report of FcRn binding analysis of an analbuminemic-like albumin variant. The Bartin-like variant lacks DIII, except the first 25 amino acids, a fact that strongly suggests that these residues are not involved in FcRn binding. Thus, despite the fact that the three HSA domains (DI-DII-DIII) are very similar in topology and three-dimensional structure [40], DIII is absolutely required for the unique FcRn pH dependent binding specificity. This was conclusively demonstrated using an engineered HSA variant consisting of only DIII that was shown to retain its FcRn binding property.

Furthermore, the data gives a molecular explanation for the clinically observed low serum levels of the Bartin splice variant, and the FcRn binding studies as reported here may be exploited for characterization of other such albumin variants. The SPR-based shFcRn binding assay may also be used to evaluate chemical modifications such as oxidation and glycosylation [31,32,34] of albumin variants isolated from blood. Genetic fusions of small therapeutics and diagnostics to HSA or molecules that bind HSA are ways to extend *in vivo* half-life and thereby clinical efficacy. This is a field of increasing interest in the biotechnological industry [41-43], and such HSA fused or targeted molecules should necessarily be evaluated for their FcRn binding properties to predict preclinical *in vivo* pharmacokinetics.

Materials and methods

PCR amplification and subcloning

The cDNA sequence encoding HSA^{Wt} (1827 bp) was PCR-amplified from a human cDNA liver library (Zyagen, CA, USA) with the primers HSA_Forw (5-taa **gaa ttc** acc atg aag tgg gta acc ttt att tcc-3) and HSA_Rev (5-ata **ctc gag** taa gcc taa ggc agc ttg act tgc-3) and the truncated Bartin-like variant (1302 bp) was PCR-amplified using the HSA-Forw primer and HSABartin_Rev (5-ata **ctc gag** acg aac taa tag cgc att ctg-3). The primers were flanked by sequences encoding recognition sites for the restriction enzymes EcoRI and XhoI (shown in bold). The PCR temperature profile was 95 °C for 60 s followed by 30 cycles of denaturation at 95 °C for 120 s and annealing and extension at 62 °C and 75 °C, respectively, for 240 s. PCR reactions were analyzed on 1% agarose gels. Amplified cDNAs were digested with EcoRI and XhoI (New England Biolabs) and incubated for 2 h at 37 °C, and subcloned on these restriction sites into the polylinker of pcDNA3-GST-oriP, in frame of a cDNA encoding the glutathione-*S*-transferase (GST) tag from *Schistosoma japonicum*. This vector system has previously been described [25]. The final vectors were denoted pcDNA3-HSA^{wt}-GST-oriP and pcDNA-HSA^{Bartin}-GST-oriP. HSA^{DII} (672 bp) was constructed using pcDNA3-HSA^{wt}-GST-oriP as template with the primers HSAsignalDIII (5-5-a taa **gaa ttc** atg aag tgg gta acc ttt att tcc ctt ctt ttt ctc ttt agc tcg gct tat tcc agg ggt gaa gag cct cag-3 and HSA_Rev. The HSA^{DII} primer includes the HSA signal sequence (encoding amino acid 1–21) that was predicted using the SignalP 3.0 Server. The PCR temperature profile was 95 °C for 30 s followed by 30 cycles of denaturation at 95 °C for 30 s and annealing at 75 °C for 30 s and extension at 77 °C for 60 s. Amplified cDNA fragments were enzyme digested and subcloned as described above. The vector was denoted pcDNA3-HSA^{DIII}-GST-oriP. All oligonucleotides were synthesized by MedProbe (Norway), and sequences were verified using in-house sequencing facility.

Production of HSA variants

Adherent human embryonic kidney (HEK) 293E cells (ATCC) were transient transfected with pcDNA3-HSA^{wt}-GST-oriP, pcDNA-HSA^{Bartin}-GST-oriP and pcDNA3-HSA^{DIII}-GST-oriP using the Lipofectamin2000 method (Invitrogen) following the protocol described [25]. Supernatants were harvested every second day for 2 weeks, pooled and filtrated, and subsequently applied to a semiautomatic Biologic workstation system (BIO-RAD Laboratories) using a GSTrap FF column (GE Healthcare) and GST-tagged proteins were purified essentially as described [25]. Eluted fractions were pooled and buffer exchanged to PBS using Amicon Ultra YM-10 columns (Millipore). Concentrated samples were analyses on a 12% Bis–Tris Criterion XT Precast gel (BIO-RAD) using the PowerPac HC apparatus (BIO-RAD Laboratories). Proteins bands were visualized using BIO-Safe Coomassie staining (BIO-RAD Laboratories). Total protein concentrations were determined using the NanoDrop Spectrophotometer.

Enzyme-linked immunosorbent assay

A monoclonal anti-human β2-microglobulin antibody (Abcam; mouse IgG2a; 2 μg/mL) was absorbed to MaxiSorp™ microtiter plate wells (Nunc) in PBS, pH 7.4 overnight at 4 °C. The wells were then blocked with 4% skimmed milk (skm) powder (Acumedia) diluted in PBS for 1 h at room temperature (RT) followed by four times washing in PBS pH 7.4 containing 0.05% Tween20 (PBSTpH7.4) (Sigma-Aldrich). Serial dilutions (5–0.04 μg/mL) of shFcRn [30] in 4% skm/PBSTpH7.4 were then added and allowed to react for 2 h at RT before four times washing in PBSTpH7.4 or PBSTpH 6.0. An anti-NIP mouse–human chimeric IgG1 antibody (consisting of mouse light chains and human heavy chains, a kind gift from Terje. E. Michaelsen, National Institute of Public Health, Norway; 2 μg/mL), HSA^{Wt} (5 μg/mL), HSA^{Bartin} (5 μg/mL) or HSA^{DIII} (5 μg/mL) diluted in 4% skm/PBSTpH7.4 or pH 6.0 were added and allowed to react for 1 h at RT before washing as described above. Subsequently, for detection of bound human IgG1, a goat HRP-conjugated antimouse lambda light chain antibody (Southern Biotech; diluted 1:4000) or, for detection of GST-tagged HSA variants, a goat HRP-conjugated anti-GST antibody (GE Healthcare: 1:4000) was diluted in 4% skm/PBSTpH7.4 or pH 6.0 and added and allowed to react for 1 h at RT. All steps were performed with 100 μL/well except the washing steps that were performed using 400 μL/well. After washing as above, the wells were developed with 100 μL TMB substrate (Calbiochem) for 45 min before 100 μL of 1 M HCl was added. The absorbance was directly read at 450 nm using a Sunrise TECAN spectrophotometer (TECAN).

Surface plasmon resonance

All surface plasmon resonance (SPR) experiments were carried out on a BIAcore 3000 instrument (GE Healthcare). Flow cells on CM5 sensor chips (GE Healthcare) were coupled with HSA^{Wt} (1560 RU) or HSA^{Bartin} (2200 RU) using amine coupling chemistry, all as described by the manufacturer. All analyses were performed in phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl) at pH 6.0 or pH 7.4, or in HBS/EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) at pH 7.4 (GE Healthcare). One hundred nanomolars of shFcRn [30] in complex or not with anti-NIP human IgG1 (200 nM) was injected over immobilized HSA variants at pH 6.0 or pH 7.4. All experiments were performed at a flow rate of 50 μL/min at 25 °C. The flow cells were 'stripped' after the dissociation-phase using phosphate buffer at pH 7.4. In all experiments, data were zero adjusted and the reference cell value subtracted. Binding analyses were performed using the supplemented BIAevaluation 4.1 wizard.

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