Research paper

A strategy for bacterial production of a soluble functional human neonatal Fc receptor

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Abstract

The major histocompatibility complex (MHC) class I related receptor, the neonatal Fc receptor (FcRn), rescues immunoglobulin G (IgG) and albumin from lysosomal degradation by recycling in endothelial cells. FcRn also contributes to passive immunity by mediating transport of IgG from mother to fetus (human) or newborn (rodents), and may translocate IgG over mucosal surfaces. FcRn interacts with the Fc-region of IgG and domain III of albumin with binding at pH 6.0 and release at pH 7.4. Knowledge of these interactions has facilitated design of recombinant proteins with altered serum half-lives and/or altered biodistribution. To generate further research in this field, there is a great need for large amounts of soluble human FcRn (shFcRn) for *in vitro* interaction studies. In this report, we describe a novel laboratory scale production of functional shFcRn in *Escherichia coli* (*E. coli*) at milligram level. Truncated wild type hFcRn heavy chains were expressed, extracted, purified from inclusion bodies under denaturing non-reducing conditions, and subsequently refolded in the presence of human β_2-microglobulin (hβ_2m). The secondary structural elements of refolded heterodimeric shFcRn were correctly formed as demonstrated by circular dichroism (CD). Furthermore, functional and stringent pH dependent binding to IgG and human serum albumin were demonstrated by ELISA and surface plasmon resonance (SPR). This method may be easily adapted for the expression of large amounts of other FcRn species and MHC class I related molecules.
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1. Introduction

FcRn is expressed in a wide variety of human tissues, including placental syncytiotrophoblasts, where it plays a role in maternofetal transport of IgG (Story et al., 1994; Kristoffersen, 1996; Simister and Story, 1997; Rojas and Apodaca, 2002). The prolonged lifespan of IgG in the circulation is the result of the IgG–FcRn interaction (Ober et al., 2004a,b). Furthermore, FcRn performs bidirectional transport of IgG or IgG–antigen complexes across epithelial cells that cover mucosal surfaces (Dickinson et al., 1999; Spiekermann et al., 2002; Yoshida et al., 2004). Recently, FcRn was found to enhance phagocytosis by neutrophiles (Vidarsson et al., 2006).

In general, FcRn binds IgG at cell surfaces or in vesicles at pH 6.0–6.5, with release at pH 7.0–7.5 (Guyer et al., 1976; Rodewald, 1976). Unbound IgG enters the lysosomal pathway and is degraded (Guyer et al., 1976; Rodewald, 1976). The interaction site on IgG has been mapped and shown to encompass conserved residues at the CH_2–CH_3 domain interface, including two histidines (Ghetie and Ward, 2000) that confer the characteristic pH dependence of the interaction with acidic residues on hFcRn.

Recently, shFcRn was purified as a ternary complex with hIgG and bovine serum albumin (BSA) (Chaudhury et al., 2003). The finding prompted interaction studies involving HSA, and again, pH dependent binding was demonstrated. A histidine residue, conserved in FcRn of 11 different species, and located opposite the IgG binding site in folded FcRn, is crucial for the interaction with albumin (Andersen et al., 2006). This may explain the prolonged half-life of HSA *in vivo*.

In all species studied, FcRn consists of a single transmembrane heavy chain non-covalently associated to soluble β_2m, and has great structural homology with MHC class I (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985; Kristoffersen, 1996). So far, shFcRn has been expressed in Chinese hamster ovary cells (Gastinel et al., 1992; West and Bjorkman, 2000), High 5 insect cells (Popov et al., 1996; Firan et al., 2001) and in human embryonic 293E cells (Berntzen et al., 2005; Datta-Mannan et al., 2007). Due to high cost and time-consuming procedures, there is a need for alternative expression systems. Bacterial production represents an attractive alternative, and MHC class I molecules have been successfully produced in *Escherichia coli*. In particular, efficient refolding was demonstrated with non-reduced heavy chains extracted from inclusion bodies (Ostergaard Pedersen et al., 2001). Large scale production of such heavy chains under non-

reducing conditions, and refolding in the presence of $h\beta_2$m and peptide was subsequently demonstrated (Ferre et al., 2003).

