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THE STOICHIOMETRY AND AFFINITY OF THE INTERACTION OF MURINE Fc FRAGMENTS WITH THE MHC CLASS I-RELATED RECEPTOR, FcRn

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Abstract—The binding of recombinant wild type and mutant Fc-hinge fragments to soluble, FcRn expressed in insect cells has been analysed. The mutant Fc-hinge fragments are derived from murine IgG1 with mutation of residues located at the CH2–CH3 domain interface (Ile253, His310, Gln311, His433 and Asn434; EU numbering). These mutant Fc-hinge fragments have previously been shown to be deficient in neonatal transcytosis in suckling mice and also have abnormally short serum half lives. The mutated residues are highly conserved in human and rodent gammaglobulins (IgGs) and are also involved in binding to staphylococcal protein A. This study demonstrates that the Fc mutants have lower binding affinities for recombinant FcRn and mutations in the CH2 domain have a greater effect than those in the CH3 domain. There is an excellent correlation between affinity and transcytosis or the control of catabolism, and this provides further evidence in support of the close overlap of the sites of IgG/Fc involved in these processes. The stoichiometry of the FcRn:Fc interaction has also been investigated and has been found to be 1:1, indicating that binding of FcRn to one CH2–CH3 domain interface site precludes an FcRn:Fc interaction at the second site. Copyright © 1996 Elsevier Science Ltd.

Key words: Fc receptor, recombinant Fc fragment, affinity, stoichiometry, transcytosis.

INTRODUCTION

Transfer of maternal IgGs across the enterocytes of the proximal intestine is the major route by which young mice and rats acquire passive immunity (Brambell *et al.*, 1964; Brambell, 1970). The IgGs are transcytosed to newborns by the neonatal transfer receptor, FcRn, and this Fc receptor is a heterodimer of two polypeptides of Mr 45–53 kDa and 12–14 kDa (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985). The 12–14 kDa polypeptide is β_2-microglobulin (β_2m), and the FcRn α chain shares homology with major histocompatibility class I molecules (Simister and Mostov, 1989). Rat and murine FcRn are highly homologous (Kandil *et al.*, 1995), and recently a human homologue of rodent FcRn has also been isolated from placenta (Story *et al.*, 1994), implicating this protein in maternofetal transfer of IgGs. Understanding the molecular mechanisms by which IgGs are transferred in rodents therefore has relevance to maternofetal transfer of IgGs in humans, and this has implications for passive immunotherapy *in utero*.

The amino acids of murine IgG1 (mIgG1) that affect binding to FcRn on the neonatal brush border have been identified using site-directed mutagenesis (Kim *et al.*, 1994a). Recombinant Fc-hinge fragments (comprising CH2, CH3 domains and the mIgG1 hinge region) derived from mIgG1 with mutation of residues located at the CH2–CH3 domain interface (Ile253, His310, Gln311, His433 and Asn434; EU numbering; Deisenhofer, 1981) were deficient in *in vivo* competition transintestinal transfer assays in suckling mice (Kim *et al.*, 1994a). Furthermore, binding studies with the least active mutant and isolated neonatal brush border showed that this mutant was defective in binding to FcRn (Kim *et al.*, 1994a). These IgG residues are highly conserved in human and rodent isotypes (Kabat *et al.*, 1991) and are also involved in binding to staphylococcal protein A (SpA; Deisenhofer, 1981). Consistent with this, human Fc complexed with SpA does not bind to FcRn (Raghavan *et al.*, 1994). The involvement of histidines of the Fc region in the FcRn:Fc interaction suggests a mechanism by which pH dependent binding of FcRn to Fc occurs (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985), as the pK_as of the imidazole side chains lie between 6 and 7.

In this study we analysed the binding of wild type and mutant Fc-hinge fragments to soluble, recombinant

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Abbreviations: β_2m, β_2-microglobulin; Fc, immunoglobulin constant region fragment; FcRn, neonatal Fc receptor; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SpA, staphylococcal protein A; SPR, surface plasmon resonance.

FcRn expressed in insect cells. The stoichiometry of the FcRn-Fc interaction has also been investigated, and in contrast to the findings with rat FcRn (Huber *et al.*, 1993) has been found to be 1:1.

MATERIALS AND METHODS

Plasmids and expression hosts

For the expression of soluble FcRn in insect cells, the α chain gene was tailored by the PCR using a plasmid containing the gene encoding the leader peptide and extracellular domains (codons 1–290) of the FcRn α chain gene (Ahouse *et al.*, 1993) as a template. This gene was isolated in our laboratory using reverse transcriptase PCR (RT-PCR) and RNA from the endothelial cell line, B10,D2.PCE (derived from lungs of B10.DBA/2 mice and a generous gift of Prof. A. Curtis). The gene has the same sequence as that isolated by Kandil *et al.* (1995) from BALB/c or C3H/HeJ mice, except for two silent changes at positions 26 and 299, and was initially ligated as an undigested PCR product into pGEM-T (Promega) and sequenced (several independent isolates were sequenced using the dideoxynucleotide method). The gene was tailored with a 5′ *Sal*I site and 3′ *Sal*I, *Bst*EII sites using the primers: FcRn5′SalI, ATC AGTCGAC AT GGG GAT GCC ACT GCC CTG G; FcRn3′BstEII-SalI, ATC AGTCGACGGTGACCA GGT CCA CAG TGA GAG GCTG (restriction sites are underlined). The tailored PCR product was then recloned into pUC119 as a *Sal*I fragment and resequenced. The pUC119-FcRn derivative was restricted with *Bst*EII and a DNA duplex encoding a polyhistidine tag (Ward, 1992) ligated into the *Bst*EII site. This resulted in tagging of the FcRn gene with an inframe his6 peptide.

