Identification and function of neonatal Fc receptor in mammary gland of lactating mice

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In addition to its proposed function in regulating serum IgG levels, the MHC class I-related neonatal Fc receptor (FcRn) is known to play a role in IgG transfer across rodent yolk sac and neonatal intestine. In contrast to humans, for which transplacental transfer of IgG appears to be the only mechanism of maternal IgG delivery, the transmission of IgG in mice occurs both antenatally (yolk sac) and neonatally (transport from mother's milk across intestinal epithelial cells). In the current study, a possible role for FcRn in regulating IgG transfer into milk has been investigated. FcRn has been shown to be present in functional form in the mammary gland of lactating mice, and is localized to the epithelial cells of the acini. Analysis of the transfer of Fc fragments and IgG which have different affinities for FcRn indicate that, unexpectedly, these proteins are transferred in inverse correlation with their binding affinity for FcRn. Thus, in the lactating mammary gland FcRn appears to play a role in recycling IgG in a mode that may have relevance to FcRn trafficking during the maintenance of constant serum IgG levels.

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1 Introduction

In rodents, maternal IgG are transmitted both across the yolk sac to the fetus and postnatally from milk via the neonatal small intestine [1–3]. This IgG transfer provides the neonate with humoral immunity for the first few weeks of life, and selective IgG transport is mediated by the neonatal Fc receptor (FcRn) [4–6]. FcRn comprises a heterodimer of two non-covalently associated polypeptide chains [3, 5]. Interestingly, isolation and sequencing of the gene encoding the heavy chain of rat FcRn revealed that it shares homology with the alpha chain of MHC class I molecules [6]. As in MHC class I molecules, the associated light chain has been identified as β2-microglobulin [6]. More recent isolation of the mouse FcRn heavy chain gene indicates that it shares a high degree of homology with rat FcRn [7].

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Abbreviations: FcRn: Neonatal Fc receptor **HRP:** Horseradish peroxidase **RT:** Reverse transcriptase **TCA:** Trichloroacetic acid **WT:** Wild type **mIgG:** Mouse IgG

The transfer of IgG from blood to milk in lactating mice is a selective process, with a decrease in plasma IgG being coincident with the accumulation in milk [8–10]. This suggests that the transport of IgG into the lactating mammary gland might involve FcRn. However, a recent report demonstrated that the concentration of IgG in maternal milk of FcRn-deficient (due to homozygous deletion of β2-microglobulin) and normal mice were similar, leading to the conclusion that the transfer of IgG into milk is not mediated by FcRn [11]. The concentration of IgG in the plasma of β2-microglobulin-deficient mice is more than 20 times lower than in milk [11], suggesting that the transport of IgG from blood to milk is independent of the concentration of IgG in the blood. However, in mice that express FcRn, the available data indicate that murine IgG traverse the mammary gland with a rate dependent on the serum IgG concentration [8] and IgG transport across the mouse mammary gland is similar to the selective transport across the neonatal intestine and yolk sac [12, 13].

The goal of the current study was therefore to investigate whether FcRn is involved in the transfer of IgG from

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maternal blood to milk in the post-parturient mammary gland of mice using approaches distinct from those described previously [8, 11]. The location of FcRn expression in the lactating mammary gland has been analyzed by *in situ* hybridization and immunohistochemistry. In addition, functional studies have been carried out to quantitate the transfer of IgG or Fc fragments which have different affinities for FcRn. The data indicate that FcRn is involved in regulating the levels of IgG/Fc in milk, but with an inverse correlation between milk IgG/Fc concentration and affinity for FcRn.

2 Results and discussion

2.1 FcRn localization

Total RNA was extracted from the mammary glands of lactating SWISS mice at 7–10 days from the date of delivery for use in reverse transcriptase (RT)-PCR with FcRn α-chain-specific primers. The presence of the FcRn α-chain mRNA was detected in mammary glands and, as a control, in small intestines isolated from neonatal mice (Fig. 1). Compared with the neonatal gut, the level of FcRn in the lactating mammary gland is lower. *In situ* hybridization with an FcRn riboprobe revealed abundant expression of FcRn mRNA in the epithelial cells of the neonatal gut (Fig. 2B), consistent with previously described Northern blotting [6, 7] and RT-PCR [14] results. In sections of mammary gland probed using the same conditions, signal was detected over the acinar epithelial cells (Fig. 2A) but at a lower level than that seen in the neonatal gut. There was no expression associated with vessels within the interstitium of the mammary gland. Control sections hybridized with a sense riboprobe derived from FcRn resulted in diffuse, nonspecific low background signal (data not shown).

