Expression and crystallization of a soluble and functional form of an Fc receptor related to class I histocompatibility molecules

(IMMUNOglobulin receptor/protein engineering/amplifiable expression system/circular dichroism)

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ABSTRACT Maternal transport of immunoglobulin to the newborn mammal is important for immune defense during the first weeks of independent life. Receptors for the Fc portion of IgG mediate the transfer of immunoglobulin from milk to the bloodstream of newborn mice and rats, by passage through intestinal epithelial cells. Neonatal Fc receptors (FcRn) isolated from intestinal epithelial cells of sucking rats bear a striking resemblance to class I histocompatibility molecules. The heavy chain of FcRn has sequence similarity in three extracellular domains to the corresponding domains of class I molecules, and the light chain of both types of molecules is B2-microglobulin. To facilitate biochemical characterization and crystallization of FcRn, we have expressed a secreted form, as well as two different lipid-linked forms solubilizable by phospholipase treatment. The lipid-linked forms are heterodimers consisting of B2-microglobulin and the extracellular portion of the heavy chain and are anchored to the membrane by a phosphatidylinositol linkage attached to either the heavy chain or B2-microglobulin. Cells expressing either lipid-linked form bind rat Fc, reproducing the known physiological pH dependence of binding. Secreted FcRn has been purified in yields up to 40 mg/liter from cell supernatants. Circular dichroism spectra of soluble FcRn appear similar to spectra of class I MHC molecules, suggesting that the similarities in primary sequence extend also to a similarity in secondary structure. Soluble FcRn crystallizes in a form amenable to a structure determination by x-ray diffraction methods, which will ultimately allow a detailed comparison of the two types of molecules.

Newborn rats acquire humoral immunity through ingestion of milk containing maternal IgG. Fc receptors on the intestinal epithelial cells of the neonate (FcRn) bind IgG from milk at acidic pH (6.0–6.5) and transport it to the basolateral membrane at the serosal side of the cell, where the IgG dissociates at a pH of 7.4. FcRn has been affinity-purified from rat intestinal epithelial cell brush borders and shown to be a heterodimer consisting of two polypeptide chains of relative molecular masses 45,000–53,000 (p51) and 14,000 (p14) (1). The light chain is B2-microglobulin (B2-m), a soluble protein also found complexed to the heavy chains of class I major histocompatibility complex (MHC) molecules. The similarity between FcRn and histocompatibility molecules also extends to the p51 subunit, which consists of three extracellular domains, all with sequence similarity to the corresponding domains of class I MHC proteins, and an unrelated cytoplasmic domain (2, 3). Class I MHC molecules bind short peptides derived from intracellular proteins, which are transported along with the MHC molecule to the cell surface, and stable cell surface expression appears to depend upon peptide binding (4–7). Crystalllographic analyses of the peptide-binding sites of three human class I MHC molecules have revealed extra electron density presumably corresponding to images of bound endogenous peptides (8–10). It would be of interest to determine whether the structurally related FcRn molecule is also transported to the cell surface with endogenous peptide(s) and whether FcRn requires a bound peptide for structural stability.

Here we report the expression of two different lipid-linked forms and one secreted form of FcRn in a glutamine synthetase-based amplifiable expression system developed at Celltech, Berkshire, U.K. (11). Both lipid-linked forms reproduce the physiological pH dependence of Fc binding and can be released as soluble molecules from the membrane with phosphatidylinositol-specific phospholipase C (PI-PLC). Soluble FcRn can be purified from cell supernatants by affinity chromatography on a rat IgG column at pH 6.5, with elution of the protein at pH 8.0. Crystals of soluble FcRn diffract to 2.9-Å resolution.

