Nonvectorial Surface Transport, Endocytosis via a Di-leucine-based Motif, and Bidirectional Transcytosis of Chimera Encoding the Cytosolic Tail of Rat FcRn Expressed in Madin-Darby Canine Kidney Cells*

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Transfer of passive immunity from the mother to the fetus or newborn involves the transport of IgG across several epithelia. Depending on the species, IgG is transported prenatally across the placenta and yolk sac or is absorbed from colostrum and milk by the small intestine of the suckling newborn. In both cases apical to basolateral transepithelial transport of IgG is thought to be mediated by FcRn, an IgG Fc receptor with homology to major histocompatibility class I antigens. Here, we analyzed the intracellular routing of a chimera encoding the rat FcRn tail fused to the ecto- and transmembrane domain of the macrophage FcγRIIB. Newly synthesized receptors were delivered in a nonvectorial manner to the apical and basolateral cell surface from where the chimera were able to internalize and transcytose. Apical to basolateral and basolateral to apical transcytosis were differently regulated. This intracellular routing of the chimera is similar to that of the native FcRn, indicating that the cytosolic tail of the receptor is necessary and sufficient to endow an unrelated FcR with the intracellular transport behavior of FcRn. Furthermore, the di-leucine motif in the cytosolic domain of FcRn was required for rapid and efficient endocytosis but not for basolateral sorting of the chimera.

Simple epithelia form barriers that allow the selective exchange of molecules between the lumen of an organ and the underlying tissue. One mechanism by which molecules can cross the epithelial barrier involves vesicular transepithelial transport (transcytosis). Our current knowledge about transcytosis of proteins is almost exclusively based on studies of the plgR, which mediates the transport of serosal polymeric IgA and IgM into mucosal secretions (for review, see Refs. 1 and 2). Transcytosis of the plgR can be faithfully reproduced in polarized kidney MDCK cells transfected with the receptor cDNA (3). Briefly, the plgR is transported from the trans-Golgi network to the basolateral surface where it binds ligand and is internalized. Basolateral targeting and internalization require signals in the form of short amino acid sequences located in the cytosolic receptor domain. Transcytosis occurs via basolateral early endosomes and an apically located endosomal recycling compartment (4, 5) and requires microtubules (6, 7) and probably BFA-sensitive coat proteins (5, 8, 9). Although free receptors transcytose, binding of ligand results in the transduction of signals (10) that stimulate the rate of transcytosis (11). It is therefore not surprising that transcytosis is regulated by a number of molecules involved in signal transduction, including CaM (12, 13).

In contrast to basolateral to apical transcytosis, little is known about transport in the opposite direction, and the scant information available is derived from proteins that are not physiologically involved in transcytosis (14–16). These studies indicate that apical to basolateral transcytosis may be independent of microtubules (6) and may also be regulated differently from transport in the opposite direction (15). In analogy to the plgR, however, apical to basolateral transcytosis of a bona fide transcytotic receptor may be subject to additional regulatory mechanisms.

One of the few known receptors to mediate the physiologically relevant apical to basolateral transcellular transport of a ligand is FcRn. FcRn, an IgG Fc receptor related to major histocompatibility complex class I molecules (17) was initially identified in the neonatal small intestine where it mediates the uptake of maternal IgG present in colostrum and milk (18–26). The receptor has also been implicated in the transport of IgG from the maternal circulation across the placental syncytiotrophoblast (27–30) or the yolk sac splanchnecle (31) into the fetal circulation. Like in the intestine, net transplacental IgG transport occurs in an apical to basolateral direction. More recently, a more general role of FcRn in the maintenance of IgG homeostasis by recycling internalized IgG and thus preventing its degradation in lysosomes has been proposed (32–34). Because binding of IgG to FcRn occurs preferentially at a slightly acidic pH of ~6.0 (for review, see Ref. 35), FcRn can bind ligand either at luminal surfaces exposed to an acidic environment such as in the intestine (24, 25), or in acidic endosomal compartments following the fluid phase internalization of IgG (31). Exposure of the receptor-ligand complex to a neutral pH following transcytosis or recycling may then lead to the dissociation of IgG into the extracellular milieu.