Immunohistochemistry using anti-FcRn $F(ab')_2$ fragment (directed against an α1-derived peptide of FcRn [15]) confirmed the *in situ* hybridization data. Epithelial cells of the neonatal gut stained positively (Fig. 2D). The epithelial brush borders were highlighted by darker staining than in the cytoplasm, consistent with some earlier studies [5, 16]. There was no staining of the endothelial cells in the lamina propria or muscular tunics. In sections of mammary glands (Fig. 2C), positive staining was seen in the acinar epithelium and within scattered histiocytes in the interstitium. Consistent with the *in situ* hybridization, no staining of the endothelium was observed. The concentration of antibody used for the detection of FcRn in the mammary gland was in the range of 20–40 μg/ml, whereas the presence of this receptor could still be detected in neonatal brush border using 1000-fold lower concentrations. However, even at the highest concentration of primary antibody, no signal could be detected in the endothelial cells of either the mammary gland or neonatal gut, indicating undetectable levels of FcRn in these cells. In contrast, endothelial expression of FcRn has been observed in mice [15] and humans [17]. In our earlier studies [15] endothelial cells in muscle and skin expressed detectable levels of this FcR, but the current studies demonstrate that endothelia in other sites such as neonatal gut and lactating mammary gland do not express FcRn or express this protein at levels that are not detectable by the methods used in this study. In this respect, FcRn has been reported to be absent in human fetal capillary endothelial cells [18], suggesting that IgG can traverse placental endothelial cells in an FcRn-independent manner.

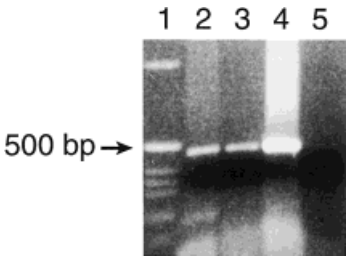

Figure 1. RT-PCR analysis of FcRn α-chain in the mammary glands of lactating mice. 1, 1 kb DNA ladder; 2, mammary gland (7 days post-delivery); 3, mammary gland (10 days post-delivery); 4, neonatal gut (7 days post-delivery); 5, negative control (no cDNA added to the reaction).

To confirm our findings that FcRn is present in the acinar epithelium in functional form, sections of mammary gland were incubated with biotinylated mouse IgG1 (mIgG1). Strong staining was apparent over the acinar epithelium at pH 6.0 (permissive for the IgG1-FcRn interaction) [4, 5, 19, 20] (Fig. 2E), whereas again no staining of the endothelium was detected. As expected for the IgG-FcRn interaction, no binding could be detected at pH 7.2 (Fig. 2F).

2.2 Functional activity of FcRn in the lactating mammary gland

The expression of FcRn in the murine mammary gland prompted us to carry out experiments to assess the transfer of Fc-hinge fragments from maternal blood into milk using both SWISS and SCID mice (Table 1). The latter mice were used since they have less than 10 μg/ml IgG in their serum and milk, and therefore the possible effects of competition by endogenous IgG are essentially absent. Transfer efficiency was calculated by dividing the amount of radiolabeled protein in 20 μl milk by the