MATERIALS AND METHODS

Reagents. Rat IgG, fluorescein-conjugated rat Fc, and phycoerythrin-conjugated F(ab')2 fragments of anti-rabbit IgG were from Jackson ImmunoResearch. Fluorescein-conjugated goat anti-mouse was from Cappel Products. Goat anti-rabbit IgG-peroxidase conjugate and purified rabbit anti-human B2-m IgG for Western blots were from Boehringer Mannheim. CNBr-activated Sepharose was from Pharmacia. Endoglycosidase F/N-glycosidase F was from Boehringer Mannheim. Methionine sulfoximine (MSX), phospholipase C, and lentil lectin-Sepharose 4B were from Sigma. Dulbecco's modified Eagle's medium (DMEM), a minimum essential medium (αMEM), and dialyzed fetal bovine serum were from Irvine Scientific and Gibco/BRL. Anti-p51 is a rabbit polyclonal antiserum against the FcRn heavy chain (2). 2B10C11, a mouse monoclonal antibody against rat B2-m, was the gift of Lennart Lögdberg (Sandoz Pharmaceutical).

Construction of Lipid-Linked and Soluble Forms of FcRn. Molecular biological experiments were performed by methods (12). The p51-DAF and B2-m-DAF chimeras were constructed by methods similar to those used for the expression of a lipid-linked form of the T-cell antigen receptor (13). The chimeric proteins consisted of the phosphatidylinositol (PI)-anchoring signal of decay-accelerating factor (DAF; residues 311–347; ref. 14) fused C-terminal to amino acid 269 of the FcRn heavy chain or to the final amino acid of B2-m. Briefly, a BamHI fragment containing the full-length FcRn (2) or rat B2-m cDNA (N.E.S., unpublished data) was inserted 5' to the DNA segment encoding the DAF PI-anchoring signal (14) cloned in pBluescript SK(−) (Stratagene). Oligonucleotide-directed in vitro deletional mutagenesis (15) generated an

Abbreviations: FcRn, neonatal Fc receptor(s); B2-m, B2-microglobulin; MHC, major histocompatibility complex; PI-PLC, phosphatidylinositol-specific phospholipase C; DAF, decay-accelerating factor; MSX, methionine sulfoximine.

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in-frame fusion between the FcRn heavy chain (p51-DAF) or βm (βm-DAF) and the PI-anchoring signal. Truncated FcRn heavy chain was created by inserting a stop codon after amino acid 269 (p51-stop) (Fig. 1A).

The modified cDNAs were introduced as Xho I–Not I fragments into the unique Xho I and Not I sites in the polylinker of the expression vector pBJ5-GS, a derivative of pBJ1-Neo (13, 16). pBJ5-GS employs the glutamine synthetase gene as a selectable marker and means of gene amplification in the presence of the drug MSX, a system developed by Celltech (11). (The glutamine synthetase minigene was removed from the expression vector pSVLGS1 obtained from Celltech and was cloned into the unique Sal I site of pBJ5 to create pBJ5-GS.) The p51-stop gene and the unmodified βm gene were also cloned into pBJ1, so that a Sal I fragment containing the desired gene as well as the SRE promoter and the simian virus 40 polyadenylation signal could be cloned into pBJ5-GS containing the appropriate partner gene. Three such vectors were created: pBJ5-GS/p51-DAF/βm, to express PI-linked FcRn heavy chain with soluble βm; pBJ5-GS/p51-stop/βm-DAF, to express truncated (soluble) FcRn heavy chain with PI-linked βm; and pBJ5-GS/p51-stop/βm, to express secreted heterodimer (Fig. 1B).

Production of the Three FcRn Forms in CHO Cells. The expression vectors were transfected into CHO cells by a calcium phosphate procedure (Stratagene). Selection and amplification of the glutamine synthetase gene were carried out according to the protocol established by Celltech. Briefly, a calcium phosphate/DNA precipitate containing 30 µg of pure DNA, or no DNA as a mock control, was added to CHO cells in 10 ml of fresh DMEM with serum. The next day, the cells were washed three times with aMEM without serum and then incubated in aMEM with 10% dialyzed fetal bovine serum and 25 µM MSX. Clones were visible after 2 weeks and were isolated and grown in 24-well plates in aMEM with 10% dialyzed fetal bovine serum and 25 µM MSX. The cloning procedure was repeated, and clones transfected with the two lipid-linked forms of FcRn were tested for expression of both protein subunits by immunofluorescence with anti-p51 and 2B10C11. Positive clones were put in six-well plates and submitted to increasing MSX concentrations from 50 to 500 µM. Clones were also tested for their ability to differentially bind rat Fc at pH 6.5 as follows: 106 cells in suspension were incubated for 1 hr at room temperature in 500 µl of phosphate-buffered saline (pH 6.5 or pH 8.0) with fluorescein-conjugated rat Fc (0.5 µM) and then washed twice. The cell pellet was resuspended in 1 ml of buffer at the appropriate pH and analyzed by flow cytometry (Ortho cell sorter). Supernatants from CHO clones transfected with pBJ5-GS/p51-stop/βm were tested for secreted FcRn heterodimer by Western blotting with anti-p51 and a rabbit anti-human βm antiserum (crossreactive with rat βm).