Characterization of the intracellular transport of human and rat FcRn in transfected epithelial MDCK cells indicates that newly synthesized receptors are transported in a nonvectorial manner.
Sorting Signals in the Cytosolic Domain of FcRn

fashion to the apical and basolateral cell surface and that they transcytose in both directions. To analyze the role of the cytosolic tail in intracellular sorting, we generated MDCK cell lines stably expressing chimera consisting of the ecto- and transmembrane domains of the macrophage FcγRIIb and wild-type or mutant cytosolic domains of FcRn. Newly synthesized FcRII/FcRn receptors were delivered in a nonpolarized manner to the apical and basolateral surface of MDCK cells. A dileucine motif in the cytosolic domain of FcRn was critical for efficient endocytosis but not for basolateral sorting. Following internalization, the chimeric receptors mediated bidirectional transcytosis. Trafficking of the FcRII/FcRn chimera closely resembles that of FcRn, consistent with a critical role of the cytosolic tail in FcRn traffic. Transcytosis of the chimera was not affected by BFA and only basolateral to apical transport required calmodulin and microtubule function, showing that transcytosis in the two directions is subject to different regulatory mechanisms.

**EXPERIMENTAL PROCEDURES**

**Materials**—Brefeldin A (Epicycle Technologies, Madison, WI), neocadzole (Sigma, Buchs, Switzerland) and W-7 (N-(6-aminohexyl)-S-chloro-1-naphthalenesulfonamide; Bio-Science Products) were purchased from Pierce Europe (Oud Beijerland, The Netherlands), prepared as a stock of 200 mg/ml Me2SO and used at a 1:1,000 dilution. 125I-NaI was from Amersham Corp. (Little Chalfont, UK), Easytag Express protein labeling mix from NEN Life Science Products (Du Pont de Nemours, Switzerland). Immunopure sulfo-NHS-biotin was purchased from Pierce Europe (Oud Beijerland, The Netherlands), prepared as a stock of 200 mg/ml Me2SO and used at a final concentration of 1.5 mg/ml. Fixed *Staphylococcus aureus* cells and protein A-negative *S. aureus* strain (Wood 46 strain) were obtained from Zymed Laboratories Inc. (San Francisco, CA) and used as a 10% suspension in phosphate-buffered saline + 0.5% Triton X-100 containing 0.5% bovine serum albumin. Streptavidin-agarose was from Sigma. Fixed *S. aureus* cells and agaroase beads were washed with phosphate-buffered saline + 0.5% Triton X-100 containing 0.5% bovine serum albumin before use. Mowiol 4-88 (Calbiochem-Novabiochem Corp.) was used at 0.1 g/ml supplemented with 0.2% (w/v) diazabicyle(2.2.2)octane (Sigma).

**Antibodies and Ligands**—Fab fragments of the monoclonal rat antiserum FcγRIIb antibody 2.4G2 (36) and a rabbit polyclonal anti-FcγRIIb serum (a gift provided by I. Meenan, Yale University, New Haven, CT) were used to detect chimeric receptors. Affinity purified labeled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). 2.4G2 Fab fragments were radioiodinated to specific activities of 2–7 × 106 cpm/μg using Iodogen (Pierce), unincorporated 125I was removed by ion exchange chromatography on Sephadex G-50 (Pharmacia) as described (41).

**Cell Culture and Transfection of MDCK Cells**—Cell culture and stable transfection of MDCK strain II cells was carried out as described (14). Two or three clones expressing each construct were analyzed. In some experiments, cells were incubated overnight with 5–10 mM butyrate (38) to induce expression. Butyrate treatment did not significantly alter the intracellular transport of the chimera under the conditions used. 125I-cane transferin bound in a polarized fashion to the basolateral side (39) and gp80 was secreted apically (40), indicating that the cell monolayers were fully polarized.

**Construction of FcRII/FcRn Chimera**—Deletion and substitution mutants of the FcRn tail were generated by PCR using rat FcRn cloned into Bluescript or pCB6 as a template. A sense primer covering the 5′ region of FcRn was combined with the different mutagenic antisense primers carrying XbaI sites in the 3′ noncoding region. PCR fragments were cut with BglII/XbaI and used to replace the BglII/XbaI fragment containing the tail domain of FcRn in Bluescript. The complete FcRn coding fragment was then excised from Bluescript with KpnI/XbaI and subcloned into the expression vector pCB6. To generate chimeras between the macrophage FcγRIIb ecto- and transmembrane domain and the wild-type or mutated FcRn cytosolic domain, a 5′ PCR primer was designed to introduce an in frame A/III site just adjacent to the C-terminal end of the transmembrane domain of FcRn. PCR products encoding wild-type and mutant FcRn tails were then appended to the luminal and transmembrane domains of a tail-less FcγRIIb2 construct carrying a unique A/III site following the transmembrane domain (38), resulting in the in frame fusion to the ecto- and transmembrane region of FcγRIIb2 (see Fig. 1). All fragments synthesized by PCR were verified by sequencing. The sequence of the primers is available upon request.