The primers used to build the baculovirus expression plasmid are: FcRnback, 5′GAT TCA GGATCC *ATG GGG ATG CCA CTG CCC TGG* 3′, Bamrev, 5′AGC GGATCC caa ttt cac 3′ (*Bam*HI sites shown underlined, region that matches the 5′ end of the FcRn leader peptide shown in italics and region that matches the pUC19 sequence upstream of the polylinker shown in lower case letters). Following the PCR, the product was digested with *Bam*HI and ligated into *Bgl*II restricted pAcUW51 (PharMingen). The orientation of the gene with respect to the p10 promoter was analysed in several of the resulting clones by PCR screening (Güssow and Clackson, 1989) and the inserts of clones with the desired orientation of FcRn checked by dideoxynucleotide sequencing. One clone (designated FcRn/pAcUW51) was used in further experiments to generate the FcRn expression plasmid.

The gene encoding murine β_2m (Kabat *et al.*, 1991) and leader peptide was isolated using RT–PCR from 1934.4 T cell hybridoma cells (Wraith *et al.*, 1989) using the primers: β2mback, 5′GAT TCA AGATCT *ATG GCT CGC TCG GTG ACC CT* 3′; β2mfor, 5′GAT TCA AGATCTT*A CAT GTC TCG ATC CCA GTA GAC* 3′ (*Bgl*II sites indicated by underlining and regions that match the 5′ and 3′ ends of the β_2m gene in italics). Following the PCR, the products were digested with *Bgl*II and ligated into *Bam*HI digested FcRn/pAcUW51. The orientation of the gene with respect to the polyhedrin promoter was analysed in several of the resulting clones by PCR screening and the inserts of clones with the desired orientation of β_2m checked by dideoxynucleotide sequencing.

Recombinant baculovirus was generated by homologous recombination using linearized *Autographa californica* nuclear polyhedrosis virus (AcMNPV) purchased from Invitrogen. Recombinant viral stocks were produced and assayed in *Spodoptera frugiperda* cells (Sf9; ATCC# CRL1711) grown in TNM-FH medium supplemented with 10% fetal calf serum and antibiotics (Summers and Smith, 1987) by cotransfection of 2×10^6 Sf9 cells with 1 μg viral wild type AcMNPV DNA and 5 μg of plasmid DNA using an Invitrogen transfection kit. Occlusion-negative viruses were identified through visual screening and plaque purified as described (Summers and Smith, 1987).

Preparation of soluble FcRn

High 5 cells (purchased from Invitrogen and maintained in Ex-cell-405 medium, JRH Biosciences) were grown in suspension culture to a density of 1×10^6/ml and infected at a multiplicity of infection of 2 using procedures described by Summers and Smith (1987). Recombinant protein was purified from the supernatant using Ni^{2+}-NTA-agarose resin (Qiagen). Purified FcRn was dialysed against PBS, pH 6.0 and stored at 4°C. An average yield was 15 mg/l supernatant. The extinction coefficients of the recombinant FcRn, determined by weight analysis, are: 1.40 ml/mg/cm and 67.9 mM/cm assuming a molecular mass of 47.5 kDa.

Radiolabeling of FcRn

The protein was radiolabeled with [^{125}I]-NaI using the Iodo-Gen reagent (Amersham) as described previously for Fc-hinge fragments (Kim *et al.*, 1994b). The specific activity of the radioiodinated protein was *ca* 10^7cpm/μg with less than 5% free iodide.

Size exclusion analysis of FcRn

Twenty microlitres of [^{125}I]-FcRn (10^7 cpm, 5 μg) were diluted into 1 ml 50 mM phosphate buffer, pH 6.0 with 0.3 M NaCl containing 10 mg BSA (68 kDa), 5 mg ovalbumin (46 kDa) and 6 mg cytochrome C (14 kDa). The mixture was chromatographed on a sephacryl S-100HR column (90 × 1.8 cm) equilibrated with 50 mM phosphate buffer, pH 6.0 containing 0.3 M NaCl. One millilitre fractions were collected and the absorbance at 280 nm and radioactivity measured. The experiment was repeated using the same conditions except that the sample was mixed with 20 μl 2% SDS and boiled for 5 min before loading onto the column, 0.3 M NaCl in the running buffer replaced by 1% SDS and the pH of the buffer adjusted to 7.5.

Preparation of Fc fragment by papain digestion of mIgG1

mIgG1 monoclonal antibody (RFB4) (Campana *et al.*, 1985) was digested with papain and the Fc fragment purified by DEAE-sepharose and protein A (SpA)-sepharose as described previously (Kim *et al.*, 1994a). This fragment was designated Fc-papain.

Preparation of the Fc-hinge fragments

Recombinant wild type and mutant Fc-hinge fragments were prepared as described previously (Kim *et al.*, 1994b). Mutants are designated as follows: I-253 = Ile-253 to Ala-253; HQ-310 = His-310 to Ala-310 and Gln-311 to Asn-311; HN-433 = His-433 to Ala-433 and Asn-434 to Gln-434; HQ-310/HN-433 = His-310 to Ala-310, Gln-311 to Asn-311, His-433 to Ala-433 and Asn-434 to Gln-434.

A recombinant Fc heterodimer which comprises one wild type Fc-hinge polypeptide (tagged with carboxyterminal his6 peptide tag) associated with one HQ-310/HN-433 mutant Fc-hinge polypeptide (tagged with carboxyterminal C-myc peptide tag) was prepared as described (Kim *et al.*, 1994c).

Size exclusion analyses of [^{125}I]-FcRn:Fc complex

Half a millilitre of [^{125}I]-FcRn (3 mg/ml) (specific radioactivity *ca.* 20 000 cpm/μg) was incubated with an equal volume of Fc fragment (obtained by papain digestion of mIgG1) containing 3, 1.5 or 1 mg/ml protein in 50 mM phosphate buffer, pH 6. The mixtures were incubated at room temperature for 2 hr and then overnight at 4°C, and were chromatographed on a sephacryl S-200HR column (90 × 1.8 cm) equilibrated with 50 mM phosphate buffer, pH 6.0, 0.3 M NaCl. One milliliter fractions were collected and the absorbance and radioactivity measured. As size standards, beta-amylase (200 kDa), transferrin (95 kDa) and ovalbumin (46 kDa) were used. The molar ratio of the [^{125}I]-FcRn:Fc complex was determined using the specific radioactivity for FcRn and extinction coefficients at 280 nm for both FcRn and Fc of 1.40 ml/mg/cm.