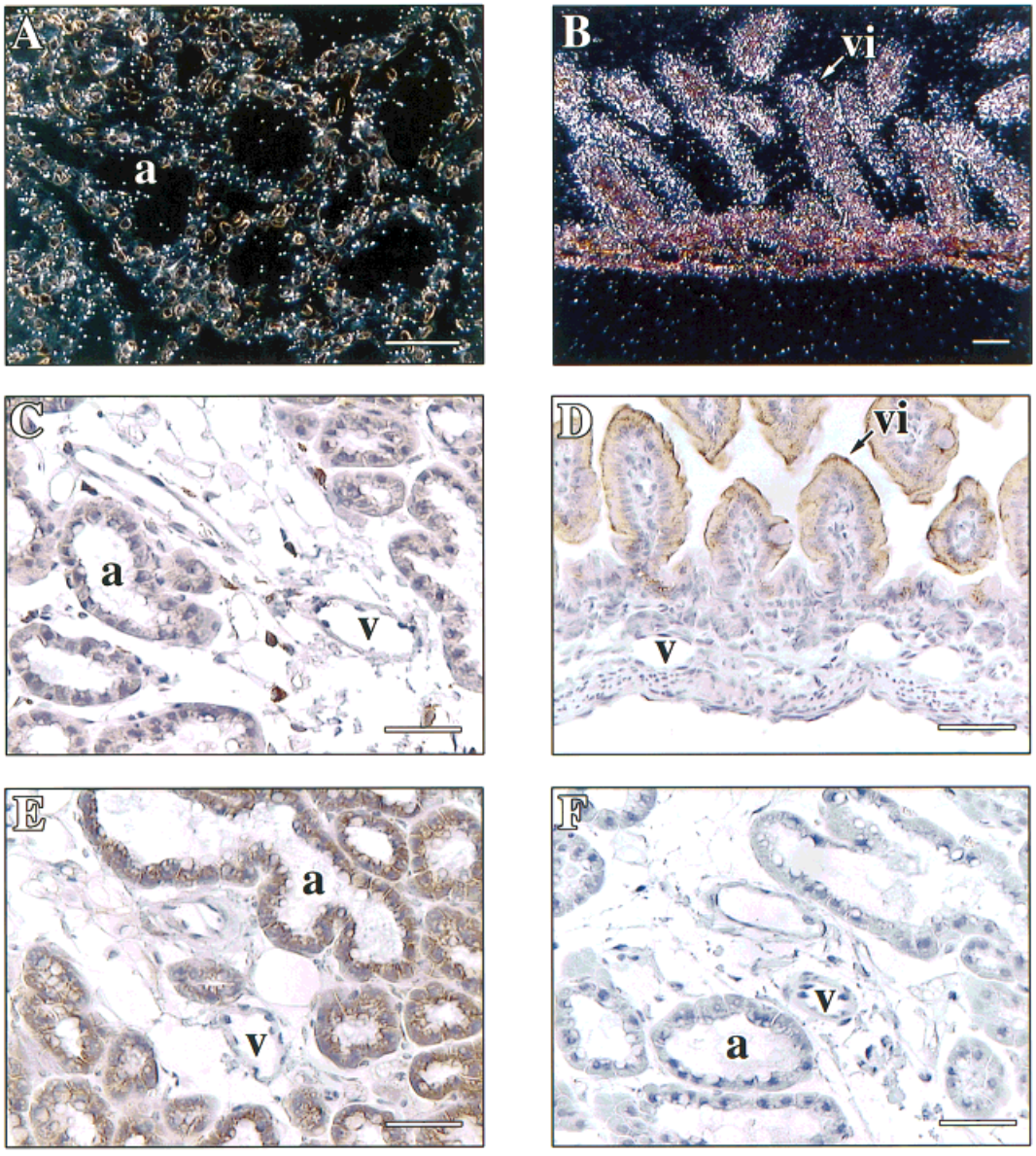

Figure 2. Dark-field images of mammary gland (A) and neonatal small intestine (B) hybridized with a riboprobe for FcRn. Silver grains are evident over the epithelial cells lining the acini of the mammary gland. Large numbers of silver grains are evident over the epithelial cells lining the intestinal villi of the intestinal gut. Immunohistochemical localization of FcRn in the mammary gland (C) and neonatal intestine (D) reveals positive staining for FcRn in the epithelium of the acini and in the cells lining the intestinal villi. Note the absence of staining in the endothelial cells lining the vessels in both the mammary gland and gut. Immunohistochemistry using mIgG1 at pH 6.0 (E) and pH 7.2 (F) indicates that IgG binds to acinar epithelium and not to endothelial cells and that the binding is pH dependent. Bar = 40 μ; a = acinus; v = vessel; vi = villus.

amount in 20 μl maternal serum at 24 h after injection of protein. Wild-type (WT) and mutated Fc-hinge fragments that have a range of affinities (from undetectable to high affinity) were used in these studies. Of significance, the absolute amount in milk of, for example, H435A mutant (which does not bind detectably to FcRn) and WT Fc-hinge at 24 h were similar, despite the lower amount of H435A in the blood due to its shorter serum half-life [21]. The data presented in Table 1 clearly indicate that in both mouse strains the WT Fc-hinge was transferred less efficiently than any of the mutants with lower affinity for FcRn. Moreover, there is an inverse correlation between

Table 1. Blood to milk transfer of recombinant mouse Fc-hinge fragments in lactating mice