**Expression, Internalization, and Transcytosis of 2.4G2 Fab Fragments**—Endocytosis and transcytosis of FcRII/FcRn was analyzed by measuring the uptake and translocation of prebound iodinated 2.4G2 Fab fragments as described previously (6, 14), except that Leibowitz 15 medium (L-15) was used instead of Dulbecco’s modified Eagle’s medium. Binding of 125I-2.4G2 Fab fragments (2–3 × 105 cpm) was specific because the addition of 100-fold excess unlabeled Fab fragments inhibited binding by 90–95%. Treatment of cells with neocadzole (6), BFA (8), or W7 (13) was carried out as described. Transcytosis was defined as the fraction of total initially bound Fab fragments present on the surface and released into the compartment opposite that from which Fab fragments had initially been allowed to bind. More than 80% of the transcytosed Fab fragments represented surface-associated material. Experiments were done several times using duplicate or triplicate filters.

**Immunofluorescence**—Staining of cells with a polyclonal anti-FcR serum or with 2.4G2 Fab fragments and internalization of prebound antibodies was carried out as described previously (14). To measure reinternalization of transcytosed receptor-Fab complexes, cells grown on Transwell filters were incubated in the presence of 2.4G2-Fab (2 μg/ml in L-15) in the apical or basolateral chamber for 45 min at 37 °C. After cooling the cells on ice and washing with ice-cold phosphate-buffered saline +, labeled secondary antibody (2 μg/ml) was allowed to bind to transcytosed molecules for 1 h. Thereafter, cells were either fixed or incubated at 37 °C for another 15 min to allow reinternalization of bound fluorescent antibodies. Following internalization, cells were washed with L-15, pH 2.5, on ice to remove surface bound antibody, fixed with 3% paraformaldehyde and mounted. Colocalization of internalized (60 min at 37 °C) biotinylated 2.4G2-Fab (1 μg/ml in L-15) with lysosomes was done using the monoclonal antibody AC17 (kindly provided by A. Le Bivic, Marseille) as described (41).

**Polarized Membrane Insertion**—Insertion of newly synthesized receptors into the apical or basolateral cell surface was analyzed as described (18, 41).

**RESULTS**

**Expression of Chimera Encoding Wild-type and Mutant Cytosolic Tails of FcRn (FcRII/FcRn)**—To determine the role of the cytosolic tail of FcRn in intracellular transport, we generated chimera in which the ecto- and transmembrane domains of FcγRIIb2 were fused to wild-type or mutant cytosolic domains of FcRn (Fig. 1). Tail-less FcγRIIb2 accumulates on the apical surface of MDCK cells because it lacks the cytosolic di-leucine motif required for basolateral sorting and endocytosis (15, 42–44), making it an attractive tag to analyze the role of cytosolic tails in targeting to different intracellular compartments or the basolateral plasma membrane (15, 38, 41). In addition to a chimera encoding the wild-type FcRn tail (FcRII/FcRn), we generated constructs encoding a deletion of the C-terminal third (CT24) or the complete tail (CT4). Because the FcRn tail encodes a di-leucine motif similar to that responsible for basolateral sorting and endocytosis of FcγRIIb2 (45, 44), we also constructed chimera encoding alanine substitutions of Leu-22 and/or Leu-23 in the context of the CT24 mutant (CT24 L22A, CT24 L23A, and CT24 L22A,L23A). The different constructs were transfected into MDCK cells, and several clones stably expressing the different receptor chimera were selected and further analyzed.

A first indication as to the subcellular distribution of FcRII/FcRn was obtained by permeabilizing cells grown on coverslips and staining with the anti-FcγRII ectodomain monoclonal antibody 2.4G2. FcRII/FcRn predominantly localized to intracellular compartments (Fig. 2A), whereas the tail minus construct was mostly observed on the plasma membrane (Fig. 2B). If cells expressing FcRII/FcRn were incubated with the antibody on ice and then fixed, plasma membrane staining was readily detectable (Fig. 2C), indicating that a fraction of FcRII/FcRn was

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Fig. 1. Amino acid sequences of the cytosolic domains of wild type and mutant FcRII/FcRn chimera. Sequences are shown in the single-letter code, and alanine substitutions in the wild-type sequence are in bold. The CKII phosphorylation consensus sequence is underlined. Residues are numbered from left to right, with position 1 corresponding to the presumed first amino acid in the cytosolic domain.

