Stoichiometry of the Interaction between the Major Histocompatibility Complex-Related Fc Receptor and Its Fc Ligand†

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ABSTRACT: The neonatal Fc receptor (FcRn) transports immunoglobulin G (IgG) across epithelia, providing passive immunity and protecting serum IgG from degradation. For both functions, FcRn binds to IgG at the acidic pH of intracellular vesicles (pH ≤ 6.5) and releases IgG at the basic pH of the bloodstream (pH ∼ 7.4). Crystallographic studies show that rat FcRn can interact with the Fc portion of IgG in a repeating array in which FcRn dimers are bridged by Fc fragments to create an "oligomeric ribbon" with a 2*n*:*n* stoichiometry. The stoichiometry of the interaction between soluble FcRn and Fc has been reported as either 2:1 for rat FcRn [Huber et al. (1993) *J. Mol. Biol. 230,* 1077−1083] or 1:1 for mouse FcRn [Popov et al. (1996) *Mol. Immunol. 33,* 521−530]. To ascertain the reasons for this difference, we analyzed complexes formed in solution between soluble rat or mouse FcRn and Fc. Using a gel-filtration assay under nonequilibrium conditions, we find that both forms of FcRn produce 2:1 receptor−ligand complexes, but that alterations of the carbohydrate moieties on mouse FcRn can result in an apparent stoichiometry of 1:1. However, under equilibrium conditions, all forms of FcRn make complexes with a 2:1 stoichiometry. We conclude that rat and mouse FcRn share the same general ligand binding properties but that small differences in affinities can produce apparent differences under nonequilibrium conditions.

The neonatal Fc receptor (FcRn)[1] transports maternal immunoglobulin G (IgG) in ingested milk to the bloodstream of newborns and protects serum IgG from a default degradative pathway (reviewed in refs *1*−*3*). For both functions, FcRn binds IgG at the acidic pH of intracellular vesicles (pH < 6.5), but shows no detectable binding at the pH of blood (pH 7.4), where it releases IgG. Previous crystallographic and biochemical studies suggested that FcRn dimerizes in response to IgG binding (*4*−*7*), which could serve as a component of a trafficking signal for FcRn/IgG complexes. An oligomeric ribbon of FcRn dimers bridged by Fc molecules was visualized in the low-resolution FcRn/Fc cocrystal structure (*5*). In intracellular vesicles, the ribbon would consist of FcRn dimers on adjacent membranes bridged by IgG molecules. We hypothesized that formation of this ribbon is required for FcRn-mediated transport of IgG (*5*, *8*).

The oligomeric ribbon observed in the FcRn/Fc cocrystal structure has a 2*n*:*n* receptor−ligand stoichiometry (*5*), consistent with the 2:1 stoichiometry observed in solution for complexes between soluble rat FcRn (rFcRn) and IgG (*9*). By contrast, a soluble version of mouse FcRn (mFcRn) produced in insect cells forms a 1:1 complex with IgG or Fc (*10*). Because the stoichiometry of the interaction between FcRn and its ligand is relevant to interpreting its function in vivo, we analyzed complexes of soluble rFcRn or mFcRn produced in mammalian cells with Fc. Using a gel-filtration assay under nonequilibrium conditions, we find that both rFcRn and mFcRn form 2:1 receptor−ligand complexes but that mFcRn containing high-mannose rather than complex N-linked carbohydrate moieties can form 1:1 complexes. Under equilibrium conditions, however, all forms of FcRn form 2:1 complexes. The ability of soluble FcRn to form 1:1 and 2:1 complexes in solution is discussed in terms of FcRn's function in vesicular trafficking.

MATERIALS AND METHODS

Expression and Purification of FcRn. A soluble version of rFcRn was expressed in Chinese hamster ovary (CHO) cells as previously described (*11*). This version is a heterodimer consisting of residues 1−269 of the mature rFcRn heavy chain associated with rat β2-microglobulin (β2m). rFcRn was isolated from the supernatants of transfected CHO cells by a functional purification involving pH-dependent binding to IgG affinity columns, in which rFcRn binds to

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[1] Abbreviations: β2m, β2-microglobulin; CHO, Chinese hamster ovary; endo H, endoglycosidase H; FcRn, Fc receptor, neonatal; hFc, Fc fragment from human immunoglobulin G; IgG, immunoglobulin G; K_D, equilibrium dissociation constant; mFc, Fc fragment from mouse immunoglobulin G; mFcRn, mouse neonatal Fc receptor; mFcRn-HM, high-mannose form of mouse neonatal Fc receptor; MHC, major histocompatibility complex; mIgG, mouse immunoglobulin G; NB-DNJ, *N*-butyldeoxynorjirimycin; rFc, Fc fragment from rat immunoglobulin G; rFcRn, rat neonatal Fc receptor; rFcRn-HM, high-mannose form of rat neonatal Fc receptor.