The molecular weight and molar ratio of the [^{125}I]-FcRn:mIgG1 complex were also determined using a sephacryl S-200HR column (90 × 1.8 cm) calibrated with ferritin (440 kDa), beta-amylase (200 kDa) and ovalbumin (46 kDa) and equilibrated with 50 mM phosphate buffer, pH 6.0, 0.3 M NaCl. The fractions of the first peak (corresponding to 215 kDa) were pooled and adjusted to pH 6.5 with 2 M Na_2HPO_4. Samples of the mixture were passed through an SpA-sepharose column (Pharmacia). The flow-through fractions and fractions eluted with 0.1 M glycine buffer, pH 3 containing 0.145 M NaCl, were collected and the molar ratio of the [^{125}I]-FcRn:mIgG1 complex determined.

Carboxypeptidase A treatment of FcRn

To remove the polyhistidine tag from the recombinant FcRn, carboxypeptidase A treatment was used. FcRn (1 mg/ml) was dialysed into 50 mM Tris–HCl, pH 8.0 and incubated with carboxypeptidase A (CA) (Sigma) at a final concentration of 40 μg/ml for 4 hr at 25°C. The mixture was passed through Ni^{2+}-NTA-agarose, the flow-through collected and Na_2EDTA added to a final concentration of 50 mM to inactivate the CA. The material was dialysed into 50 mM phosphate buffer, pH 6. As a control, FcRn was treated in the same way except that no CA was added. Following dialysis into 50 mM sodium phosphate buffer, pH 6 an aliquot of CA-treated FcRn (CA-FcRn) was analysed in competition binding using mIgG1-sepharose. In these assays the ability of CA-FcRn or control FcRn to inhibit the binding of ^{125}I-labeled FcRn to mIgG1-sepharose was determined and found to be not significantly different. This indicated that CA treatment does not affect the functional activity of FcRn.

Analysis of the FcRn:Fc stoichiometry using CA treated FcRn

CA-FcRn (100 μg) was mixed with an equimolar amount of FcRn (100 μg: with his6 peptide) plus Fc-papain (100 μg). The mixture was incubated for 1 hr at 25°C with agitation and passed through Ni^{2+}-NTA-agarose (0.2–0.3 ml packed volume). The flow-through was collected and the column washed with *ca* four column volumes (two fractions) of 50 mM phosphate buffer, pH 6.0. Fc-papain, bound to the column *via* FcRn, was eluted with five column volumes (three fractions) of 100 mM Tris–HCl, pH 7.4 and bound FcRn subsequently eluted with a total of four column volumes (two fractions) of 250 mM imidazole, pH 8.0. Fractions were analysed on 12% SDS–PAGE run under reducing conditions followed by staining with Coomassie brilliant blue.

Analysis of binding of soluble FcRn to wild type and mutant Fc-hinge fragments in competition assays

Wild type and mutant Fc-hinge fragments were dialysed into 50 mM phosphate buffer, pH 6.0. 350 μl of 10% w/v mIgG1-sepharose (3 mg IgG1/ml gel) were incubated in 50 mM phosphate buffer, pH 6.0 with 100 μg Fc-hinge fragment and 0.5 μg ^{125}I-labeled FcRn. Following incubation (2 hr at 25°C followed by 16 hr at 4°C), beads were washed four times and bound radioactivity determined. The experiments were repeated several times and for each Fc-hinge fragment, triplicate samples were used.

SPR measurements

A BIAcore biosensor system (Pharmacia LKB Biosensor, Inc.) was used for real time surface plasmon resonance (SPR) experiments. Immobilization of FcRn was performed using amine coupling chemistry as recommended by the manufacturer (BIAtechnology Handbook). The following sample injections were made using a sensor chip with carboxymethylated dextran surface: a mixture of 15 μl volumes of 0.1 M N-hydroxysuccinimide and 0.1 M 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide; 10–35 μl of 10–30 μg/ml FcRn in 10 mM NaOAc, pH 5.0; 30 μl 1 M ethanolamine. For binding

experiments the FcRn coupled to the sensor chip was exposed to different concentrations of ligand in phosphate buffered saline (PBS), pH 6.0 containing 0.005% Tween 20 with a flow rate of 5 μl/min at 20°C. At pH 6.0, FcRn binds wild type Fc-hinge fragment in a concentration-dependent manner, whereas in 50 mM HEPES buffer, pH 7.4 (used to recover the chip) FcRn binding to Fc-hinge is undetectable. Complete activity of FcRn is recovered after the pH is lowered to 6.0 and FcRn is stable during repeated cycles of binding-elution in BIAcore experiments for several hours at 20°C (data not shown).

The determination of rate and equilibrium binding constants involved transformations of the primary data using the manufacturer's BIAevaluation software. The curves representing nonspecific binding obtained using a chip with coupled ethanolamine were subtracted from the corresponding curves for a chip with coupled FcRn. For nearly all data a non-linear fit algorithm was used to calculate first order apparent dissociation (k_d) and association (k_a) rate constants from the most representative parts of kinetic curves as described in the BIAcore manual. The kinetic curves influenced by diffusion limitation effects were excluded from the analysis. For each sensorgram a value for the apparent equilibrium dissociation constant (K_d) was calculated. The K_d values were then used to determine mean values and standard deviations.

RESULTS

Purified, radiolabeled FcRn was analysed by chromatography on sephacryl S-100HR in the presence or absence of SDS (Fig. 1). FcRn runs as a single peak corresponding to a molecular mass of *ca* 50 kDa, and in the presence of 1% SDS the FcRn dissociated into two components with molecular masses of 37 kDa (α chain) and 12–14 kDa (β_2m), respectively. SDS–PAGE analysis of FcRn gives molecular masses of the two polypeptides

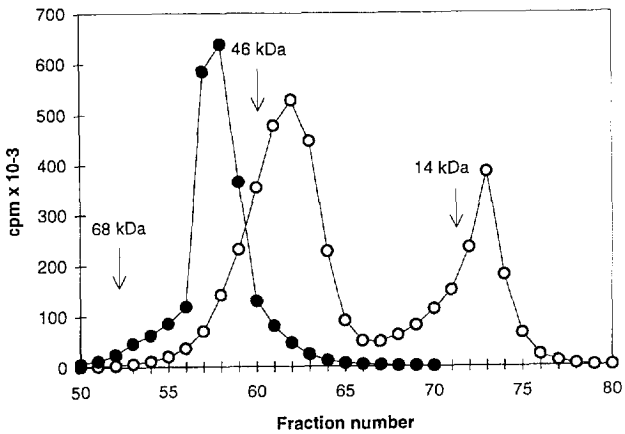

Fig. 1. Sephacryl S-100HR chromatography of [^{125}I]-FcRn.Closed circles, elution with 50 mM phosphate buffer. pH 6.0, 0.3 M NaCl. Open circles, elution with 50 mM phosphate buffer, pH 7.5, 1% SDS. Arrows indicate elution positions for molecular weight standards: BSA (68 kDa), ovalbumin (46 kDa) and cytochrome c (14 kDa).