Fig. 2. Steady state distribution, surface expression and internalization of FcRII/FcRn and a tail-minus mutant. Cells expressing FcRII/FcRn (A) or CT4 (B) were fixed, permeabilized, and stained with Fab fragments of the mouse monoclonal anti-Fc\(\alpha\) antibody prebound125I-labeled 2.4G2 followed by a labeled second antibody. In panels C—F, 2.4G2 was bound to cells expressing FcRII/FcRn (C and E) or CT4 (D and F) at 4 °C. Cells were then washed and either fixed (C and D) or incubated at 37 °C for 30 min to allow for the internalization of bound antibody (E and F). After internalization, 2.4G2 remaining on the cell surface was removed by washing with acid (pH 2.5) before fixation, permeabilization, and staining with the labeled second antibody.

Fig. 3. A di-leucine containing cytosolic region of FcRn is critical for efficient endocytosis. MDCK cells expressing the indicated chimera were allowed to internalize prebound radioiodinated 2.4G2 Fab fragments for the periods of time shown. After cooling the cells on ice, Fab fragments present in the medium, on the cell surface (pH 2.5 wash) or inside the cells was determined. The fraction of the initially bound Fab fragments that have been internalized at the different time points is shown. Values were determined in triplicate and differed by less than 15%.

Sorting Signals in the Cytosolic Domain of FcRn

**FCRn**

- NRMRSGLPAWLSLSDGDSGDL P QGNIPLPEAPQGVNAFPATS
- NRMRSGLPAWLSLSDGDSGDLP
- NRMRSGLPAWLSLSDGDSGDLT
- NRMRSGLPAWLSLSDGDSGDALP
- NRMRSGLPAWLSLSDGDSGDLAP
- NRMRSGLPAWLSLSGSDDSAAP

**CT24**

- NRMRSGLPAWLSLSDGDSGDL P QGNIPLPEAPQGVNAFPATS
- NRMRSGLPAWLSLSDGDSGDLP
- NRMRSGLPAWLSLSDGDSGDLT
- NRMRSGLPAWLSLSDGDSGDALP
- NRMRSGLPAWLSLSDGDSGDLAP
- NRMRSGLPAWLSLSGSDDSAAP

**CT24 L22A**

- NRMRSGLPAWLSLSDGDSGDL P QGNIPLPEAPQGVNAFPATS
- NRMRSGLPAWLSLSDGDSGDLP
- NRMRSGLPAWLSLSDGDSGDLT
- NRMRSGLPAWLSLSDGDSGDALP
- NRMRSGLPAWLSLSDGDSGDLAP
- NRMRSGLPAWLSLSGSDDSAAP

**CT24 L23A**

- NRMRSGLPAWLSLSDGDSGDL P QGNIPLPEAPQGVNAFPATS
- NRMRSGLPAWLSLSDGDSGDLP
- NRMRSGLPAWLSLSDGDSGDLT
- NRMRSGLPAWLSLSDGDSGDALP
- NRMRSGLPAWLSLSDGDSGDLAP
- NRMRSGLPAWLSLSGSDDSAAP

**CT24 L22,23A**

- NRMRSGLPAWLSLSDGDSGDL P QGNIPLPEAPQGVNAFPATS
- NRMRSGLPAWLSLSDGDSGDLP
- NRMRSGLPAWLSLSDGDSGDLT
- NRMRSGLPAWLSLSDGDSGDALP
- NRMRSGLPAWLSLSDGDSGDLAP
- NRMRSGLPAWLSLSGSDDSAAP

**CT4**

- NRMRSGLPAWLSLSDGDSGDL P QGNIPLPEAPQGVNAFPATS
- NRMRSGLPAWLSLSDGDSGDLP
- NRMRSGLPAWLSLSDGDSGDLT
- NRMRSGLPAWLSLSDGDSGDALP
- NRMRSGLPAWLSLSDGDSGDLAP
- NRMRSGLPAWLSLSGSDDSAAP

**FcRII-B2 TM & ectodomain**

**FcRII / FcRn**

**Fig. 3.** A di-leucine containing cytosolic region of FcRn is critical for efficient endocytosis. MDCK cells expressing the indicated chimera were allowed to internalize prebound radioiodinated 2.4G2 Fab fragments for the periods of time shown. After cooling the cells on ice, Fab fragments present in the medium, on the cell surface (pH 2.5 wash) or inside the cells was determined. The fraction of the initially bound Fab fragments that have been internalized at the different time points is shown. Values were determined in triplicate and differed by less than 15%.