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immobilized IgG at pH 6 and is released from the matrix by raising the pH to 8 (*11*). A gene encoding truncated mFcRn (stop codon inserted after the codon for residue 269 of the mature protein) was constructed from the mFcRn cDNA (kindly provided by Dr. Neil Simister, Brandeis University) as described for the rFcRn construct (*11*). The truncated mFcRn gene was inserted into the expression vector PBJ5-GS, which carries the glutamine synthetase gene as a selectable marker and means of gene amplification in the presence of the drug methionine sulfoximine (*12*). The mFcRn expression vector was cotransfected into CHO cells with an expression plasmid containing the complete cDNA sequence of mouse β2m (*a* allele) (*13*). Transfection, selection, amplification, isolation of cell lines secreting mFcRn, and functional purification of secreted mFcRn heterodimers on an IgG affinity matrix were done as described previously for rFcRn (*7*, *11*, *14*). N-Terminal sequence analysis of purified mFcRn revealed the first 17 residues of the mature forms of mFcRn and mouse β2m in approximately equimolar amounts (data not shown). Thus mouse β2m does not exchange for endogenous hamster β2m or bovine β2m present in the medium.

Preparation of High-Mannose Forms of rFcRn and mFcRn. To generate FcRn molecules with high-mannose rather than complex carbohydrate moieties, CHO cells expressing rFcRn or mFcRn were treated with *N*-butyldeoxynorjirimycin (NB-DNJ) (kind gift of R. A. Marks, Searle, Skokie, IL). NB-DNJ inhibits N-linked oligosaccharide processing enzyme α-glucosidase I, and its effects on recombinant proteins expressed in CHO cells have been previously characterized *(15)*. FcRn-expressing cells were grown in glutamine-free α minimal essential medium (Irvine Scientific) supplemented with 5% dialyzed fetal bovine serum (Gibco−BRL), 100 mM methionine sulfoximine (Sigma), 100 units/mL penicillin, and 100 μg/mL streptomycin. When the cells became confluent, 2 mM NB-DNJ was added to the medium. Harvest of cell culture supernatants for FcRn purification was initiated 2 days after the first application of NB-DNJ. Samples of purified FcRn (3 μg in 20 mM piperazine, pH 5.5) isolated from NB-DNJ-treated and untreated cells were incubated with 0.05 milliunit of endoglycosidase H (endo H) (Boehringer Mannheim) following the manufacturer's protocol and analyzed on an SDS−polyacrylamide 13% gel.

Soluble forms of mFcRn/mouse β2m heterodimers expressed in insect cells (*10*) were kindly provided by Drs. S. Popov and E. Sally Ward (University of Texas Southwestern Medical Center, Dallas, TX). One form contained a polyhistidine tag attached to the C-terminal residue of the truncated FcRn polypeptide chain. The other form had been treated with carboxypeptidase A to remove the C-terminal polyhistidine tag (*10*).

Fc Reagents. Experiments were done with Fc fragments of either human, mouse, or rat IgG. rFcRn binds to human, mouse, and rat IgG and their Fc fragments with similar affinities (*16*); thus the IgG and Fc used for the rFcRn experiments were chosen on the basis of gel-filtration properties and/or availability. The hFc was derived from a CD4−Fc fusion protein (kindly provided by Steven Chamow, Genentech, South San Francisco, CA) in which CD4 domains were fused to the Fc region from a human IgG1 (*17*). The hFc fragment of the chimeric protein was released by treatment with papain and purified as described (*17*). The mFc was derived from a mouse IgG1 monoclonal antibody against human Zn-α_2-glycoprotein called 1B5 (*18*), whose purification and interactions with rFcRn have been described previously (*7*, *16*). mFc was prepared by incubating the 1B5 antibody with 1:100 (w/w) papain in the presence of 10 mM Cys and 2 mM EDTA. After 1 h at room temperature, the digestion was stopped with 50 mM iodoacetamide and the mFc fragment was purified by a combination of anion-exchange, cation-exchange, and size-exclusion chromatography. Intact 1B5 IgG was used in some experiments. Recombinant rFc was purified by FcRn affinity chromatography (*9*) from the supernatants of CHO cells expressing secreted IgG2a rFc fragments (W. L. Martin and P.J.B., submitted for publication).