The protection of IgG and albumin from degradation as well as maternofetal transfer of IgG are essential biological phenomena, which depend on the interaction with FcRn. Several publications have demonstrated that HSA is a suitable drug carrier (Kurtzhals et al., 1995; Makrides et al., 1996; Peters, 1996; Warnecke and Kratz, 2003; Wunder et al., 2003; Melder et al., 2005). Furthermore, the development of IgG variants as well as therapeutic albumin or Fc fusion molecules or other selected binding molecules with increased or decreased affinity for FcRn (Dall'Acqua et al., 2002; Spiekermann et al., 2002; Bitonti et al., 2004; Kenanova et al., 2005) will need to be tested *in vitro*, which will require large amounts of purified shFcRn.

We constructed a prokaryotic pET28 vector containing cDNA encoding a truncated form of the hFcRn wild type (WT) heavy chain in frame of a modified hexahistidine tag (HAT). Here we demonstrate hFcRn heavy chain expression as inclusion bodies in *E. coli*. Extracted and purified chains were denatured and refolded *in vitro* under non-reducing conditions in the presence of an excess amount of $h\beta_2$m, using the established refolding strategy (Ferre et al., 2003). The yield of functional heterodimeric receptor was at milligram levels, and it had the folding characteristics and ligand binding properties of the native receptor.

2. Materials and methods

2.1. Cloning of hFcRn heavy chain into prokaryotic pET28+ expression vector

A truncated hFcRn heavy chain WT encoding cDNA, without leader sequence (aa 1–268), was PCR amplified with primers, FcRnSeam.forw and FcRnSeam.rev (Table 1). The pET28+ vector (Novagen, Darmstadt, Germany) was modified to encode a HAT-tag in the polylinker between the restriction sites *Hin*dIII and

Table 1
Primer sequences

Primer	Sequence [a]
FcRnSeam. forw	5′-AGT TA*C TCT TC*A AGA GCA GAA AGC CAC CTC TCC CTC C-3′
FcRnSeam.rev	5′-AGT TA*C TCT TC*A TTA CAG CTC CAC CCT GAG GGG CTG-3′
ForwpETSeam	5′-AGT TA*C TCT TC*A GCT TGC GGC CGC ACT CGA GCA CCA C-3′
RevpETSeam	5′-AGT TA*C TCT TC*A TCT ACC CTC GAT GGA TCC GTT GTT GTG GGC-3′

[a] Eam 1104 I restriction sites are in italic.

*Bam*HI to create pET28-HAT as follows: pET28-HAT was amplified by PCR with the primers pET28Seam.forw and pET28Seam.rev (Table 1), and the inserts ligated in frame with the HAT-tag using Seamless cloning with the restriction enzyme Eam 1104 I (Stratagene; La Jolla, CA). The construct made was denoted pET28-HAT-hFcRn (heavy chain WT). The plasmid was subsequently transformed into *E. coli* BL21 (DE3), BL21-CodonPlus-(DE3)-RIL and BL21-CodonPlus-(DE3)-RP cells as described in the BL21 CodonPlus-(DE3) Competent Cell Introduction Manual by Strategene. The two latter strains were plated on LB-agar plates containing both kanamycin (50 µg/ml) and chloramphenicol (34 µg/ml). Recombinant hβ_2m cDNA was introduced and expressed in the pET28 system as previously described (Ostergaard Pedersen et al., 2001).