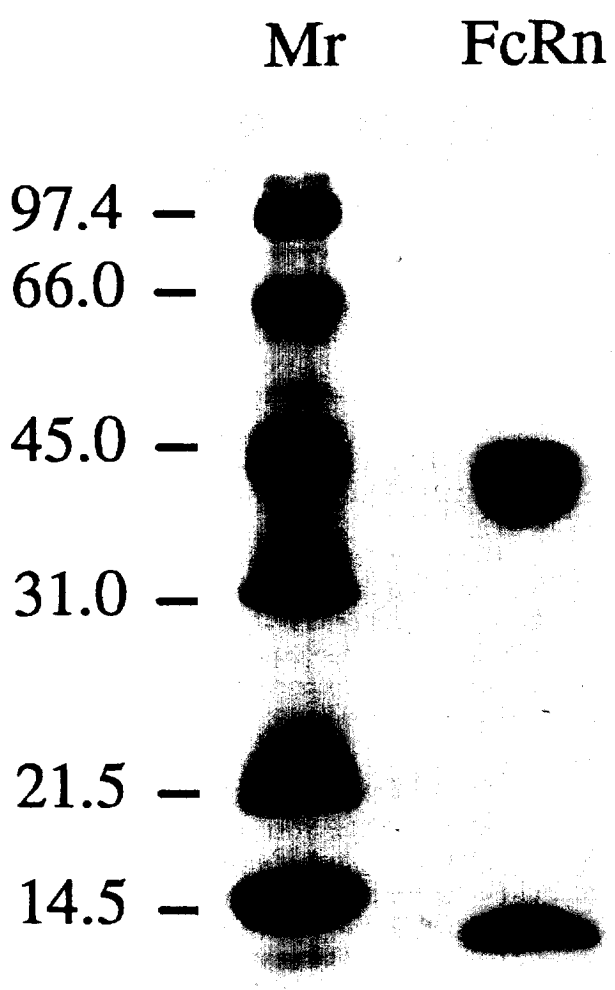

Fig. 2. 15% SDS–PAGE analysis of purified soluble FcRn followed by staining with Coomassie brilliant blue.

close to that found by size exclusion chromatography (Fig. 2), and immunoblotting was used to confirm that the 12–14 kDa protein is β_2m (data not shown).

As Fc or IgG has two potential binding sites for FcRn, prior to carrying out affinity measurements using SPR, it was necessary to determine the stoichiometry of the FcRn–Fc interaction. Chromatographic analysis indicated that the molecular mass of the complex formed at pH 6.0 between radiolabeled FcRn and Fc-papain was *ca* 100 kDa irrespective of the molecular ratio of the components in the reaction mixture (Fig. 3). This molecular mass indicates that one Fc-papain molecule (50 kDa) is bound to one FcRn (*ca* 50 kDa) molecule in a 1:1 complex. Using the specific radioactivity of FcRn and optical density for the pooled fractions of the first peak, a FcRn:Fc molar ratio of 1.15 ± 0.11 ($n=10$) was also found. The complex formed at pH 6.0 can be dissociated into FcRn and Fc by chromatography at pH 7.5 on sephacryl S-200HR (Fig. 3).

To analyse whether the 1:1 stoichiometry for the Fc-papain:FcRn interaction was also observed for the more physiologically relevant mIgG1:FcRn interaction, chromatography of a mixture of mIgG1 and radiolabeled FcRn on a sephacryl S-200HR column was carried out

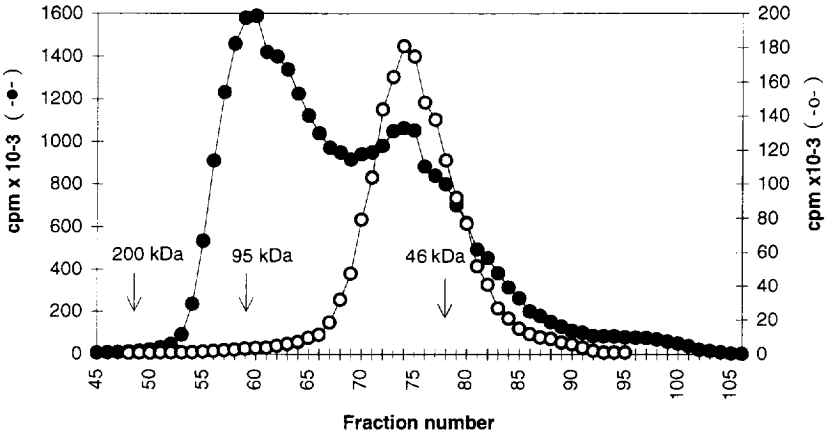

Fig. 3. Sephacryl S-200HR chromatography of [^{125}I]-FcRn:Fc complex. Closed circles, elution of a mixture [^{125}I]-FcRn plus Fc (2:1 molar [^{125}I]-FcRn:Fc ratio) in 50 mM phosphate buffer, pH 6.0. Open circles, second chromatographic analysis of the combined fractions (#55–60) of the first peak in 50 mM phosphate buffer, pH 7.5. Arrows indicate elution positions for molecular weight standards: β-amylase (200 kDa), transferrin (95 kDa) and ovalbumin (46 kDa).