Fc-hinge fragment	Strain of mice	Number of mice	Transfer coefficient (blood to milk[a])	Percent of TCA-insoluble material in milk[b]	Affinity for FcRn[c]	Half-life[d] (h)
WT	SCID	9	0.089 ± 0.019	56.6 ± 6.0	67.0 ± 5.4	119.1
	SWISS	5	0.147 ± 0.027	86.3 ± 9.0		
H435A	SCID	7	0.540 ± 0.056	54.4 ± 6.5	3.6 ± 2.9	17.4
	SWISS	4	0.652 ± 0.090	75.6 ± 5.1		
H433A-N434Q	SCID	4	0.309 ± 0.068	N. D.	53.7 ± 3.6	76.9
H310A-Q311N/ H433A/N434Q	SCID	5	0.788 ± 0.153	70.8 ± 5.1	21.5 ± 2.4	15.6
Fabγ1 (control)	SWISS	3	5.127 ± 0.400	51.9 ± 23.0		ND

a) Radioactivity in milk/radioactivity in blood at 24 h after injection of ^{125}I-labeled protein.
b) The percent of TCA-insoluble material in serum was in all cases over 95 %.
c) Measured as percent inhibition of ^{125}I-FcRn binding to mIgG1-Sepharose relative to the binding in absence of Fc-hinge fragment [20, 21].
d) Previously published results [21].

transfer, affinity for FcRn and half-life of these Fc fragments (Table 1). The Pearson correlation coefficients were –0.948 for transport into milk and half-life, and –0.833 for transport into milk and affinity for FcRn. To assess the integrity of the Fc-hinge fragments, serum and milk samples (with fat removed) from mice injected with WT or H435A Fc fragment were analyzed by SDS-PAGE followed by autoradiography. This resulted in two bands at 25 and 50 kDa corresponding to Fc-hinge fragments that are non-covalently and covalently linked (via hinge region – S-S bridges), respectively. Taken together with the trichloroacetic acid (TCA) insolubility of the transferred material, this indicates that intact Fc-hinge fragments rather than lower molecular weight metabolites are transferred. The transfer of an Fab fragment, which has a molecular weight similar to that of an Fc-hinge fragment but does not interact with FcRn was also analyzed (Table 1). This fragment was transferred more efficiently than any of the Fc-hinge fragments, reinforcing the inverse correlation between FcRn binding and transfer. This is reminiscent of earlier data in which it was shown that the average transmammary transfer of Bence Jones proteins is 40 times greater than that of human IgG [8]. This difference in transfer cannot be accounted for by diffusion alone [8] since the molecular size of these two proteins differs by only about threefold.

The transfer studies were extended to the analysis of several rat IgG (IgG1, IgG2a, IgG2b) which are known to have different affinities for FcRn [22]. Again, an inverse correlation between transfer, affinity for FcRn and half-life was observed (Table 2). Thus, the data obtained for Fc-hinge fragments do not reflect artifacts due to the use of recombinant nonglycosylated Fc-hinge molecules.

The transfer data in Tables 1 and 2 represent the transfer at 24 h after injection of radioiodinated proteins, *i.e.* a static time point. In addition, the contribution of transfer during the time for which the pups were present with lactating mothers (21 h after injection) was not assessed. Therefore, to analyze the dynamics of transfer, a different experimental approach based on that described previously by Gitlin and colleagues [8] was taken. Mothers were injected with radiolabeled IgG or Fc-hinge fragments and, at varying time points after injection, whole body radioactivity of the pups was compared with the radioactivity of the maternal blood (Fig. 3). Thus, instead of manually milking the mothers (which could not be carried out as a time course), pups were used as receptacles to quantitate transfer of radiolabeled protein into milk. In this approach, technical limitations precluded the analysis of the amount of radiolabeled protein in serum and intestine of the pups at these time points and complete body counts were determined. These counts there-

Table 2. Blood to milk transfer of rat IgG subclasses in lactating mice

IgG subclass	Transfer coefficient (blood to milk)[a]	Affinity (K_d) for FcRn (nM)[b]	Half-life[c] (h)
IgG1	0.244 ± 0.053	210	223.2
IgG2a	0.260 ± 0.056	140	234.7
IgG2b	0.550 ± 0.150	1067	57.2

a) Three SWISS mice for each group.
b) The affinities were derived from surface plasmon resonance experiments [22].
c) Previously published results [22].