- **A** di-leucine containing cytosolic region of FcRn is critical for efficient endocytosis. MDCK cells expressing the indicated chimera were allowed to internalize prebound radioiodinated 2.4G2 Fab fragments for the periods of time shown. After cooling the cells on ice, Fab fragments present in the medium, on the cell surface (pH 2.5 wash) or inside the cells was determined. The fraction of the initially bound Fab fragments that have been internalized at the different time points is shown. Values were determined in triplicate and differed by less than 15%.

- **B** di-leucine containing cytosolic region of FcRn is critical for efficient endocytosis. MDCK cells expressing the indicated chimera were allowed to internalize prebound radioiodinated 2.4G2 Fab fragments for the periods of time shown. After cooling the cells on ice, Fab fragments present in the medium, on the cell surface (pH 2.5 wash) or inside the cells was determined. The fraction of the initially bound Fab fragments that have been internalized at the different time points is shown. Values were determined in triplicate and differed by less than 15%.

- **C** di-leucine containing cytosolic region of FcRn is critical for efficient endocytosis. MDCK cells expressing the indicated chimera were allowed to internalize prebound radioiodinated 2.4G2 Fab fragments for the periods of time shown. After cooling the cells on ice, Fab fragments present in the medium, on the cell surface (pH 2.5 wash) or inside the cells was determined. The fraction of the initially bound Fab fragments that have been internalized at the different time points is shown. Values were determined in triplicate and differed by less than 15%.

- **D** di-leucine containing cytosolic region of FcRn is critical for efficient endocytosis. MDCK cells expressing the indicated chimera were allowed to internalize prebound radioiodinated 2.4G2 Fab fragments for the periods of time shown. After cooling the cells on ice, Fab fragments present in the medium, on the cell surface (pH 2.5 wash) or inside the cells was determined. The fraction of the initially bound Fab fragments that have been internalized at the different time points is shown. Values were determined in triplicate and differed by less than 15%.

- **E** di-leucine containing cytosolic region of FcRn is critical for efficient endocytosis. MDCK cells expressing the indicated chimera were allowed to internalize prebound radioiodinated 2.4G2 Fab fragments for the periods of time shown. After cooling the cells on ice, Fab fragments present in the medium, on the cell surface (pH 2.5 wash) or inside the cells was determined. The fraction of the initially bound Fab fragments that have been internalized at the different time points is shown. Values were determined in triplicate and differed by less than 15%.

- **F** di-leucine containing cytosolic region of FcRn is critical for efficient endocytosis. MDCK cells expressing the indicated chimera were allowed to internalize prebound radioiodinated 2.4G2 Fab fragments for the periods of time shown. After cooling the cells on ice, Fab fragments present in the medium, on the cell surface (pH 2.5 wash) or inside the cells was determined. The fraction of the initially bound Fab fragments that have been internalized at the different time points is shown. Values were determined in triplicate and differed by less than 15%.

As shown in Fig. 3, chimera encoding the wild-type tail rapidly and efficiently endocytosed Fab fragments and after 10 min of warming the cells, up to 50% of the Fab fragments had been internalized. Although deletion of the C-terminal half of the tail in CT24 led to a reduction in the rate and extent of endocytosis when compared with FcRII/FcRn, this mutant was still capable of significant internalization. In contrast, substitutions of either Leu-22, Leu-23, or both to alanines led to a dramatic reduction in endocytosis of CT24. Nevertheless, internalization by the leucine substitutions was still above that observed for the tail-minus construct. Similar results were obtained for the different mutants if endocytosis from the apical or basolateral cell surface was analyzed in cells grown as polarized monolayers (data not shown). These results indicate that the di-leucine motif in the cytosolic tail plays a critical role for rapid and efficient endocytosis of FcRn.
Sorting Signals in the Cytosolic Domain of FcRn

Table 4

| Construct                  | Apical | Basolateral |
|----------------------------|--------|-------------|
| FcRII/FcRn                 | 75%    | 25%         |
| FcRII/FcRn CT24            | 70%    | 30%         |
| FcRII/FcRn CT24 LL22,23A   | 76%    | 24%         |
| FcRII/FcRn CT4             | 60%    | 40%         |