2.2. Prokaryotic expression of recombinant hFcRn heavy chains

Human FcRn heavy chains were expressed in a 2 l fermentor using IPTG induction followed by extraction of inclusion bodies as previously described for expression of MHC class I heavy chains (Ferre et al., 2003). SDS-PAGE analysis of samples taken during fermentation was done according to the procedure described by Chen and Christen (1997). Approximately 300 ml of Ni^{2+} activated nitrilotriacetic acid (NTA) matrix was packed in a standard column. Samples of solubilized inclusion bodies (approximately 160–190 ml) were applied to the column, which was equilibrated with 8 M urea, 25 mM Tris–HCl and 0.2 M NaCl (pH 8.0) (loading buffer). Unbound material was removed with 2 column volumes of loading buffer. Bound proteins were eluted at a flow rate of 1–5 ml/min using a 0–25% gradient of the loading and elution buffers. The elution buffer consisted of 8 M urea, 50 mM Tris–HCl, 0.2 M NaCl and 0.25 M imidazole. Prior to the subsequent size exclusion chromatography (SEC) step, collected fractions from the Ni^{2+} NTA column purification were pooled and concentrated on a 10 kDa nominal molecular weight limit ultrafiltration membrane (Millipore) in a nitrogen pressure Amicon cell (Millipore), with stirring to a final volume of 10–30 ml. SEC was done on a Sephacryl 200 high resolution column equilibrated with 8 M urea and 25 mM Tris–HCl (pH 8.0). Collected fractions were pooled and stored at −20 °C.

2.3. Refolding of shFcRn

Purified hFcRn WT heavy chain, in 8 M urea buffer, was diluted into 50 mM Tris–glycine (pH 8.5) buffer

containing a 4-fold M excess of hβ_2m (final concentration of heavy chain was 30 µg/ml). After incubation at room temperature for 1 h and at 4 °C for 72 h, the mixtures were concentrated and applied to a 1 l Superdex 200 PG column. The collected fractions were analyzed by SDS-PAGE followed by silver staining as described in the silver staining kit protocol from Amersham Pharmacia Biotech. All the steps were performed with freshly made solutions. Fractions containing heterodimeric shFcRn were pooled and concentrated. The protein concentrations were measured with BCA Protein Assay Reagent (Pierce Biotechnology, Inc., Rockford, IL) or by protein molecular epsilon at 280 nm 86,120 (l/mol/cm).

2.4. Circular dichroism

Circular dichroism (CD) spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo Japan) calibrated with ammonium *d*-camphor-10-sulfonate (Icatayama Chemicals, Tokyo Japan). Measurements were performed at 23 °C using a quartz cuvette (Starna, Essex, UK) with a pathlength of 0.1 cm. All measurements were performed with a protein concentration of 0.15 mg/ml in either 10 mM PBS (pH 7.4) or in pure water (pH 7.0). Samples were scanned 7 times at 20 nm/min, using a bandwidth of 1 nm. The response time was 1 s, and the wavelength range was 190–260 nm. The data were averaged and the spectrum of a sample-free control was subtracted. The α-helical content of the proteins was calculated after smoothing (means-movement, convolution width 5) from ellipticity data, using the neural network program CDNN version 2.1 and the supplied neural network based on the 33-member basis set (Bohm et al., 1992). Thermal denaturation curves were determined as the change in CD signal at 208 nm during heating. The temperature was controlled with a TPC-423S/L system (Jasco International Co.) and a heating rate of 1 °C/min. After baseline correction, the unfolding curve was smoothed (means-movement method; convolution width 11) and normalized, and the apparent melting temperature (*Tm*) was determined from the transition midpoint visible in the first derivative of the unfolding curve, using the computer program Origin 7.0 (OriginLab Corporation, Northampton, MA, USA). All measurements were conducted at least twice.

2.5. Biotinylation of shFcRn WT

A preparation of shFcRn WT dissolved in PBS at 1 mg/ml, was added to 125 µl of *N*-hydroxysuccinimido

(NHS)-biotin (Sigma) at 1 mg/ml in distilled water and incubated on a rotator for 4 h at room temperature followed by 4 °C overnight. The mixture was then spun through a centricon YM-10 (Millipore) 5 times to remove unconjugated NHS-biotin.