Determination of Protein Concentrations. FcRn and Fc concentrations were determined spectrophotometrically in 6 M GuHCl by using extinction coefficients at 280 nm of 87 130 M^{-1} cm^{-1} (rFcRn), 89 690 M^{-1} cm^{-1} (mFcRn), 69 280 M^{-1} cm^{-1} (hFc), 67 620 M^{-1} cm^{-1} (mFc), and 61 720 M^{-1} cm^{-1} (rFc). Extinction coefficients were calculated from the protein sequences as described (*19*). For measurements of protein concentrations in aqueous solution, A_{280} measurements for a fixed amount of protein were compared in 6 M GuHCl and aqueous solution and the coefficient was adjusted if necessary.

Nonequilibrium Gel-Filtration Analyses of the FcRn/Fc Stoichiometry. FcRn and Fc were incubated at molar ratios between 3:1 and 0.5:1 in 20 mM MES and 150 mM NaCl, pH 6.0, keeping the concentration of Fc fixed at 5 μM. After 20 min at room temperature, 25 μL was injected onto a Superdex 200 HR 32/30 gel-filtration column (Pharmacia) equilibrated in the incubation buffer. The chromatography was performed at a flow rate of 0.1 mL/min and the absorbance of the eluent was monitored at 280 nm. Fractions were analyzed by SDS−PAGE (data not shown). In some experiments, fractions corresponding to the FcRn/Fc complex were pooled and run on a Superdex 75 HR 32/30 gel-filtration column at pH 8 to separate FcRn and Fc. The area under each of the peaks was determined by photocopying the recorder trace and weighing the peak, and the molar ratio of FcRn to Fc in the complex was calculated after correction for the difference in extinction coefficients between FcRn and Fc.

Equilibrium Gel-Filtration Analyses of the FcRn/Fc Stoichiometry. The equilibrium column chromatography method of Hummel and Dreyer *(20)* was used to characterize the interaction between FcRn and Fc. A Superdex 200 PC 3.2/30 gel-filtration column (inclusion volume of 2.4 mL) was equilibrated and run in 20 mM sodium cacodylate and 150 mM NaCl, pH 6.0, containing 5 μM FcRn (equilibration buffer). Samples (20 μL) including 5 μM Fc and various concentrations of FcRn ($\geq$5 μM) were incubated for 20 min at room temperature in equilibration buffer that contained 5 μM FcRn and then injected onto the column. Chromatography was performed at a flow rate of 0.1 mL/min with use of a SMART micropurification system (Pharmacia), and the absorbance of the eluent was monitored at 280 nm.

RESULTS

Gel-filtration experiments are commonly used in stoichiometric assays because they do not require protein labeling