Fig. 4. Polarized steady state distribution and insertion of newly synthesized chimeric receptor. A. steady state distribution of wild-type and mutant FcRII/FcRn. Cells expressing the indicated chimera were cultured with 2.4G2 Fab added either from the apical or the basolateral compartment on ice. Quantitation of the binding for each clone was obtained from five to nine independent experiments, each carried out using duplicate or triplicate filters. Values differed by less than 5% and similar distributions were obtained for several clones expressing different levels of the chimeric proteins. B, cell surface transport of newly synthesized chimeric receptors. MDCK cells expressing the indicated chimera were metabolically labeled for 15 min. Labeled proteins were chased for 30 or 45 min and the apical (ap) or basolateral (bl) cell surface was then biotinylated. Cells were lysed and total chimeric receptors were immunoprecipitated. An aliquot of the immunoprecipitate was used to determine total labeled receptor (Total); the rest was incubated with immobilized streptavidin to isolate biotinylated cell surface receptors (Surface). Precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The bands on the autoradiograph were quantitated by densitometry, and the amount of surface receptor was normalized to the total amount of labeled chimera. The fraction of each mutant inserted into the apical or basolateral domain is shown for a typical experiment. Similar results were obtained if cells were chased for 30 min in the continuous presence of the anti-FcRII antisera to detect labeled receptors that would only transiently be exposed on the cell surface (not shown).

receptors was not because of a lack of polarization of the different clones.

Thus, FcRII/FcRn preferentially localized to the apical surface at equilibrium, and the localization was not significantly altered for the different tail mutants analyzed.

Newly Synthesized FcRII/FcRn Are Transported in a Nonvectorial Fashion to the Apical and Basolateral Domain—To determine whether the apical steady state localization of FcRII/FcRn reflected the direct delivery of newly synthesized receptors to the apical domain, we monitored the appearance of a cohort of newly synthesized receptors on the apical and basolateral cell surface. Cells grown on Transwell units were pulse labeled for 15 min with 35S)methionine/cysteine, and labeled proteins were chased to the cell surface for 30 or 45 min. Cells were then cooled on ice, and the apical or basolateral surface was then biotinylated. Following cell lysis, total receptors were immunoprecipitated. An aliquot of the immunoprecipitated receptors was used to determine the total amount of receptor labeled, the rest was precipitated with streptavidin-agarose to isolate molecules that had appeared on the cell surface. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography and the amount of biotinylated surface receptors was normalized to the amount of total labeled receptors for each filter.

As shown in Fig. 4B, an equal fraction of pulse-labeled FcRII/FcRn was biotinylated from the apical and basolateral side, indicating that cell surface transport was not vectorial. Also CT24 and CT24 LL22,23A were inserted to similar extents into the apical and basolateral surface, indicating that the mutations did not alter the nonvectorial transport of FcRII/FcRn to the plasma membrane. In contrast, however, the CT4 tail-less construct was preferentially transported to the apical domain, consistent with previous studies (15, 42–44). Similar results were obtained if cells were chased for 30 min in the continuous presence of the anti-FcYRII antisera (42) to detect labeled receptors, which may only transiently be exposed on the cell surface (data not shown).

These experiments thus show that regardless of the predominant apical localization at equilibrium, FcRII/FcRn and the mutants are nonvectorially transported from the trans-Golgi network to the apical and basolateral surface.

FcRII/FcRn Transcytoses in Both Directions—Despite the nonpolarized membrane insertion, FcRII/FcRn was enriched on the apical domain at steady state, indicating that transcytosis may play a role in receptor distribution. We therefore analyzed transcytosis of 125I-2.4G2 Fab fragments prebound at 4 °C from the apical or basolateral surface of MDCK cell monolayers. After washing nonbound Fab fragments, the cells were incubated at 37 °C for different periods of time and returned on ice. The fraction of Fab fragments released into the apical or basolateral compartment or present on the apical and basolateral plasma membrane or inside the cells was quantitated. Fab fragments present on the membrane or in the medium opposite the compartment of addition at a given time were defined as having undergone transcytosis.

Fab fragments prebound to the basolateral cell surface were internalized by FcRII/FcRn as evidenced by their removal from the apical surface (Fig. 5A) and their intracellular appearance (Fig. 5B). After 30–60 min, Fab fragments started to appear on the opposite cell surface and by 60 min more than 30% of the basolateral surface had been transcytosed (Fig. 5D). In contrast, cells expressing CT4 transcytosed significantly less prebound Fab fragments. The transcytosed Fab fragments, more than 80% remained membrane associated (i.e., were removed by the acid wash, not shown). A small fraction of Fab fragments was released basolaterally (Fig. 5C) and less than 20% was still on the basolateral surface after 60 min (Fig. 5A). Because transcytosed receptors are able to reinternalize (see below) and our biochemical transcytosis assay only detects Fab fragments present on the opposite surface at any given time, the actual extent of transcytosis is most likely underestimated.