Phospholipase C Treatment. Cleavage of lipid-linked proteins was done as described (13), but using a stock of phospholipase C containing PI-PLC (Sigma P6135; 1 mg/ml), which was added to 105 cells at a 100-fold dilution. Cells were incubated for 2 hr at 37°C.

Purification of Soluble FcRn by Affinity Chromatography and Biochemical Analysis. Rat IgG (70 mg) was covalently linked to 7 ml of CNBr-activated Sepharose (10 mg/ml) according to the manufacturer’s directions. The pH of 100–500 ml of supernatant from a CHO clone secreting FcRn heterodimer growing in 100 µM MSX was decreased to pH 6.5 and put on the column at a constant flow rate of 20 ml/hr at 4°C, and the column was washed with 300 ml of 50 mM sodium phosphate, pH 6.5/0.05% NaN3. Elution of FcRn was initiated with 50 mM sodium phosphate, pH 8.0/0.05% NaN3. Fractions of 2 ml were collected and their optical density at 280 nm measured. Typically, ~10 µg of FcRn heterodimer [quantified by bicinchoninic acid (BCA) assay; Pierce] was eluted in six fractions from 250 ml of supernatant harvested from 106–107 cells. The protein was concentrated by Centricon 10000 filters. Deglycosylation of 10 µg of purified protein was done for 16 hr in 50 mM Hepes (pH 7.5) at 37°C with 0.5 unit of endoglycosidase F/N-glycosidase F.

N-Terminal Sequencing. N-terminal sequencing was performed on 20–40 µg of purified soluble FcRn in a phosphate buffer dried on a poly(vinylidene difluoride) membrane (17) and inserted into an Applied Biosystems model 4778 sequencing reaction cartridge.

CD Spectra. A Jasko J-600 spectropolarimeter was used in the wavelength range of 190 to 260 nm with a 0.1-mm cell. Purified HLA-B40 (gift of D. Wiley and Anastasia Haykov, Harvard University) and soluble FcRn heterodimer were concentrated to 0.1–0.5 mg/ml in 20 mM sodium phosphate.
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Crysalization of Soluble FcRn. Crystals were grown by vapor diffusion from protein solutions (20 mg/ml) in 10 mM Pipes (pH 6.5/0.05% NaN3, in 2-μl droplets with 24.0% (wt/vol) polyethylene glycol 3350, 2.0% (wt/vol) saturated ammonium sulfate, and 100 mM Pipes (pH 6.5).

RESULTS

Expression of Two Lipid-Linked Forms of FcRn. An expression vector (Fig. 1B) containing the hamster glutamine synthetase gene (11) (for selection and amplification), the gene encoding the extracellular portion of the FcRn heavy chain fused in-frame to the cDNA from DAF encoding its lipid attachment signal (14), and the rat β2m gene was transfected into CHO cells. Stable lines were generated and the transfected gene was amplified by selection with MSX. Cell surface expression of FcRn heavy and light chains was detected by immunostaining with anti-p51 and anti-rat β2m antibodies and was reduced after treatment with PI-PLC (Fig. 2 B1 and B2). CHO cells expressing lipid-linked FcRn heterodimers bound rat Fc, and the physiological pH dependence of Fc binding was reproduced, in that binding was observed at pH 6.5 but not at pH 8.0 (Fig. 2D2). Binding was inhibited by addition of unlabeled rat IgG or rat Fc at 100 μg/ml, but not by the equivalent amount of an unrelated protein (data not shown). Expression of the lipid-linked FcRn heavy chain in the absence of rat β2m was at a level comparable to the expression in the presence of rat β2m (data not shown), but the binding of fluorescein-conjugated rat Fc was diminished (Fig. 2D4).