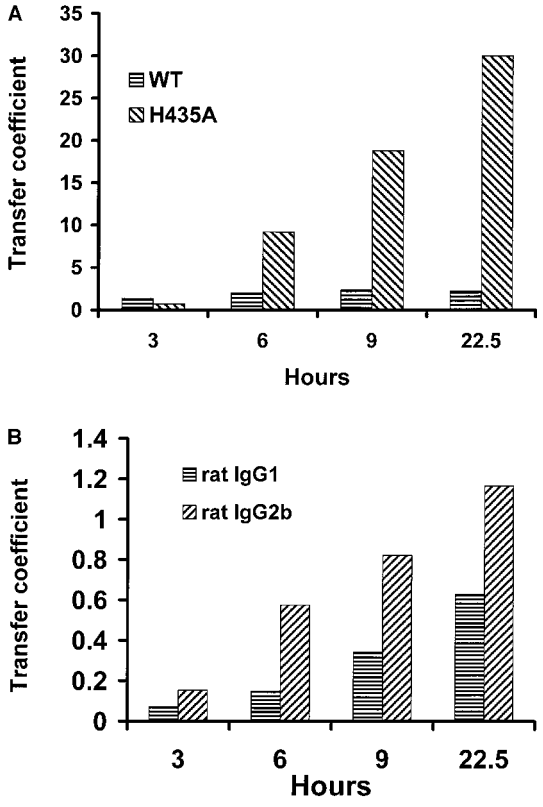

Figure 3. Transfer of WT and H435A mutant Fc fragments (A) and of rat IgG1 and rat IgG2b (B) from blood to milk of lactating mice. The transfer coefficient is the total amount of radioactivity of the pups divided by the radioactivity in maternal blood at the indicated times.

fore not only represent transferred protein in the ingested milk (in the stomach/intestine) but also protein that has been transferred to other body compartments such as the serum via neonatal, FcRn-mediated transcytosis. In addition, radiolabeled protein (or metabolites thereof) will be lost by excretion during the course of the experiment. As a result, the amount of radiolabeled protein in the pups is an underestimate of the amount transferred into the milk that the neonate has ingested. Despite these limitations, the data shown in Fig. 3 confirm and extend the observations for the analysis of transfer into milk at the 24-h time point (Tables 1 and 2) for both Fc-hinge and complete IgG molecules and clearly show an inverse correlation between affinity for FcRn and transfer.

The behavior of the Fc-hinge fragments and IgG contrasts with their activity in transfer across both the maternofetal barrier and the neonatal intestine, where FcRn binding shows a direct correlation with transfer [21]. The data in Tables 1, 2 and Fig. 3 indicate that molecules which interact with FcRn with high affinity appear to be recycled away from the milk gland, whereas proteins with low/undetectable affinity traverse the acinar epithelium into the milk. Thus, although FcRn is present in mammary gland, it appears to play a "reverse" role in IgG/Fc transport. This is similar to our observations for IgG transport into the gall bladder, where WT Fc fragments accumulated less well than their mutated derivatives with lower affinity for FcRn [15]. The mode of operation of FcRn in the acinar epithelia of the lactating mammary gland appears to be similar to that proposed for FcRn when acting as the receptor that regulates serum IgG levels [23], *i.e.* FcRn binds and returns its ligands to the vascular space. As a result of this recycling, IgG or Fc fragments with higher affinity for FcRn persist in the circulation for longer than those with lower affinity [21].

The precise route of FcRn trafficking in both serum IgG homeostasis and mammary transfer requires further investigation and could involve FcRn-mediated recycling and/or bidirectional transcytosis. Thus, FcRn could either play a dual role in transporting IgG both into and away from milk (bidirectional transcytosis), or act solely in the recycling mode. However, in rodents the data indicate that FcRn is saturable and unable to return all IgG, resulting in transfer of IgG in inverse correlation with their affinity. Of significance, the observation that proteins with low/undetectable affinity for FcRn accumulate in milk indicates that for these proteins, FcRn-mediated transcytosis is not required for delivery from the vascular space into milk.

Consistent with the proposed role of FcRn in limiting the transport of IgG into milk in an affinity-dependent manner, mice which lack functional FcRn due to homozygous deletion of β2-microglobulin have a higher IgG concentration in milk (200 μg/ml) than in serum (5–10 μg/ml) [11]. In β2-microglobulin-deficient animals, IgG therefore appear to be concentrated in the milk [11] in contrast to normal lactating mice where the concentration of IgG in milk is significantly lower than in the serum (our unpublished data and [9]).