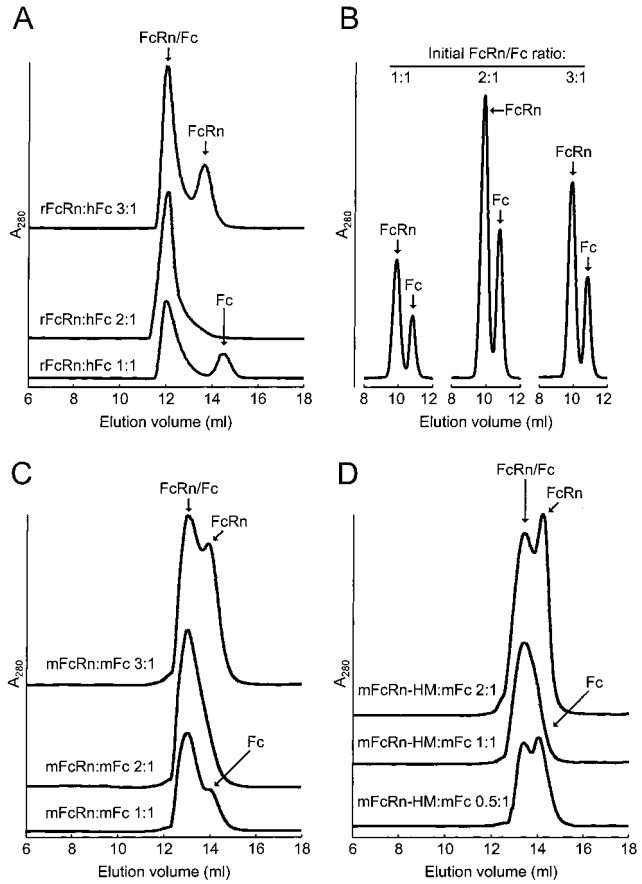

FIGURE 1: Nonequilibrium gel-filtration analyses of FcRn/Fc complexes. FcRn and Fc were incubated at pH 6.0 at the indicated molar ratios and then passed over a gel-filtration column to separate FcRn/Fc complexes from the free proteins (panels A, C, and D), or the FcRn/Fc complex was passed over the column at pH 8 to separate the components (panel B). The identities of the proteins in each of the peaks were confirmed by SDS−PAGE analysis (data not shown). (A) rFcRn and hFc. The apparent stoichiometry is 2:1. (B) rFcRn/hFc complexes isolated after separation from uncomplexed proteins (panel A) were dissociated and separated by gel filtration at pH 8. The amount of protein in each peak was calculated by integrating the peak area. Regardless of the initial input ratio of the two proteins, the rFcRn/hFc complex consists of 2 rFcRn per hFc. (C) mFcRn and mFc. The apparent stoichiometry is 2:1. Results similar to those presented in panel B were obtained for mFcRn/mFc complexes chromatographed at pH 8. The results cannot be accurately quantitated, however, because mFcRn and mFc cannot be completely separated by gel filtration (data not shown). (D) mFcRn-HM and mFc. The apparent stoichiometry is 1:1. A 1:1 stoichiometry was also found for the interaction of mFcRn produced in insect cells (*10*) with mFc (data not shown).

and provide additional information such as peak shape and relative mobility. Since isothermal titration calorimetry had already established that rFcRn and IgG form a 2:1 complex in solution under equilibrium conditions (*9*), we first investigated the interaction between rFcRn and Fc. Various ratios of rFcRn and Fc were incubated and the rFcRn/Fc complex was separated from free rFcRn or free Fc on a gel-filtration column (Figure 1A). Maximal separation between rFcRn, the rFcRn/Fc complex, and Fc was achieved with hFc, but similar results were obtained for mFc and rFc (data not shown). At pH 6.0, the complex runs at the same position on the gel-filtration column regardless of the initial molar ratio of rFcRn and hFc that was injected (Figure 1A). At a 2:1 molar ratio of rFcRn to hFc, virtually all of the protein chromatographed as the complex. When the input ratio of rFcRn to hFc is greater than 2:1, there is an additional peak corresponding to free rFcRn; when the input ratio of rFcRn to hFc is less than 2:1, there is an additional peak corresponding to free hFc (verified by SDS−PAGE; data not shown). When each of the complexes was dissociated at pH 8 and separated by gel filtration, the resulting free components in each complex were present at a 2:1 ratio (Figure 1B).

Having verified that the rFcRn exhibited 2:1 stoichiometry with Fc using the gel-filtration assay, we used the assay to investigate the stoichiometry between mFcRn and Fc. Using a soluble form of mFcRn produced in CHO cells, we found a 2:1 stoichiometry for the complex between mFcRn and mFc (Figure 1C) as well as between mFcRn and rFc or hFc (data not shown). These results contrast with those obtained with a soluble form of mFcRn produced in insect cells, in which it was found that mFcRn bound to mFc and mIgG with a 1:1 stoichiometry (*10*). Using the insect-derived form of mFcRn either with or without the attached polyhistidine tag (*10*), we also derived a 1:1 stoichiometry for its interaction with mFc (data not shown), suggesting that the insect-derived and the mammalian-derived forms of mFcRn differ in their interactions with mFc.