(data not shown). The 215 kDa peak represents a mixture of the mIgG1:FcRn complex and free mIgG1 and, as FcRn blocks SpA binding (our unpublished data and Raghavan *et al.*, 1994), the complex can be separated from mIgG1 using SpA-sepharose. In the flow-through fraction the FcRn:mIgG1 molar ratio was 1.1, in contrast to 0.1 for the bound fraction corresponding to free mIgG1.

Our results are in contrast to the rat FcRn:Fc interaction which has been reported to have a stoichiometry of 2:1 (Huber *et al.*, 1993). Further analyses were carried out to assess whether the 1:1 stoichiometry indicated by chromatography could be verified for murine FcRn using independent approaches. Thus, FcRn with a carboxyterminal his6 peptide was treated with carboxypeptidase A, which resulted in removal of the his6 peptide with retention of functionally active FcRn (designated CA-FcRn). CA-FcRn retained full activity in mIgG1 binding (data not shown) and can be distinguished from untreated FcRn (with his6 peptide) using 12% SDS–PAGE (Fig. 4). Following incubation, the mixture of equimolar amounts of CA-FcRn, FcRn and Fc-papain was passed through Ni^{2+}-NTA-agarose to retain FcRn plus bound proteins. The rationale behind this experiment is shown in Fig. 5. Gel analysis (Fig. 4) of the flow-through from the Ni^{2+}-NTA-agarose, washes at pH 6.0 and pH 7.4 and imidazole elutions indicate that only Fc:FcRn complexes bind to the column and there is no evidence for the binding of CA-FcRn *via* an "Fc-bridge". CA-FcRn appears either in the flow-through

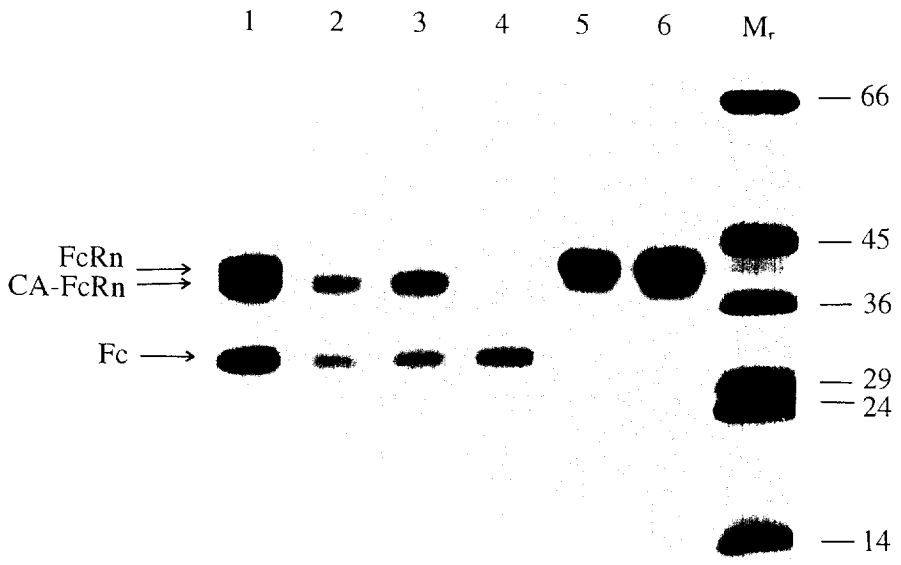

Fig. 4. SDS–PAGE analysis of the fractions eluted from Ni^{2+}-NTA agarose column. An equimolar mixture of FcRn, CA-FcRn and Fc-papain was loaded onto the column and the eluted proteins analysed. Lane 1, starting mixture; lane 2, flow-through at pH 6.0; lane 3, wash with pH 6.0; lane 4, eluate at pH 7.4; lane 5, eluate with 250 mM imidazole; lane 6, purified FcRn; M_r, molecular weight standards.

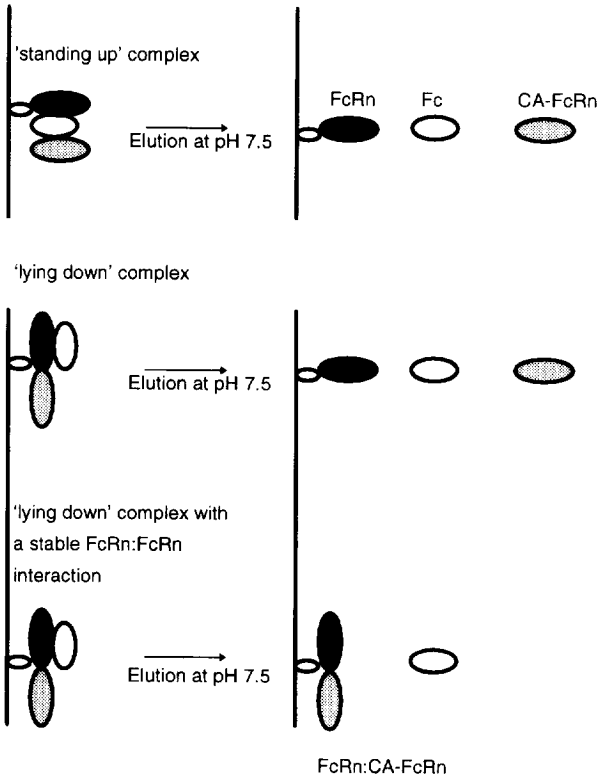

Fig. 5. A hypothetical scheme of the "lying down" and "standing up" complexes formed between FcRn, CA-FcRn and Fc, and their elution patterns from Ni^{2+}-NTA agarose column.

or washes at pH 6.0. Furthermore, Fc-papain bound to FcRn elutes at pH 7.4 (lane 4) and only FcRn elutes with 250 mM imidazole (lane 5) (12% SDS–PAGE was used to optimize the separation of CA-FcRn and FcRn; however, under these conditions β_2m runs-off the end of the gel).

Control experiments were also carried out in which the following mixtures were passed through Ni^{2+}-NTA-agarose and treated in the same way: (i) CA-FcRn plus Fc-papain; (ii) FcRn plus Fc-papain. For (i) CA-FcRn and Fc-papain flowed directly through the column and for (ii) Fc:FcRn complexes were retained, with Fc-papain being washed off at pH 7.4 and FcRn eluted with 250 mM imidazole. Thus, the data show that hybrid CA-FcRn:Fc:FcRn complexes do not form, indicating a stoichiometry of 1:1. These observations also exclude the formation of FcRn dimer in solution, consistent with the report of Huber *et al.* (1993).