2.6. ELISA detection of hIgG1 and mIgG1 binding to shFcRn

Wells were coated with 100 μl of BSA-5-iodo-4-hydroxy-3-nitro-phenacetyl (NIP) conjugate ($NIP_{16}BSA$) at 1 μg/ml, and incubated overnight at 4 °C. They were then blocked with 2% skimmed milk for 1 h and washed four times with PBS/0.005% Tween 20 (PBS/T). Anti-NIP human (hIgG1) or anti-NIP mouse IgG1 (mIgG1) was added in concentrations ranging from 0.04 to 10 μg/ml, incubated for 1 h at room temperature and washed four times with PBS/T at pH 5.5. Biotinylated shFcRn WT was pre-incubated with streptavidin-ALP (Amersham) for 30 min at room temperature before the samples were dissolved into PBS/T at pH 5.5. The complexes were added to the wells and incubated for 1 h at room temperature. The plates were then washed four times with PBS/T at pH 5.5. 100 μl of the substrate ABTS/H_2O_2 (Sigma) was added to each well. The absorbance was measured at 405 nm using a Sunrise TECAN spectrophotometer (TECAN, Maennedorf, Switzerland). The same ELISA was done with PBS/T at pH 7.4 to observe the pH dependent interaction or at pH 5.5 with different concentrations (6.25–50 μg/ml) of *Staphylococcus aureus* protein A (Sigma).

2.7. Binding studies using surface plasmon resonance

SPR experiments were carried out using a Biacore 3000 (Biacore AB, Uppsala, Sweden). Flow cells of CM5 sensor chips were coupled with hIgG1 (~1200 RU) or shFcRn (~630 RU) using amine coupling chemistry as described in the protocol provided by the manufacturer. The coupling was performed by injecting 10 μg/ml of the proteins in 10 mM sodium acetate, pH 5.0 (Biacore AB). For all experiments, phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl, 0.005% Tween 20) at pH 5.5 or pH 7.4, or HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) at pH 7.4 (Biacore AB) were used as running buffer and dilution buffer, respectively. ShFcRn or HSA were injected over the flow cells in concentrations in the range of 0.012–4 μM and 0.135–20 μM, respectively, at a flow rate of 30–50 μl/ml at 25 °C. The same running buffer at pH 7.4 was used to "strip" the flow cells at the end of each dissociation phase. In all experiments, data were zero adjusted and the reference cell subtracted. All binding analyses were performed using BIAEvaluation.

3. Results

3.1. Cloning, prokaryotic expression and purification of hFcRn WT heavy chains

To establish a prokaryotic expression system, hFcRn encoding cDNA was amplified from the eukaryotic vector, such that the eukaryotic leader sequence was omitted, and the PCR product subcloned into pET28-HAT vector in frame of a 19 amino acid hexa-histidine tag (HAT-tag). A map of the hFcRn heavy chain construct is shown in Fig. 1A. The pET28-HAT-hFcRn construct was transformed into *E. coli* strains BL21-CodonPlus-(DE3)-RP, BL21-CodonPlus-(DE3)-RIL and DE21 (DE3). Pilot expression experiments showed high expression in all strains, but no significant increase in the modified strains compared to the BL21 (DE3) (data not shown). Thus, large scale fermentations were performed using the latter strain.

Human FcRn heavy chains were produced in a 2 l fermentor as recently described for the production of MHC class I heavy chains (Ferre et al., 2003). Expression was initiated with IPTG, and the temperature was set to 42 °C to increase the amount of protein expressed as inclusion bodies in the cytoplasmic space. Bacterial samples collected prior to and during induction were reduced and analyzed by SDS-PAGE as seen in Fig. 1B. The level of expression of hFcRn heavy chains before induction and after 1 h, 2 h and 3 h is shown. Human FcRn heavy chains migrated as bands corresponding to a molecular size of ~32 kDa. This corresponds well with the theoretical molecular mass calculated using the Antheprot 2000 program (data not shown). The fermentation yield obtained for the hFcRn heavy chain was estimated to be ~1.5 g per 2 l fermentation.