Both mFcRn and rFcRn contain four potential N-linked glycosylation sites in their extracellular regions (*21*, *22*). The crystal structure of rFcRn produced in CHO cells reveals that at least three of the sites contain attached carbohydrate moieties (*4*), of which one (attached to Asn-225) is definitively a complex carbohydrate (*23*). Most glycoproteins expressed in insect cells carry high-mannose type or truncated trimannosyl N-glycans, as compared to proteins expressed in mammalian cells, which usually contain complex carbohydrate antennae (*24*, *25*). To investigate the effects of differences in FcRn carbohydrate structure on its interaction with Fc, we treated CHO cells expressing mFcRn or rFcRn with NB-DNJ. NB-DNJ stops the conversion of high-mannose carbohydrates to complex forms and thus will induce the production of rFcRn and mFcRn with high-mannose carbohydrates (rFcRn-HM and mFcRn-HM). Treatment with the drug did not affect FcRn expression, β2m association, or pH-dependent binding to an IgG affinity matrix (data not shown). FcRn purified from the treated and untreated cells was compared by SDS−PAGE analysis before and after treatment with endo H, a glycosidase that cleaves high-mannose, but not complex, forms of carbohydrate (*26*). rFcRn-HM and mFcRn-HM show altered mobilities and differential sensitivity to endo H treatment compared with rFcRn and mFcRn (Figure 2), consistent with a shift toward attachment of high-mannose rather than complex carbohydrates as a result of the treatment with NB-DNJ. The stoichiometries of the interactions of the high-mannose forms of FcRn with Fc were determined by the gel-filtration assay. rFcRn-HM, like rFcRn, exhibited a 2:1 stoichiometry with hFc and mFc (data not shown), whereas the stoichiometry of the interactions of mFcRn-HM and mFcRn differed, in that mFcRn-HM exhibited a 1:1 stoichiometry with mFc (Figure 1D). However, a 2:1 stoichiometry was found for both forms of mFcRn with hFc or rFc instead of mFc (data not shown). Thus alteration of the carbohydrates attached to mFcRn, but not rFcRn, results in formation of 1:1 complexes with mFc in this assay.

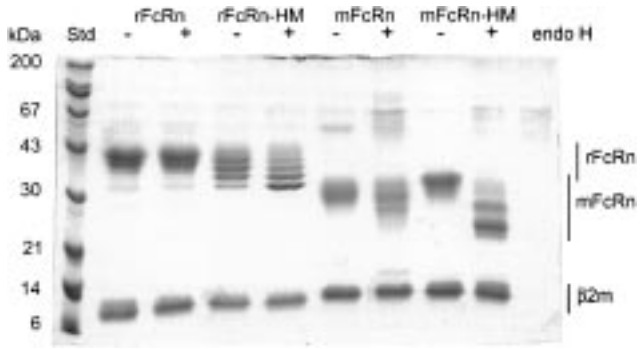

FIGURE 2: SDS−PAGE analysis of FcRn and FcRn-HM treated with endo H. Samples were incubated in the presence (+) or absence (−) of endo H and analyzed on an SDS−13% polyacrylamide gel under reducing conditions.

The stoichiometry determined by conventional gel filtration cannot be considered definitive, since the binding interaction is being assayed under nonequilibrium conditions. If some of the complexes dissociate during the experiment, their constituents can fail to rebind because they are being separated from each other by the chromatographic procedure. Therefore, depending of the kinetics of the binding and the separation efficiency of the column, one could observe an artificially low binding or no binding at all. To avoid this problem, we used the equilibrium gel-filtration assay of Hummel and Dreyer (*20*). In this method, a gel-filtration column is equilibrated with buffer containing a uniform concentration of one binding partner (FcRn for our experiments). The other binding partner (Fc or IgG) is injected with different amounts of the first protein (FcRn) over the gel-filtration column, which is run in the equilibration buffer that contains FcRn. When the amount of additional FcRn injected is less than or greater than the amount required for formation of the FcRn/ligand complex, a trough (in the case of too little FcRn) or a peak (in the case of excess FcRn) is observed at the position that free FcRn migrates. When the amount of additional FcRn injected is equal to that required for formation of the FcRn/ligand complex, a flat baseline is observed at the position that free FcRn migrates; thus all of the added FcRn is complexed with the ligand. Depending on the K_D of the interaction and the protein concentrations, however, the peak corresponding to the complex will contain some amount of free ligand that is in equilibrium with the bound form. Therefore, unless the protein concentrations greatly exceed the K_D, the ratio of the concentration of added FcRn to the concentration of ligand will be a nonintegral value, from which the integral value corresponding to the stoichiometry can be extracted by rounding up to the next integer.