Using SPR, the FcRn interaction with the wild type Fc-hinge fragment and the Fc-hybrid was compared as a further test of the stoichiometry. The Fc-hybrid comprises a wild type Fc-hinge polypeptide associated with the HQ-310/HN-433 mutant polypeptide (Kim *et al.*, 1994c) and therefore has only one functional FcRn interaction site per molecule. FcRn was coupled to the BIAcore chip and sensorgrams of the binding of the Fc fragments are shown in Fig. 6. The amount of Fc-hinge bound can be quantitated (Table 1) and is similar to that of the Fc-hybrid at saturating concentration (note that the concentrations of Fc-hinge fragments used are considerably higher than the estimated dissociation constant; see affinity data in Table 3, footnote).

To analyse whether FcRn could bind to either the preformed 1FcRn:1Fc complexes or unbound FcRn on the chip, FcRn was injected over the chip shortly after the beginning of the dissociation phase. Using FcRn at a concentration of up to 6 μM, no significant binding of FcRn was observed indicating the absence of interaction of FcRn with either the preformed complex or free FcRn present on the chip (Fig. 6). This observation is consistent with the data obtained using other methods (Fig. 3, Fig. 4).

To measure the relative affinities of the FcRn interaction with the wild type and mutant Fc-hinge fragments that have mutations of residues at the CH2–CH3 domain interface (Kim *et al.*, 1994b), competition binding experiments were carried out (Table 2). In these experiments Fc-hinge fragments competed with mIgG1 coupled to sepharose for binding to radiolabeled, soluble FcRn. To investigate the interaction of FcRn with wild type and mutant Fc-hinge fragments further, SPR was used to determine apparent kinetic constants (Table 3). The observed kinetics of binding to FcRn coupled to the biosensor chip were assumed to follow a second order equation for a bimolecular association of one FcRn molecule with one Fc molecule, and a first order equation for a monomolecular dissociation of the complex. Analysis of the dissociation regions of the sensorgrams showed that for most of the data, the kinetic model of two parallel dissociation reactions gave the best fit of the observed sensorgrams with a probability of 0.99–1 when compared with a model of a single dissociation reaction. A similar biphasic dissociation phenomenon was reported previously in a number of studies involving antigen-antibody

Table 1. Parameters of the dissociation curves of FcRn:Fc–hinge complexes

Wild type Fc–hinge				Fc–hybrid			
R1 (RU)	$k_{d1} \times 10^3$ (sec^{-1})	R2 (RU)	$k_{d2} \times 10^4$ (sec^{-1})	R1 (RU)	$k_{d1} \times 10^3$ (sec^{-1})	R2 (RU)	$k_{d2} \times 10^4$ (sec^{-1})
68	13.2	505	2.69	59	13.7	541	2.25
34	13.8	454	3.0	50	8.34	446	2.39
43	8.04	376	2.52	39	8.09	337	3.15

Calculations were made for an interval 450–900 sec (30 sec after the start of the dissociation phase). RU, response unit; R1 and R2, SPR signals corresponding to the fast and slow dissociation processes respectively.

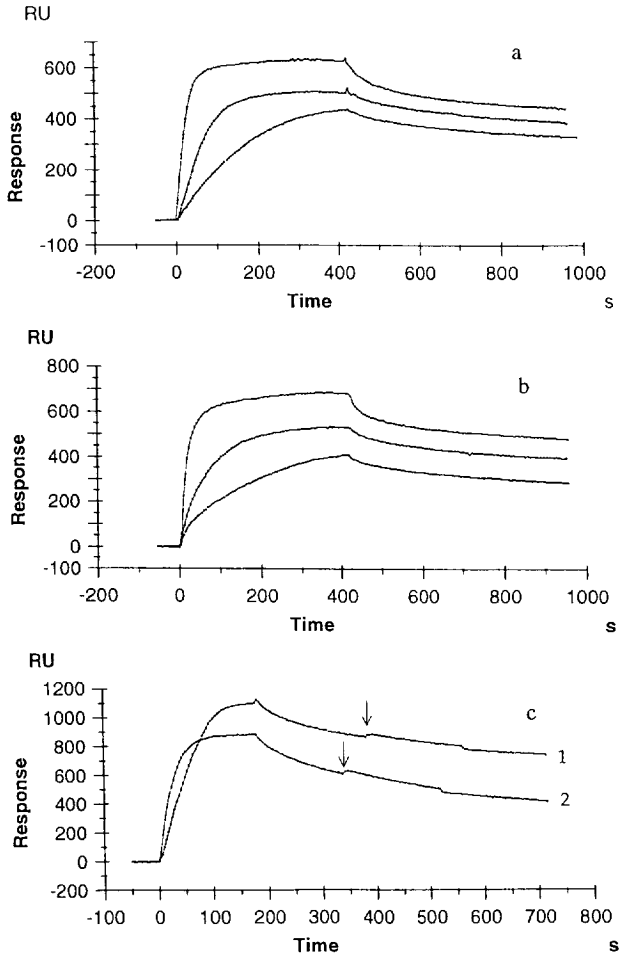

Fig. 6. SPR analyses of the FcRn interaction with wild type Fc-hinge (a), Fc-hybrid (b), the complex preformed at the chip (c). FcRn was immobilized (a, b, 1440 RU; c, 2290 RU) and the corresponding Fc-hinge fragment injected. Concentrations of the analytes were: a, wild type Fc-hinge, 55 nM, 110 nM and 375 nM; b, Fc-hybrid, 60 nM, 120 nM and 390 nM; c, wild type Fc-hinge, 320 nM (curve 1), Fc-papain, 157 nM (curve 2), FcRn in solution, 0.8 μM.

interactions (Borrebaeck *et al.*, 1992; Malmborg *et al.*, 1992; O'Shannessy *et al.*, 1993) and allows a number of different kinetic interpretations (Glaser, 1993; O'Shannessy *et al.*, 1993; Wohlhueter *et al.*, 1994). Typically, the slow dissociation reaction comprised *ca.* 80% or more of the net SPR response (Table 1), and this was used in the calculations of affinity (Table 3). Taking into account a rather small amplitude of the SPR signal for the fast dissociation phase, we did not study this phenomenon further.