The inverse correlation between transfer of an IgG or Fc fragment into milk and affinity for FcRn has interesting implications for the levels of IgG in the milk that the suckling neonatal mice ingest. The IgG1 isotype is present in milk in lower amounts relative to maternal serum levels than IgG2b, which has a lower affinity for FcRn (milk to blood ratios of 0.25 and 0.31 for murine IgG1 and mIgG2b, respectively; unpublished observation). However, the relative efficiency of transfer across the neonatal intestine, for which a direct correlation with affinity is seen [21], would be predicted to reverse this. The preferential transfer into milk of poor FcRn ligands followed by more efficient neonatal transfer of higher affinity FcRn ligands might therefore provide a mechanism by which

the levels of maternally derived IgG of different isotypes in the neonate are balanced.

Similar mechanisms of IgG transfer into the mammary gland may occur in other species. For example, ruminants deliver maternal IgG to newborn by intestinal transmission via milk [13] in a selective way [24]. The transfer works against a steep concentration gradient (*e. g.* the concentration of IgG is ten times higher in cow colostrum than in serum) [24] and requires the Fc fragment of IgG [25, 26]. The mammary gland of cows transmits the IgG1 subclass better than IgG2, with an IgG1/IgG2 ratio of greater than 10/1 in colostrum [24]. Interestingly, the serum half-life of bovine IgG2 is longer (17.7 days) than that of bovine IgG1 (9.6 days) (reviewed in [25]), again indicating an inverse correlation between serum persistence and transfer into milk (Tables 1 and 2). Although little is known about the existence of a bovine ortholog of FcRn [27], this suggests that similar mechanisms for IgG transfer into milk may occur in both mice and cows. However, in contrast to mice, intestinal transfer of IgG in ruminants does not show selectivity [28].

The extremely low concentration of IgG in human milk [29] suggests that either human FcRn is absent from the mammary gland or, if expressed, functions extremely efficiently to recycle IgG from milk back into serum. We favor the second explanation, since the absence of FcRn in the mammary gland of β2-microglobulin-deficient mice increased rather than decreased the concentration of IgG in milk [11]. By extension, IgG mutants that do not bind to FcRn would be predicted to concentrate in human milk. Regardless of the mechanism by which low milk IgG levels are attained in humans, IgG transfer occurs solely by transplacental transfer via a process in which FcRn has been implicated [18, 30–33]. It appears that the selective transmission of IgG from mother to young is regulated at distinct sites in different species, for example placenta for humans and monkeys, yolk sac for guinea pigs and rabbits, mammary gland for ruminants and yolk sac, mammary gland and gut for rodents [13, 34].

In summary, the data indicate that FcRn is expressed in the epithelial cells of lactating mammary gland where it functions to regulate IgG transfer into milk. However, in contrast to earlier reports in which analogies between maternofetal/intestinal transfer and transport into milk were drawn [8, 12], the current data demonstrate an inverse correlation for these two modes of IgG transport. This suggests that FcRn appears to function in the mammary gland primarily as a recycling, rather than transcytotic, receptor. It will be of interest to investigate the molecular mechanisms by which these different routes of FcRn trafficking are regulated.

3 Materials and methods

3.1 Mice

Outbred SWISS Webster mice were purchased from Charles River (Wilmington, MA) and Harlan Sprague-Dawley (Pratville, AL). Outbred SCID mice were purchased from Taconic (Germantown, NY). The animals were maintained in the UT Southwestern Medical Center animal facility. The mice were either mated in this facility, or untimed pregnant females were purchased (Harlan Sprague-Dawley). Mammary glands were harvested from mothers at 7–10 days after birth. Neonatal intestines were isolated from 7–10-day-old neonates for use as a control. The tissues were either used immediately or frozen at –80 °C.

3.2 Proteins and radiolabeling procedure

Recombinant WT Fc-hinge and mutant fragments derived from mIgG1 were expressed in *Escherichia coli* and purified using Ni^{2+}-NTA-Agarose as previously described [21, 35]. The following mutants were used: H435A, H433A-N434Q and H310A-Q311N/H433A-N434Q. Nomenclature of mutants used is as follows: H435A = His435 to Ala, etc. These fragments have been analyzed previously in FcRn interaction studies [21]. Fab fragment was obtained by digestion of mIgG1 with papain at pH 7.0 (Sigma, St. Louis, MO) followed by purification using protein A-Agarose and HPLC on a TSK3000SW preparative column. Rat IgG1, IgG2a and IgG2b were purchased from Zymed Laboratories (San Francisco, CA) and mIgG1 was obtained from Abbott Laboratories (Needham, MA). Proteins were radiolabeled with $Na^{125}I$ (Amersham, Arlington Heights, IL) using the Iodo-Gen procedure as previously described [35].