Gel-filtration columns were equilibrated with 5 μM rFcRn, mFcRn, or mFcRn-HM. Samples containing 5 μM ligand (either rFc or mFc) were incubated with different amounts of the appropriate FcRn and injected over the column. In solution, the K_D of the interaction between rFcRn and IgG is 0.5 μM (*9*); thus most of the injected ligand should be in the bound form. As can be seen in Figure 3, in all cases in which equimolar amounts of Fc and additional FcRn are injected, there is a trough at the position where free FcRn migrates, indicating a stoichiometry higher than 1:1. A flat baseline is observed when the concentration of added FcRn is between 8.5 and 9.5 μM; thus the stoichiometry is 2:1, even for the interaction of mFcRn-HM with mFc (Figure 3C), which showed an apparent 1:1 stoichiometry under nonequilibrium conditions (Figure 1D). Similar results were obtained for the analysis of the interaction between mFcRn-HM and mIgG (data not shown).

DISCUSSION

The stoichiometry of the interaction between FcRn and its IgG ligand is important for understanding the mechanism

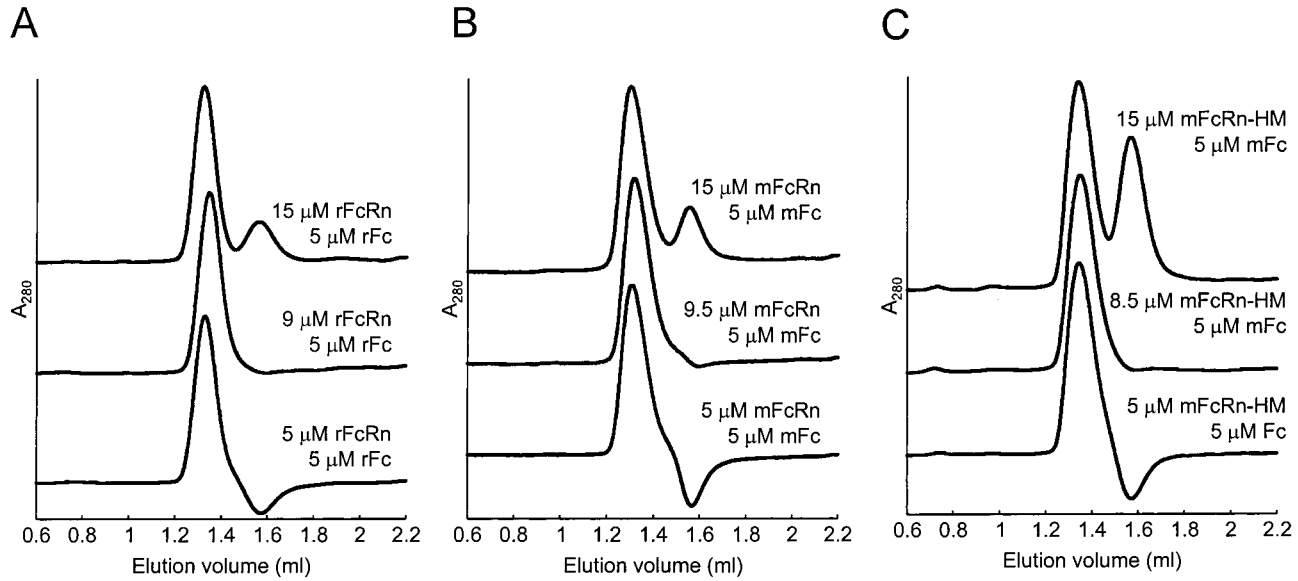

FIGURE 3: Equilibrium gel filtration analyses of FcRn/Fc complexes. FcRn was incubated with 5 μM Fc at the indicated FcRn and Fc concentrations in buffer containing 5 μM FcRn (equilibration buffer). Samples were then injected onto a column equilibrated in the equilibration buffer. The peak that elutes first corresponds to an FcRn/Fc complex in equilibrium with free Fc. The second peak or trough is at the elution volume of free FcRn. When a flat baseline is observed at the position where free FcRn migrates, all of the FcRn in addition to the amount in the equilibration buffer is complexed with ligand; thus the stoichiometry can be determined from the ratio of FcRn and Fc in the complex. In all cases, the concentration of bound Fc in the complex is slightly lower than 5 μM because the peak formed by the complex contains free Fc in equilibrium with bound Fc. (A) rFcRn and rFc. (B) mFcRn and mFc. (C) mFcRn-HM and mFc.