Inclusion bodies were solubilized in 8 M urea under non-reducing conditions to preserve the oxidation state of the extracted heavy chains. The extracted proteins were purified by Ni^{2+} NTA column purification and SEC as described in Section 2. Fractions collected after 8 M urea solubilization (lane 1) and after SEC purification (lane 2) were analyzed by SDS-PAGE (Fig. 1C). The resulting yield for the hFcRn WT heavy chain was ~200–300 mg per 2 l fermentation. $H\beta_2m$ was expressed in *E. coli* and purified as previously described (Ostergaard Pedersen et al., 2001; Ferre et al.,
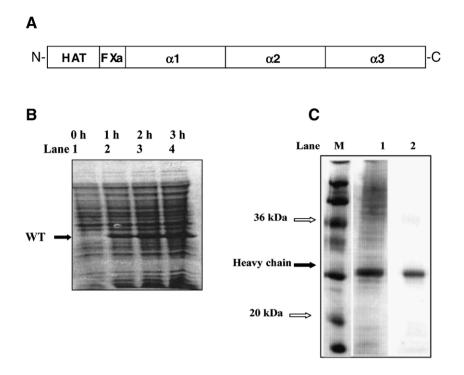
Fig. 1. Bacterial expression of the hFcRn heavy chain. (A) Schematic illustration showing the truncated hFcRn heavy chain fused N-terminally to a modified histidine tag (HAT). An FXa cleavage site is inserted between the HAT-tag and the hFcRn heavy chain. Locations of the three domains (α1, α2 and α3) are indicated. (B) Boiled and reduced 12% SDS-PAGE analyses of fermentation samples from bacterial expression of hFcRn heavy chain. Sample pellets were resuspended in buffer. After centrifugation, portions of supernatant were loaded directly on the SDS-PAGE gel. Lane 1, expression before induction; lanes 2–4, samples taken 1, 2 and 3 h after induction with IPTG, respectively. The position of the hFcRn heavy chain monomers is shown with black arrows. (C) Non-reducing SDS-PAGE analyses of 8 M urea solubilized inclusion body preparations containing hFcRn heavy chain WT (lane 1) and after purification on Ni^{2+} NTA and SEC (lane 2). Lane M, protein marker.

2003). The yield was ~300 mg per 2 l fermentation of refolded hβ_2m.

3.2. Large scale production of shFcRn WT by assisted in vitro refolding

To produce complete heterodimeric shFcRn, purified WT heavy chains were added to the refolding solutions containing excess purified and previously refolded hβ_2m (Burmeister et al., 1994a,b; Ferre et al., 2003). Fig. 2A shows the UV trace from the SEC separation of shFcRn WT. The results demonstrate SEC separation of three main peaks denoted EL1, EL2 and EL3. The corresponding fractions were analyzed by non-reducing SDS-PAGE as seen in Fig. 2B. The in vitro refolded protein was eluted as heterodimeric complexes containing FcRn heavy chain and hβ_2m (EL2) as well as free aggregated heavy chains (EL1) and residual hβ_2m (EL3). The total yield of shFcRn WT was 10–15 mg per fermentation, which corre-

sponds to 5% of the heavy chain added to the refolding mixture. The refolded and SEC purified heterodimeric shFcRn molecules corresponding to EL2 were pooled, concentrated and tested in SDS-PAGE (data not shown). To determine the correct molecular mass of both purified receptors, we performed mass spectrometry analyses. The mass spectra yielded two intense ion signals at mass values of ~11.7 kDa and ~32.6 kDa (data not shown). These correspond to the calculated values of the hβ_2m and hFcRn heavy chains, respectively.

3.3. Determination of structure and thermal stability

To obtain an indication as to whether the bacterially expressed and in vitro refolded shFcRn receptor was correctly folded, structural features and thermal stability were determined by CD analyses. As shown in Fig. 3A, the CD spectra for shFcRn WT exhibits the classical β-sheet signal designated by a negative peak
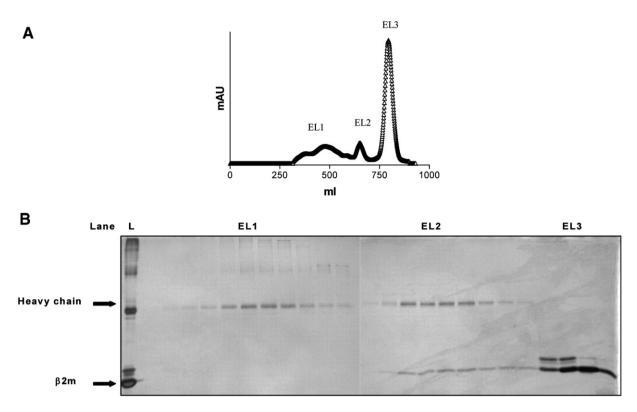
Fig. 2. Refolding of shFcRn. (A) The large scale *in vitro* refolding of shFcRn was concentrated and applied to SEC. The UV trace shows three main elution fractions. (B) Non-reducing SDS-PAGE analyses of collected fractions from SEC separation (EL1, EL2 and EL3). Lane L protein marker. The positions of heavy chains and hβ_2m are indicated by black arrows.