Although Fab fragments internalized from the basolateral side efficiently transcytosed, only 15–20% of the Fab fragments internalized from the apical side transcytosed (Fig. 6D) and ~60% were released into the apical medium or still on the apical surface after 60 min (Fig. 6, A and C). Transcytosis by
FcRII/FcRn, however, was still significantly higher than that by the tail-minus mutant, where less than 5% of the Fab fragments transcytosed (Fig. 6D). As discussed above, also transcytosis in the apical to basolateral direction is probably underestimated, because translocated receptors can reinternalize from the basolateral cell surface (see below).

More than 90% of the Fab fragments recovered in the medium after 60 min at 37 °C were trichloroacetic acid precipitable and thus represented intact Fab fragments that had either dissociated after warming the cells or following recycling or transcytosis. Consistent with the lack of Fab degradation, little if any colocalization of internalized Fab fragments with late endosomes and lysosomes (labeled with the anti-canine lamp-2 antibody AC17, see Ref. 45) was observed (not shown).

To visualize transcytosis, cells expressing FcRII/FcRn were incubated for 45 min at 37 °C in the presence of 2.4G2 Fab fragments added to the apical or basolateral compartment. Cells were then cooled on ice, and a labeled goat anti-rat antibody was added to the opposite chamber. As shown in Fig. 7, A and D, cells incubated with Fab fragments in the apical chamber showed the typical labeling of the basolateral surface, whereas addition of Fab fragments to the basolateral chamber resulted in apical plasma membrane staining, indicating that Fab fragments had transcytosed in both directions. As expected for plasma membrane labeling, the staining was sensitive to acid pH wash (not shown), and no labeling was observed in the absence of Fab fragments (not shown), if cells were incubated with Fab fragments at 4 °C (Fig. 7, C and F) or if nontransfected cells were used (not shown). If cells carrying labeled second antibody bound to transcytosed Fab fragments (as shown in Fig. 7, A and D) were warmed for 15 min at 37 °C, labeled antibodies were internalized into a vesicular compartment (Fig. 7, B and E), showing that transcytosed FcRII/FcRn could indeed reinternalize from the opposite cell surface.

In summary, these experiments show that Fab fragments bound to apical or basolateral FcRII/FcRn are able to transcytose to the opposite plasma membrane domain where they can reinternalize.
compartment and then cooled on ice. A labeled second antibody was the opposite cell surface. MDCK cells expressing FcRII/FcRn were 37 °C for 15 min to allow for endocytosis of bound labeled antibodies (A, B, D, and E) or 4 °C (C and F) with 2.4G2 Fab fragments present in the basolateral (A–C) or apical (D–F) compartment and then cooled on ice. A labeled second antibody was allowed to bind from the opposite compartment for 60 min on ice. Cells were then either fixed and stained (A, D, C, and F), or transferred to 37 °C for 15 min to allow for endocytosis of bound labeled antibodies (B and E). Membrane staining (A and D) indicates transcytosis of Fab fragments and vesicular labeling (B and E) shows reinternalization of transcytosed Fab fragments. No binding of second antibody was seen if cells were incubated with Fab fragments at 4 °C (C and F).

Fig. 7. Transcytosed Fab fragments are reinternalized from the opposite cell surface. MDCK cells expressing FcRII/FcRn were incubated for 45 min at 37 °C (A, B, D, and E) or 4 °C (C and F) with 2.4G2 Fab fragments present in the basolateral (A–C) or apical (D–F) compartment and then cooled on ice. A labeled second antibody was the opposite cell surface. MDCK cells expressing FcRII/FcRn were 37 °C for 15 min to allow for endocytosis of bound labeled antibodies (A, B, D, and E) or transferred to 37 °C for 15 min to allow for endocytosis of bound labeled antibodies (B and E). Membrane staining (A and D) indicates transcytosis of Fab fragments and vesicular labeling (B and E) shows reinternalization of transcytosed Fab fragments. No binding of second antibody was seen if cells were incubated with Fab fragments at 4 °C (C and F).

As shown in Fig. 8, BFA had no significant effect on transcytosis in either direction. Apical to basolateral transport was also not affected if microtubules were depolymerized or if calmodulin function was impaired using W-7. In contrast, W-7 and nocodazole severely interfered with basolateral to apical transport and reduced transcytosis to 40 and 60% of control values, respectively. These results show different requirements for calmodulin activity and microtubules in apical to basolateral and basolateral to apical transcytosis of the same protein.