3.3 RT-PCR

Tissues were homogenized in 1 ml of TRIZOL (Gibco-BRL Life Technologies, Grand Island, NY) using a tissue grinder and the RNA extracted by chloroform followed by isopropyl alcohol precipitation. RNase-free DNase (Boehringer Mannheim, Indianapolis, IN) was added to the purified RNA to digest residual genomic DNA. Complementary DNA was synthesized using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and a forward primer which is complementary to the 3' of the gene encoding the cytoplasmic domain of the mouse FcRn α-chain (5' TCA GGA AGT GGC TGG AAA GGC ATT 3' [7]). The cDNA was used in the PCR with the same forward primer and back primer which primes in the α3 domain of murine FcRn (bases 640–656 of the coding sequence: 5'-TCT GGC TCC TCC GTG CT-3') resulting in a fragment of about 420 bp. PCR products were sequenced to ensure that the products were mouse FcRn gene segments.

3.4 Generation of riboprobes

An FcRn-derived PCR product was generated using the following primers: FcRn 481 back (anneals to bases 479–501 of FcRn coding sequence): 5' ATC A AAGCTT GC TAA TCT GTG GAT GAA GCA G 3', and FcRn for (anneals to bases 700–721 of FcRn coding sequence): 5' TAG T AAGCTT TA GCC CAT TGC GCA GGA ATC G 3'. Hind III restriction sites are underlined, and PCR products were digested with Hind III and ligated into Hind III-restricted pBluescript II KS phagemid. Plasmid DNA was isolated from clones harboring both orientations of the insert, and used in reverse transcription reactions with T7 RNA polymerase (Maxiscript kit, Ambion Inc., TX) in the presence of ^{35}S-labeled UTP (400–800 Ci/mmol, Amersham) to generate sense (negative control) and anti-sense riboprobes. Unincorporated radiolabeled ribonucleotides were removed using SELECT-D(RF) spin chromatography columns (5 Prime-3 Prime, Inc., Boulder, CO). Riboprobe integrity was analyzed by Southern transfer onto a Hybond-N membrane (Amersham) followed by exposure on a phosphorimager screen (Molecular Dynamics, CA).

3.5 *In situ* hybridization

In situ hybridization was performed as described [36] with some modifications [37]. Following euthanasia, adult lactating mice were perfused via cardiac puncture with heparinized saline followed by a 4 °C cold 4 % paraformaldehyde solution. The mammary gland was harvested and immersion-fixed in 4 % paraformaldehyde overnight at 4 °C. Sections of small intestines of euthanized neonatal mice were flushed in 4 % paraformaldehyde and immersion-fixed overnight at 4 °C. The tissues were then dehydrated through graded ethanols, cleared in xylene and embedded in paraffin. Sections of lactating mammary glands and neonatal intestines were cut at 5 μm. Paraffin was removed from the sections with xylene, followed by graded ethanol hydration, post-fixation in 4 % paraformaldehyde, pronase digestion (20 μg/ml for 7.5 min) and acetylation (0.1 M triethanolamine-HCl, pH 7.5, 0.25 % acetic anhydride for 5 min). Tissue sections were incubated with radiolabeled riboprobe (7.5×10^6 cpm/ml) for 12 h at 55 °C in 50 % formamide, 0.3 % dextran sulfate, 1 × Denhardt's solution, 0.5 mg/ml tRNA. Following hybridization, slides were washed in a series of increasing stringency buffers, treated with RNase A, washed and coated with K.5 nuclear emulsion (Ilford, GB) and exposed for 14 days. The slides were developed, counterstained with hematoxylin and examined using bright and dark field optics.