at 217–218 nm and a positive peak at 195–197 nm, suggesting that the receptor is primarily composed of β-structure with a minor α-helical contribution. The calculated secondary structural elements are shown in Table 2. The data are in good agreement with those previously reported for soluble rat FcRn (Burmeister et al., 1994a,b) and shFcRn (West and Bjorkman, 2000). Thus, the receptor seems to be correctly folded.

To determine the thermal stability, the procedure was performed at 200 nm while the temperature was increased from 25 °C to 97 °C. The melting profile is shown in Fig. 3B, and confirm the presence of a single unfolding transition for the receptor in this temperature range with a midpoint unfolding temperature of 58.5±1 °C.

3.4. Functional characterization of the refolded shFcRn

To investigate the functional integrity of the *in vitro* refolded shFcRn, we performed binding studies using ELISA and SPR. Binding to hIgG1 was detected in a pH dependent sandwich ELISA using BSA-NIP and NIP-specific hIgG1 as described in Section 2. The assay was performed under acidic conditions (pH 5.5) as well as at physiological pH (7.4). Biotinylated shFcRn was diluted in buffer with pH 5.5 or pH 7.4 and added to the pre-washed wells. Bound shFcRn was visualized by targeting the biotinylated receptor with streptavidin-ALP. Fig. 4A shows that bacterially produced and *in vitro* refolded shFcRn bound to hIgG1 in a concentration dependent manner at pH 5.5. In contrast, and as expected, no binding was detected at pH 7.4.

To prove that the binding of the bacterially produced shFcRn to hIgG1 was mediated by direct interaction with the hIgG1 Fc part, shFcRn was added together with different concentrations of the *Staphylococcus aureus* protein A. Protein A is capable of binding with high affinity to the Fc CH_2–CH_3 interface of immunoglobulins from a large number of species, including human IgGs. Fig. 4B demonstrates the competitive binding as decreased pH dependent binding to hIgG1 of shFcRn in the presence of an increasing amount of protein A.

Recent data show that hFcRn binds IgG from a limited number of species while mouse FcRn does not show this stringency (Ober et al., 2001; Zhou et al., 2005). The bacterially produced shFcRn preparation also shows stringent species dependent binding as

binding to NIP-specific mIgG1 was demonstrated in the manner described above (Fig. 4C). The ELISA shows the expected stringency with no functional binding at pH 5.5.

To obtain more quantitative data, surface plasmon resonance was performed. Initially, shFcRn samples were tested by size exclusion high performance liquid chromatography and no aggregates were observed (results not shown). Functional binding to anti-NIP hIgG1 was measured by injecting 1 μM of shFcRn WT over immobilized hIgG1 (Fig. 4D). The sensorgram demonstrates reversible pH dependent binding at pH 5.5. The same experiment was performed at pH 7.4 with no detectable binding. Furthermore, the equilibrium constants (K_D) for the interaction between the receptor and ligands were calculated from the resonance profiles for near equilibrium or equilibrium binding levels using BIAEvaluation. Here, a series of concentrations (0.012–4 μM) of shFcRn was injected over immobilized IgG at

Table 2
Secondary structures of the shFcRn WT

	shFcRn (WT) (%)
Helix	14.6
Anti-parallel	46.1
Parallel	6.5
Beta-turn	11.7
Random coil	21.1
Total	100.0

pH 6.0 as described above. The K_D of the interaction of hIgG1 with shFcRn was determined to be $(1.7 \pm 2.5) \times 10^{-6}$ M, which agrees well with the values determined by others (Firan et al., 2001; Zhou et al., 2005). To mimic the physiological situation where FcRn is a transmembrane receptor binding circulating soluble IgG, shFcRn was immobilized and hIgG injected. This gave the same pH dependent binding profile. Moreover, the affinity was dramatically increased to the nanomolar range (data not shown). This phenomenon has previously been reported by others (West and Bjorkman, 2000; Bitonti et al., 2004).