The second lipid-linked form of FcRn was expressed by transfecting a plasmid containing the DNA encoding the DAF PI-attachment signal (14) fused to the rat β2m gene, together with the gene encoding the FcRn heavy chain truncated with an in-frame stop codon after amino acid 269 (p51-stop), and the glutamine synthetase gene (Fig. 1B). Stable CHO lines expressing this lipid-linked form of FcRn were generated by selection with MSX and stained with the anti-p51 and anti-rat β2m antibodies. Staining was reduced after treatment with PI-PLC (Fig. 2 C1 and C2). The FcRn/β2m-DAF heterodimers also showed the expected pH dependence of Fc binding (Fig. 2D3). Expression levels of the two forms of lipid-linked FcRn heterodimers appeared comparable (Fig. 2 B1 and C1).

Expression of Secreted FcRn. An expression vector (Fig. 1B) containing the glutamine synthetase gene, the truncated FcRn heavy chain gene, and the rat β2m gene was transfected into CHO cells. Media collected from individual clones were assayed for secretion of FcRn heterodimers by Western blotting aliquots of supernatants with antibodies specific for the heavy and light chains. Positive clones were amplified with increasing amounts of MSX. Medium (250 ml) from a high-expressing clone growing in 100 μM MSX was adjusted to pH 6.5 and passed over a rat IgG affinity column. The column was extensively washed, and FcRn heterodimers were eluted by changing the pH to 8.0, yielding ~10 mg of purified protein. The high-expressing CHO clone was grown in a Cell Pharm II hollow-fiber bioreactor device (Unisyn Fibertec, San Diego) in the presence of 100 μM MSX. The yield of soluble FcRn was typically 7–10 mg per day harvest.

Purified FcRn was analyzed by SDS/17.5% PAGE (Fig. 3). Under either reducing or nonreducing conditions, two bands were detected: a sharp band of apparent molecular mass 13 kDa, corresponding to β2m, and a broad diffuse band at ~43 kDa, corresponding to the truncated FcRn heavy chain. Treatment of purified FcRn with a mixture of endoglycosidase F and N-glycosidase F had no effect on the apparent molecular mass of β2m, but the majority of the heavy chain shifted its position of migration to 30 kDa, in close agreement with the predicted molecular mass of the unmodified truncated heavy chain (30,274 daltons). Glycosidase-treated FcRn did not bind to a goat anti-rat IgG antibody (data not shown), also suggesting that all carbohydrate moieties were removed. These data suggest that ~13 kDa of the extra molecular mass was due to N-linked glycosides, a figure that is not inconsistent with the utilization of all four potential N-linked glycosylation sites in the FcRn heavy chain sequence (2). Deglycosylated soluble FcRn retained its ability to bind to the Fc affinity column (data not shown), suggesting that the

![Fig. 2](image-url) Flow cytometric analyses of CHO cells transfected with lipid-linked forms of FcRn. (A1, B1, and C1) Staining of cells with anti-p51 (secondary antibody is phycoerythrin-conjugated F(ab')2 fragment of anti-rabbit IgG). (A2, B2, and C2) Staining by 2B1OC11 (anti-rat β2m; secondary antibody is fluorescein-labeled goat anti-mouse IgG). —, Primary antibody alone. —, Primary and secondary antibodies. —, Both antibodies, cells treated with phospholipase C. (Because the anti-p51 serum is cytotoxic for the transfected cells, the difference in staining after phospholipase C treatment is difficult to detect and is shown only for staining by 2B1OC11.) Cells were untransfected (A1 and A2) or were transfected with p51-DAF/β2m (B1 and B2) or p51-stop/β2m-DAF (C1 and C2). (D1–D4) Binding of fluorescein-labeled rat Fc to transfected cell lines at pH 8 (----) versus pH 6.5 (-----). Cells were untransfected (D1) or were transfected with p51-DAF/β2m (D2), p51-stop/β2m-DAF (D3), or p51-DAF (D4).
carbohydrate moieties were not involved in the interaction between FcRn and Fc.