The data shown in Tables 2 and 3 clearly demonstrate that mutation of the CH2 domain residues Ile-253 and His-310, Gln-311 influence the affinity of the Fc-hinge fragment to a greater extent than mutation of the CH3 domain residues His-433, Asn-434. This is consistent with the effects of these mutations on *in vivo* transcytosis in neonatal mice (Kim *et al.*, 1994a). Furthermore, the decreases in affinity for the I-253, HQ-310 mutants are due both to increased off-rates and decreased on-rates. The Fc-hybrid and the wild type Fc-hinge have similar off-rates, whereas the on-rate for Fc-hybrid is 1.5 times lower and this is most likely due to the presence of only one functional site, rather than two, per molecule. These data support the observation that only one FcRn molecule is involved in a direct interaction with either wild type Fc-hinge or Fc-hybrid. Furthermore, the affinities of mIgG1 and Fc-papain are similar to that of the wild type Fc-hinge. The correlation between percentage inhibition of binding (Table 2) and neonatal transcytosis (Kim *et al.*, 1994a) or β phase half life (Kim *et al.*, 1994b) are excellent (Pearson coefficients of 0.98 and 0.99, respectively) (Fig. 7).

DISCUSSION

In this report our recent studies on catabolism and transcytosis of IgGs (Kim *et al.*, 1994a,b,c, 1995) are extended to an analysis of the binding of wild type and mutant Fc-hinge fragments to the neonatal Fc receptor, FcRn. In order to characterize the FcRn:Fc interaction further, murine FcRn was expressed in soluble form in insect cells. The recombinant FcRn binds IgG in a pH dependent way both in solution (Fig. 3) and on the surface of the BIAcore chip (Fig. 6), and this is consistent with the findings of others for soluble fragments of rat FcRn (Gastinel *et al.*, 1992).

Table 2. Mutant Fc-hinge fragments and their inhibition of binding of recombinant soluble FcRn to mIgG1-sepharose

IgG or Fc-hinge fragment	% inhibition of binding
mIgG1	70.7 ± 5.4
Wild type Fc-hinge	67.0 ± 5.4
I-253	31.1 ± 7.3
HQ-310	26.1 ± 3.1
HN-433	53.7 ± 3.6
HQ-310/HN-433	21.5 ± 2.4

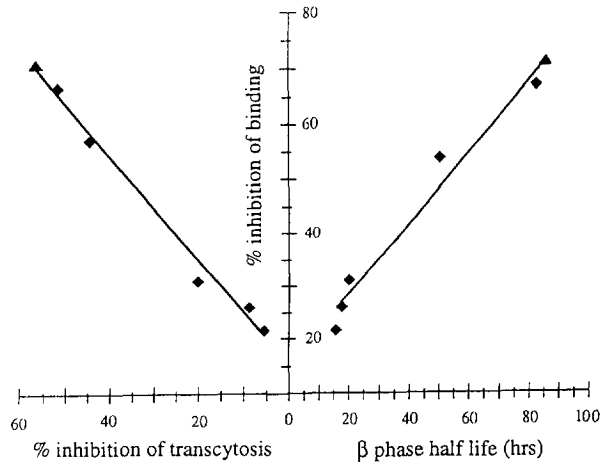

Fig. 7. Correlation between percentage inhibition of neonatal transcytosis, β phase half life and percentage inhibition of binding of ^{125}I-labeled FcRn to mIgG1-Sepharose (Table 2) for mIgG1 (filled triangles) and wild type/mutant Fc-hinge fragments (filled squares).

Fc residues that are involved in the control of IgG catabolism (Kim *et al.*, 1994b,c), transcytosis and binding to neonatal brush borders (Kim *et al.*, 1994a) were previously identified using a number of mutant Fc-hinge fragments. Although the wild type Fc-hinge fragment used in this and earlier studies is aglycosylated it has the same β phase half life and neonatal transcytosis activity as glycosylated mIgG1 (Kim *et al.*, 1994a,b). This indicates that for mIgG1, lack of glycosylation does not affect these two processes although this has been shown not to be the case for other isotypes (Tao and Morrison, 1989). Consistent with the data concerning neonatal transcytosis of the mutant Fc fragments (Kim *et al.*, 1994a), X-ray crystallographic studies suggested that amino acid residues located at the CH2–CH3 domain interface were involved in the FcRn:Fc interaction (Burmeister *et al.*, 1994). Mutation of conserved residues at positions 253 and 310, 311 within the CH2 domain have a considerable effect on both the association and dissociation rate constants (Table 3). The mutations at positions 433, 434 influence the kinetics and the affinity of the interaction to a lesser extent, whereas the mutant HQ-310/HN-433 that encompasses CH2 and CH3 domain residues has an affinity below the level of detection using SPR measurements. The affinities of the FcRn:Fc interaction for the wild type and mutant Fc-hinge fragments clearly correlate with the β-phase half lives and neonatal transcytosis activities (Fig. 7). This close overlap between the FcRn interaction site and the region that regulates IgG catabolism further supports the concept that the Fc receptors involved in neonatal transcytosis and catabolism control might be related, as originally proposed by Brambell *et al.* (1964).