To asses whether the bacterially produced shFcRn binds HSA, shFcRn was covalently immobilized on a CM5 chip and increasing concentrations of HSA (0.13–20 μM) were injected. Fig. 4E shows a typical reversible binding profile when 20 μM HSA was injected at pH 5.5. No binding was observed at pH 7.4. The binding data was fitted to the simple Langmuir 1:1 model supplemented with the BIAEvaluation Wizard, and this calculation gave a K_D of $(5.6 \pm 0.1) \times 10^{-6}$ M. This is in agreement with reported affinity (Andersen et al., 2006; Chaudhury et al., 2006). The derived K_D for the shFcRn–HSA interaction is in the same range as for the hFcRn–hIgG interaction when IgG is immobilized on the chip. Thus, the bacterially expressed and *in vitro* refolded shFcRn is functional as shown by pH dependent and reversible binding to its ligands.

4. Discussion

FcRn is functional as a soluble molecule, making shFcRn suitable for interaction studies and structural analyses. Thus, the aim of this study was to establish a cost-effective bacterial expression system to generate large amounts of such shFcRn molecules that display the biochemical and immunological properties of molecules produced in eukaryotic cells.

Initially, we expressed the two subunits of shFcRn in *E. coli* as inclusion bodies, and they were extracted and
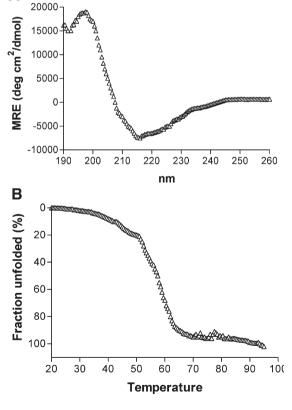

Fig. 3. CD structure and stability analyses of shFcRn. (A) Analyses of the secondary structural elements of recombinant refolded shFcRn WT (△) was monitored by CD measurements. (B) Thermal denaturation profiles of shFcRn WT (△) was monitored by CD measurements at 200 nm. The graph is normalized and shows T_m value for the refolded receptor at 58.5±1 °C.
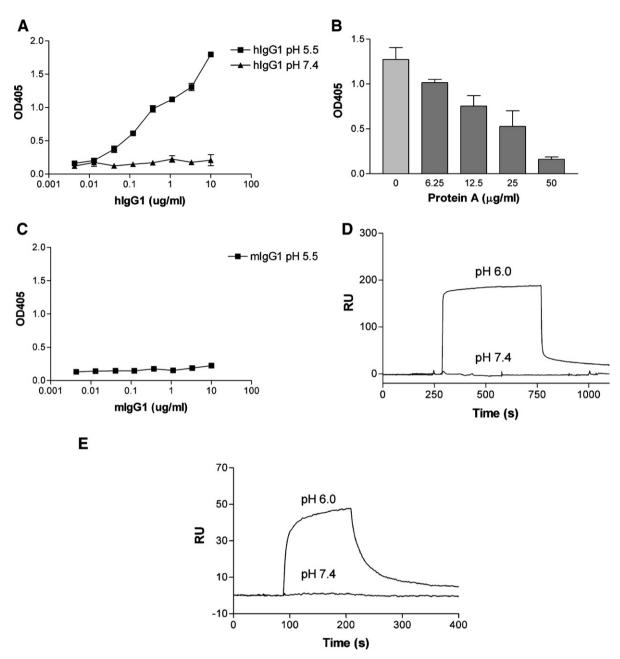
Fig. 4. Functional characterization of refolded shFcRn. (A) ELISA analyses of refolded shFcRn with hIgG1 at pH 5.5 and pH 7.4. (B) ELISA binding analyses of refolded shFcRn with hIgG1 at pH 5.5 in the presence of different concentrations of *Staphylococcus aureus* protein A. (C) ELISA binding analyses of refolded shFcRn with mIgG1 at pH 5.5. (D) SPR analyses of shFcRn binding to hIgG1 at pH 5.5 and pH 7.4. (E) SPR analyses of shFcRn binding to HSA at pH 5.5 and pH 7.4.