by which FcRn binds and transports IgG. For example, a stoichiometry higher than 1:1 is compatible with models in which the presence of bound ligand is detected by clustering or bridging of receptors. Although determining a stoichiometry from the crystal structure of a receptor/ligand complex is usually straightforward, the FcRn/Fc cocrystal structure reveals a repeating structure of FcRn molecules binding Fc, from which several possibilities for a physiologically relevant complex can be extracted (*5*). One possibility is the repeating structure itself, in which FcRn dimers are bridged by Fc molecules to form an oligomeric ribbon with a 2*n*:*n* stoichiometry. If the oligomeric ribbon exists in vivo, IgG molecules would bridge FcRn dimers tethered to parallel adjacent membranes, which are present in acidic transport vesicles. Other possibilities for a physiologically relevant complex include the two different 2:1 complexes that are built from the 1:1 interaction of FcRn with Fc. In the first of these, one molecule of an FcRn dimer binds to one side of Fc. In the second, FcRn monomers bind to both sides of Fc. A final possibility for a physiologically relevant complex is a 1:1 complex in which a single FcRn binds to one side of Fc.

In considering which of the complexes are biologically relevant, one must differentiate between the in vivo and in vitro situations. In vivo, receptors are tethered to a membrane under conditions of high local protein concentrations in which receptor dimerization and oligomeric ribbon formation could be facilitated. These conditions might be mimicked by the high protein concentration in crystals made with soluble FcRn (11 mM in the FcRn/Fc cocrystals) (*5*). For other biochemical experiments, soluble FcRn is studied at lower protein concentrations (usually micromolar) that would not be expected to favor formation of receptor dimers or the oligomeric ribbon. Indeed, FcRn/Fc and FcRn/IgG complexes isolated on nonequilibrium gel-filtration columns are relatively low molecular weight complexes (*9*, *10*) that presumably represent either 2:1 or 1:1 portions of the oligomeric ribbon observed in the FcRn/Fc cocrystals.

Whereas soluble rFcRn forms 2:1 complexes with IgG (*9*), 1:1 complexes were found for mFcRn under nonequilibrium conditions (*10*). To determine if this discrepancy represents a fundamental difference in how rFcRn and mFcRn interact with ligand, we examined the solution behavior of both proteins under equilibrium and nonequilibrium conditions.

We find that rFcRn forms 2:1 receptor/ligand complexes on gel-filtration columns run under both equilibrium and nonequilibrium conditions. By contrast, mFcRn can form 2:1 or 1:1 complexes with Fc under nonequilibrium conditions. The mammalian cell-derived form of mFcRn containing complex N-linked carbohydrates forms only 2:1 complexes, whereas the high-mannose carbohydrate-containing forms of mFcRn produced either in insect cells or in NB-DNJ-treated mammalian cells formed 1:1 complexes with mFc and 2:1 complexes with hFc and rFc. These results are compatible with a model in which the two FcRn molecules that can interact with Fc bind with different affinities, with the first binding event representing a higher affinity interaction, as previously suggested (*1*, *5*, *8*, *10*, *27*). In the equilibrium gel-filtration experiments, all forms of FcRn bind ligand with a 2:1 stoichiometry, suggesting that the K_D for binding the second FcRn to Fc is less than 5 μM, the concentration of FcRn in the equilibration buffer of the columns. If it were possible to use higher concentrations of FcRn in the equilibration buffer, we presume that larger FcRn/Fc complexes corresponding to longer pieces of the FcRn/Fc oligomeric ribbon could be revealed. These experiments are difficult to perform, however, due to the large amount of protein required to equilibrate the column at extremely high (e.g., millimolar) concentrations.

We conclude from these experiments that rFcRn and mFcRn share the same general properties for recognizing IgG. These include the tendency to form 2:1 receptor/ligand complexes in solution, with the potential to form larger oligomeric ribbons with a 2*n*:*n* stoichiometry.

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