Purified soluble FcRn was subjected to N-terminal sequence analysis to verify the origin of the β2m species associated with the FcRn heavy chain and to look for possible peptides associated with the FcRn heavy chain. Since the sequence of hamster β2m was previously unknown, a cDNA library was constructed from CHO cell RNA, and a hamster β2m clone was isolated and sequenced (L.N.G., unpublished data; EMBL data library, accession no. X57112). The first 16 residues of bovine (20) and rat (21) β2m differ at amino acid residues 3, 4, and 6 (of the mature protein), and the sequences of hamster and rat β2m differ at residues 3, 4, 7, and 11. Two N-terminal sequences in equimolar amounts were identified in the FcRn sample: the sequence AEPLRPLMYH-LAAVSD, corresponding to the first 16 amino acids of the mature FcRn heavy chain (3), and the sequence IQKTPQIQVYSKHPPE, corresponding to the sequence of the first 16 residues of mature rat β2m (21). No evidence of sequences corresponding to bound peptides was seen.

**CD Spectral Comparison of Soluble FcRn and MHC Class I Proteins.** Analysis of CD spectra of HLA-B40 and soluble FcRn (Fig. 4) by a nonrestrained least squares fitting procedure indicated a dominant β-strand component with a minor helical contribution, similar to that reported previously (22, 23) for HLA-B7 and HLA-A2, although lower amounts of β-structure were predicted for other class I MHC molecules from CD spectral analyses (23, 24). The percent helix estimated from our analysis of the spectrum of HLA-B40 agrees well with the amount found in class I MHC crystal structures (8–10) (~20% helix), but our CD analysis predicts a higher content of β-structure than is found in the crystal structures (8–10) (~42% β-structure). We have noted a discrepancy in calculated β-structure by using another method of analysis (28), but both methods suggest that FcRn and HLA-B40 have similar percentages of secondary structural elements.

**Crystals of Soluble FcRn.** Soluble FcRn protein forms crystals of approximate dimensions 0.3 mm × 0.1 mm × 0.1 mm in space group C222 (Fig. 5). The unit cell dimensions are a = 126.4 Å, b = 191.7 Å, and c = 149.6 Å. The asymmetric unit of the crystal is estimated to contain two to four molecules based on average volume to mass ratios (V/m) of protein crystals (25), representing solvent contents between 73% (if two molecules per asymmetric unit) to 32% (if four molecules per asymmetric unit). The crystals diffract to 3.5-A resolution using nickel-filtered CuKα radiation from a rotating-anode x-ray generator but suffer from radiation damage such that data can be collected only to ~4-A resolution. The use of a synchrotron x-ray source (SSRL, Stanford University) greatly improves the resolution limit, and a complete native data set to 2.9-A resolution has been collected. Single crystals of a complex between FcRn and Fc have also been obtained (A. H. Huber, L.N.G., and P.J.B., unpublished results).

**DISCUSSION**

As a first step in a biochemical and structural analysis to address questions raised by the intriguing structural similarity between FcRn and MHC class I molecules, we have expressed large quantities of three different forms of soluble FcRn. A strategy for secretion of a normally membrane-bound heterodimeric glycoprotein by deletion of the transmembrane region through introduction of an in-phase translation stop codon has not always proved successful. Therefore we initially constructed two different lipid-linked forms of FcRn, which can be solubilized by treatment with PI-PLC. The first form was anchored to the membrane by attaching the lipid-anchoring signal from DAF (14) following amino acid 269 of the FcRn heavy chain and was expressed together with rat β2m in CHO cells, using a glucose synthetase-based amplifiable expression system (11). [Residue 269 is the counterpart of the MHC class I residue 274, which is the last residue of the class I a3 domain (2).] The second lipid-linked form of FcRn consisted of the FcRn heavy chain truncated after residue 269, which was expressed together with rat β2m anchored to the membrane via a lipid-anchoring signal attached to its C-terminal residue. With this form of FcRn, we hoped to address the potential problem of the exchange of rat β2m for bovine β2m in the medium or for hamster β2m inside the CHO cells. Class I MHC molecules on the surface of cultured cells associate with heterologous β2m from serum.
(26). It is thus conceivable that the structurally similar FcRn molecules might do the same, resulting in a heterogenous population of cells expressing a truncated class I MHC heavy chain asso- ciated with a PI-PLC anchored form of β2m might also be a method of producing a homogeneous population of soluble class I MHC heterodimers.