purified independently. Surprisingly, the fermentations gave rise to gram levels per litre of hFcRn heavy chains without prior codon optimization. High yields were obtained using a fermentation strategy previously described for MHC class I heavy chains (Ferre et al., 2003). Produced inclusion bodies were extracted and purified in 8 M urea buffer. This process disrupts the

tertiary structure of the proteins, but keeps the preformed disulfide bonds intact. The refolding process is complicated by the fact that hFcRn heavy chains cannot fold to their native state in the absence of $h\beta_2m$. Thus, they were diluted into a solution containing an excess of $h\beta_2m$. After SEC separation, clear fractions of heterodimeric shFcRn appeared. Thus, the

heterodimeric receptor refolded efficiently when a reducing step was omitted.

The total yield of refolded shFcRn WT was ~7 mg/l. In comparison, Gastinel et al. (1992) described production of soluble rat FcRn in Chinese hamster ovary cells using gene amplification in the presence of methotrexate, followed by clone selection where the selected clone produced 40 mg/l supernatant. Alternatively, the yield from the baculovirus system based on production in High 5 insect cells was 5 mg/l after virus amplification and screening (Popov et al., 1996). Both approaches are costly and time-consuming, while the bacterial system described here is cost-effective and rapid (~2 weeks). Notably, it gives functional refolded shFcRn without the need for stable transfections.

The secondary structure elements of the *in vitro* refolded molecule were determined using CD measurements and showed a similar composition to native purified shFcRn as described previously (Burmeister et al., 1994a,b; West and Bjorkman, 2000). Furthermore, we were able to reproduce the characteristic pH dependent reversible binding to hIgG1 at pH 6.0, with no specific binding at pH 7.4 in ELISA. The same result was achieved using Biacore technology, where shFcRn was injected over immobilized hIgG that yielded affinity in the micromolar range. Likewise, injected IgG bound immobilized shFcRn with the same affinity as previously described for soluble receptor expressed in a eukaryotic system. In addition, we show pH dependent binding to HSA in the same fashion as for the FcRn–IgG interaction.

To investigate whether the HAT-tag used for purification influenced ligand binding, we attempted to remove the HAT-tag using the FXa cleavage site inserted in frame and between the HAT-tag and the N-terminal of the hFcRn heavy chain. Surprisingly, the WT FcRn heavy chain itself was cleaved. Following this observation, the same procedure was applied to a shFcRn mutant (C48S/C251S) produced in the same system (manuscript in preparation). In this case, the tag was removed, and the cleaved shFcRn variant showed the same pH dependent IgG and HSA binding profiles as the tagged versions. Furthermore, the same affinities were derived from surface plasmon resonance evaluations. This indicates that the tag does not contribute to ligand binding.

The three-dimensional structures of hFcRn and rodent hFcRn are very similar (Burmeister et al., 1994a,b; West and Bjorkman, 2000). However, there are a number of differences at the interface of FcRn that contact the IgG Fc part. The most notable is a substitution of L135 (hFcRn) for a D137 (mouse

FcRn). This residue and surrounding residues have recently been shown to contribute to the characteristic stringent binding of hFcRn to different IgG species in contrast to the mouse form of FcRn (Ober et al., 2001; Zhou et al., 2005). We were able to reproduce this stringency with bacterially produced shFcRn. Importantly, a significant difference between hFcRn in eukaryotic cells and the bacterially produced receptor is that the latter preparation necessarily is non-glycosylated, due to lack of a glycosylation system in bacteria. However, the preparations were fully functional.

Thus, the hFcRn preparation described here can be used to measure the binding properties of recombinant antibodies or other FcRn binding molecules to predict their *in vivo* pharmacokinetics. In conclusion, heterologous expression and *in vitro* refolding proved a convenient method. Importantly, the strategy may prove successful as a platform for the production of other eukaryotic molecules, such as MHC class I related molecules.

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