3.6 Immunohistochemistry

Sections of lactating mammary glands and neonatal intestines were cut at 5 μm, deparaffinized with xylene and rehydrated through graded ethanols. The slides were microwaved in Citrate Antigen Retrieval Buffer (BioGenex, San Ramon, CA) and then equilibrated in PBS. The sections were permeabilized in 0.3 % Triton X-100/PBS, washed and non-specific secondary binding was blocked with 3 % normal goat serum in PBS. The endogenous peroxidases were quenched in 0.3 % H_2O_2/CH_3OH. The primary antibody was a polyclonal anti-mouse FcRn F(ab')$_2$ fragment, obtained by immunizing rabbits with an FcRn-derived peptide (YCLN-GEEFMKFNPRIG), as described previously [15]. The antibody was diluted in 1.5 % normal goat serum/PBS at a final concentration of 20–40 μg/ml and incubated overnight with the sections at 4 °C. The bound anti-FcRn antibody was detected by sequential incubation with a biotinylated secondary antibody [goat anti-rabbit F(ab')$_2$ (ICN Biochemicals, Costa Mesa, CA), at 12 μg/ml in 1.5 % normal goat serum/PBS], followed by horse radish peroxidase-conjugated streptavidin at a 1/500 dilution (HRP-streptavidin; Vector, Burlingame, CA). Bound HRP-streptavidin was detected by addition of diaminobenzidine (Dako, Carpinteria, CA). The sections were counterstained with hematoxylin.

Biotinylated mIgG1 was also used to detect FcRn in tissue sections. mIgG1 was biotinylated at pH 8.0 with a 20-fold molar excess of biotin-3-sulfo-N-hydroxysuccinimide ester (Sigma), followed by gel filtration on Sephadex G-25 to remove excess biotin. Slides were microwaved as previously described, equilibrated in PBS, blocked with normal goat serum, incubated overnight with 25 μg/ml biotinylated mIgG1 in 1.5 % normal goat serum/PBS and the reaction developed with HRP-streptavidin (1/500 dilution). These experiments were carried out at pH 6.0 (permissive for the mIgG1-FcRn interaction) [4, 5, 19] and pH 7.2 (non-permissive for this interaction).

3.7 Quantitation of blood to milk transfer of rat IgG and mouse Fc fragments

Three days after delivery of pups, lactating SWISS or SCID mice were given 0.1 % Lugol solution (Sigma) in sweetened drinking water and throughout the entire period of the experiment. Twenty-four hours later, the animals were injected with 150 μl ^{125}I-labeled protein ($2–5 \times 10^7$ cpm) in the tail vein and bled retroorbitally with a 20-μl capillary micropipette, 3 min after injection. Twenty-one hours later, the mothers were separated from the litters and after 4 h 2 I.U. oxytocin (Sigma) in 200 μl saline was administered i.p. The animals were bled again (20 μl) and after anesthesia with metofane (Pitman-Moore, Mundelein, IL), milk was manually expressed. The radioactivity of 20 μl whole milk and of 20 μl blood harvested at 3 min and 24 h was measured in a gamma counter. The transmission was calculated by dividing the radioactivity of milk by the radioactivity of the blood at 24 h. The TCA-insoluble radioactivity of both blood and milk was measured by precipitation of the serum and milk (with fat removed) using 10 % TCA.

3.8 Analysis of the time dependence of blood to milk transfer of rat IgG and mouse Fc fragments

Following delivery, litter sizes were reduced to 3–5 pups of comparable weights and sizes. After 4–5 days, mothers were injected i.v. with radioiodinated IgG or Fc fragment. At 3, 6, 9 and 24 h after injection, the whole body radioactivity of the pups (individually) and mothers were determined. In addition, the radioactivity in 25 μl of blood harvested by retroorbital bleeding of mothers was determined at the same time intervals. Radioactivity measurements were determined using either an LKB-Wallac (Stockholm, Sweden) gamma counter (blood or whole body radioactivity of pups) or an AtomLab 100 dose calibrator (Atomic Products Corporation) (whole body radioactivity of pups and mothers). The "Transfer coefficient" for each protein was calculated by dividing the total body radioactivity of the pups by the total body radioactivity of the mother (both measured in μCi), or the total radioactivity of the pups divided by the radioactivity of mother's blood, both measured in cpm. The results obtained by both procedures were comparable and therefore only the data calculated by the second method are presented. The cpm of each individual pup differed from the average cpm for that litter by a SD of less than 20 %.

3.9 SDS-PAGE and autoradiography

Serum samples and milk (with fat removed) collected from lactating SWISS mice were analyzed by SDS-PAGE using nonreducing conditions and a Pharmacia-LKB PhastSystem. The radioactivity was detected by autoradiography.

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