Expression of the FcRn heavy chain and rat β2m was detected at the cell surface of CHO clones expressing either bovine or hamster PL-PI-PLC treated with rat β2m or with bovine or hamster PI-PLC treatment of cells expressing a truncated class I MHC heavy chain associated with a PI-anchored form of β2m might also be a method of producing a homogeneous population of soluble class I MHC heterodimers.

Expression of the FcRn heavy chain and rat β2m was detected at the cell surface of CHO clones expressing either anti-p51 antisemur (2) and a monoclonal antibody specific for rat β2m. Staining was diminished by treatment of cells with PI-PLC (Fig. 2 B1, B2, and C2). In the case of the first lipid-linked form of FcRn, it is possible that some of the cell surface rat β2m detected is associated with hamster class I MHC molecules, but not at pH 8.0 (Fig. 2D–4), reproducing the physiological pH dependence of Fc binding, and confirming that the presence of the PI anchor on either of the polypeptide chains does not interfere with proper heterodimer formation or Fe binding. Diminished binding of rat Fe to lipid-linked heavy chains transfected in the absence of rat f32m (Fig. 2D–4) suggests that the FcRn molecule is not fully functional in the absence of rat β2m, even when paired with a hamster or bovine β2m light chain.

Because the truncated p51 FcRn heavy chain was capable of being transported to the surface of the cell, and because little or no exchange of rat β2m with bovine or hamster β2m occurred, we next attempted to make a secreted form of FcRn. The gene encoding the truncated FcRn heavy chain was transfected together with the rat β2m gene, again using the Celltech glutamine synthetase-based amphilic expression system. With this expression system, a high level of expression can be obtained after an initial selection, and gene amplification is rapidly achieved. Clones secreting soluble FcRn heterodimer were evaluated for relative expression levels by analysis of supernatant samples on Western blots, and a high-expressing clone secreting FcRn at 40 mg/liter was selected at 100 μM MSX. FcRn was purified on a rat IgG affinity column by passing supernatants over the column at pH 6.5 and eluting protein at pH 8.0. This purification scheme is gentle, taking advantage of the physiological pH dependence of Fc binding, thereby avoiding the harsh elution conditions necessary for elution of immunoaffinity columns.

SDS/PAGE analysis of purified FcRn showed two bands corresponding to truncated heavy chain and β2m. No evidence of covalent dimerization of the FcRn heavy chain mediated by a disulfide bond (1) was seen by comparison of the mobility under reducing and nonreducing conditions, and purified FcRn was eluted from a gel filtration column at the position expected for a complex of a single heavy and light chain (data not shown). Microsequencing of FcRn heterodimer revealed the expected N-terminal residues of the heavy chain and rat β2m in equimolar amounts, suggesting that β2m exchange either was minimal or did not occur. Because the structurally related class I MHC molecules are transported to the cell surface with peptides derived from intracellular proteins and appear to depend upon the presence of bound peptide for structural stability (4–7), we were interested to see whether FcRn heterodimers showed evidence of bound peptide. No other sequences were detectable, suggesting that the FcRn heterodimer was not complexed with a unique peptide. A mixture of peptides would probably not have been detected and cannot be ruled out as a possibility.

Samples of soluble FcRn and the human class I MHC molecule HLA-B40 were analyzed by CD spectroscopy (Fig. 4). Analysis of CD spectra of the class I molecule was reasonably consistent with the known class I x-ray structures (8–10) and with previously reported CD spectra (22, 23), suggesting that the molecule is primarily composed of β-structure with a minor α-helical contribution. The CD spectrum of soluble FcRn appears similar, which together with the primary sequence similarity (2) further suggests that the two types of molecules may adopt similar tertiary structures. X-ray structural analysis of single crystals of soluble FcRn should ultimately allow the comparison of the two related molecules at atomic resolution and facilitate greater knowledge of the function of FcRn.

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