The stoichiometry of binding in solution of rat FcRn to the Fc fragment of IgG was reported to be 2:1 (Huber *et al.*, 1993). The co-crystal stucture of rat FcRn and rat Fc revealed the presence of two distinct 2:1 FcRn:Fc

Table 3. Affinities of the interaction of the recombinant Fc fragments with FcRn derived from SPR measurements

Protein	$k_d \times 10^{3}$ [a] (sec^{-1})	$k_a \times 10^{-5}$ [b] (M sec^{-1})	K_d [c]
Fc-hinge	0.24 ± 0.04	1.4 ± 0.2	1.7 ± 0.1 nM
Fc-hybrid	0.26 ± 0.05	0.9 ± 0.2	2.5 ± 0.7 nM
mIgG1	1.4 ± 0.2	2.9 ± 0.6	4.8 ± 0.3 nM
Fc-papain	1.5 ± 0.2	2.6 ± 0.8	6.0 ± 1.5 nM
HN-433	0.4 ± 0.1	1.1 ± 0.3[d]	4.2 ± 2.5 nM[d] (fast)
		0.069–0.47	8.2–57 nM (slow)
HQ-310	7.6 ± 3.2	0.053 ± 0.019	1.4 ± 0.1 μM
I-253	8.5 ± 3.5	0.016 ± 0.003	5.1 ± 1.9 μM
HQ-310/HN-433		undetectable binding	

[a] Mean value and standard deviation of apparent dissociation kinetic constant.
[b] Mean value and standard deviation of apparent association kinetic constant.
[c] Mean value and standard deviation of equilibrium dissociation constant.
[d] Both slow and fast association process take place with a gradual increase in apparent K_d values.

To measure the affinities, FcRn was immobilized to a response of *ca* 1500 RU and the corresponding Fc-hinge fragment injected. Concentrations of the analytes were: wild type Fc-hinge, 55–750 nM; Fc-papain, 80–320 nM; Fc-hybrid 60–390 nM; HN-433, 29–570 nM; HQ-310, 2.3–9.3 μM; I-253, 5.0–8.0 μM; HQ-310/HN-433, 0.8–8 μM.

complexes (Burmeister *et al.*, 1994) and raised the question as to which complex is physiologically relevant. The two types of complexes can be distinquished, since in the "lying down" complex (Burmeister *et al.*, 1994) one FcRn molecule of an FcRn homodimer interacts with only one CH2–CH3 domain interface of the Fc fragment, whereas in the symmetric "standing up" complex each FcRn molecule binds one of two CH2–CH3 domain interfaces of the Fc fragment (Fig. 5).

In contrast to previous data (Huber *et al.*, 1993), in this study only 1FcRn:1Fc and 1FcRn:1mIgG1 complexes were detected using size exclusion chromatography, and this stoichiometry was not affected by the molar ratio of FcRn:Fc in the reaction mixture. In order to confirm the 1:1 stoichiometry of the FcRn:Fc interaction in solution, Fc was incubated with a mixture of both FcRn and CA-FcRn (FcRn without his6-tag). We reasoned that if they exist in solution, both "lying down" and "standing up" 2:1 FcRn:Fc complexes formed between equimolar amounts of Fc, FcRn and CA-FcRn would be retained on the Ni^{2+}-NTA-agarose column at pH 6.0, and the complex formation can be subsequently detected by elution at pH 7.5 of Fc plus CA-FcRn ("standing up" complex) or Fc plus CA-FcRn ("lying down" complex with a weak FcRn:FcRn interaction) or Fc only ("lying down" complex with a stable FcRn:FcRn interaction). However, no evidence for the existence of any of these complexes was obtained (Fig. 4), and the exclusion of the formation of FcRn dimers in solution is consistent with the observations of Huber *et al.* (1993). The stoichiometry of the FcRn-Fc interaction was also analysed using SPR (Karlsson *et al.*, 1991). A comparison of the data for binding of Fc-hinge and Fc-hybrid to FcRn on a sensor chip for different Fc concentrations (Table 1) shows that only one of two potential interaction sites of the wild type Fc-hinge is utilized for its high affinity interaction with FcRn.

The reasons for the difference in FcRn:Fc stoichiometry between our data and those of Huber *et al.* (1993) are not clear, although it may be due to a species difference (rat vs mouse). It is also possible that FcRn expressed in insect cells is glycosylated differently to the rat FcRn expressed in CHO cells (Gastinel *et al.*, 1992), and this may result in recombinant FcRn with different properties (Reilly *et al.*, 1992).

In this study the Fc-hybrid has similar affinity for binding to FcRn as the wild type Fc-hinge, and yet the Fc-hybrid is not transcytosed in neonatal mice (Kim *et al.*, 1994a). This led to the suggestion (Kim *et al.*, 1994a) that two functional sites are required for IgG transcytosis, and this is therefore apparently in contradiction with the stoichiometry of 1:1 for the FcRn:Fc interaction. This discrepancy may, however, be resolved if the interaction of the membrane bound FcRn with Fc/IgG differs to that of soluble FcRn with its ligands. For example, it may be possible for a membrane bound FcRn to form dimers that can be stabilized by interaction between either the FcRn transmembrane regions, cytoplasmic tails or both in a trimeric 2FcRn:1Fc complex which "sandwiches" one Fc fragment. In such a structure, the existence of a slow exchange of the Fc between two closely apposed FcRn molecules would explain the 1:1 stoichiometry in solution because at any particular moment only one FcRn molecule interacts with one site of the Fc. This model is consistent with the high flexibility of the CH2–CH3 domain interface in Fc (Deisenhofer, 1981; Romans *et al.*, 1977; Seegan *et al.*, 1979; for reviews see, Edmundson *et al.*, 1995; Nezlin, 1990) and also with the data of Simister and Rees (1985) who observed that in the presence of IgG, FcRn was dimeric. This model would explain why the Fc-hybrid, despite having an affinity similar to wild type Fc-hinge for FcRn binding (Table 2 and Table 3), is not transcytosed *in vivo*, as a second binding site is not available for interaction with FcRn in the dimer. The dichotomy between the *in vitro* and *in vivo* properties of the Fc-hybrid clearly requires further investigation.

In summary, Fc mutants that are defective in transcytosis and catabolism control also have lower binding affinity for interacting with recombinant FcRn. This extends further the close overlap between the site of IgG involved in these two processes and provides information concerning the molecular details, including the stoichiometry, of the FcRn:Fc interaction. Understanding the latter at the molecular level has relevance to the passive transfer of immunity in rodents, and the identification of a related Fc receptor in human placenta (Story *et al.*, 1994) suggests that our findings may be extrapolated to maternofetal transfer. In agreement with the data for murine IgG1, recent size exclusion experiments with an Fc fragment derived from murine IgG2a indicated a 1:1 stoichiometry for its interaction with murine FcRn.

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