DISCUSSION

FcRn is implicated in the luminal to serosal transepithelial transport of IgG in the small intestine, the placental syncytiotrophoblast and the yolk sac (2, 46). When expressed in MDCK cells, newly synthesized human and rat FcRn are delivered in a nonpolarized manner to the apical and basolateral surface from where they transcytose to the opposite domain.2 Using chimera encoding the cytosolic tail of rat FcRn fused to the ecto- and transmembrane domain of FcyRIIb2, we analyzed the contribution of the cytosolic tail of FcRn to its intracellular routing. Interestingly, the overall intracellular traffic of the chimera was very similar to that of native FcRn, indicating that the cytosolic domain of FcRn can confer the intracellular transport behavior of FcRn to an unrelated FcR. Furthermore, a di-leucine motif in the tail of FcRn was identified that is critical for rapid and efficient endocytosis but not for basolateral sorting.

Biosynthetic Surface Transport—Newly synthesized FcRII/FcRn was delivered to the cell surface in a nonpolarized fashion. Because FcRn can shuttle between the apical and basolateral surface following endocytosis, efficient sorting to one or the other domain during biosynthesis may not be required. In contrast, the pIgR, which transcytoses in the basolateral to apical direction and is then proteolytically cleaved upon arrival on the apical domain (3), requires efficient basolateral sorting to ensure that all receptor molecules are available for transcytosis. The nonvectorial transport of FcRII/FcRn may be because of a weak basolateral sorting signal in the cytosolic domain of FcRn in conjunction with a putative recessive apical determinant in the transmembrane and/or ectodomain of FcyRIIb2. The presence of a weak basolateral sorting activity in the FcRn tail is implicated by the larger fraction of FcRII/FcRn transcytosed basolaterally as compared with the tail-minus construct (50% versus 20%). Di-leucine-based signals can mediate basolateral sorting of several proteins, including FcyRIIb2 (43, 44). Although FcRn encodes a di-leucine motif at a similar position in the tail as FcyRIIb2, cell surface insertion of FcRn/FcRn was not affected by inactivating the di-leucine motif in the context of a truncated tail construct (CT24 L22A,L23A), suggesting that an unknown feature in the membrane proximal half of the tail is responsible for the more pronounced basolateral surface transport of FcRII/FcRn as compared with CT4. Alternatively, because the di-leucine motif is located upstream from a serine residue within a CKII site (see Fig. 1) that is subject to phosphorylation,3 its basolateral sorting activity may be subject to regulation. CKII phosphorylation of serines has been implicated in modulating the traffic of several proteins (47–50). Also the pIgR encodes a phosphorylated serine (Ser-664) in the vicinity of the basolateral sorting signal and substitution of Ser-664 by an aspartic acid to mimic the negative charge of a phosphoserine results in the nonpolarized transport of the normally basolaterally sorted receptor (51). Although it is conceivable that CKII phosphorylation plays a direct role in basolateral sorting or indirectly regulates the activity of a basolateral signal, it has to be noted that the human FcRn lacks the CKII phosphorylation site, but it is also transported in a nonvectorial fashion in the biosynthetic route.4

Endocytosis and Transcytosis—In contrast to basolateral sorting, efficient internalization of FcRII/FcRn requires the presence of the di-leucine motif. As observed for FcyRIIb2 (43, 44, 52), endocytosis of FcRn/FcRn was only slightly reduced by deleting the C-terminal half of the tail (CT24) but was dramatically inhibited if one or both leucines were mutated to alanine. Nevertheless, CT24 L22A,L23A retained some residual internalization activity when compared with a tail-minus construct. A CKII site plays a role in internalization of furin (53–55), and efficient endocytosis of the pIgR requires, in addition to two tyrosine determinants (56), a serine within a CKII consensus sequence (57). Thus, a possible involvement of the CKII site in the cytosolic domain of rat FcRn in regulating the endocytic activity of the di-leucine signal remains to be analyzed.

FcRn/FcRn transcytosed in both directions. Although receptors internalized from the apical domain mostly recycled back to the apical domain, 15–20% of apically prebound Fab fragments transcytosed to the basolateral surface after 60 min. Fab fragments internalized from the basolateral surface transcytosed more efficiently (30%) and a smaller fraction recycled. The amount of transcytosis is likely underestimated because transcytosed Fab fragments are rapidly reinternalized and the biochemical assay only detects Fab fragments present on the surface at any particular time point. For comparison, 20 and 40% of antibodies prebound to a mutant low density lipoprotein receptor (15) or FcyRIIb2 (6) are transcytosed in the apical to basolateral direction, but less than 5% of prebound dIgA is

3 I. Stefaner and W. Hunziker, unpublished observation.
translocated basolaterally by the pIgR (6). In the opposite direction, 25–50% (depending on the nature of the ligand or the assay) of the pIgR transcytoses (3, 6, 11), but less than 5% of the low density lipoprotein receptor, FcγRIIb2, or the transferrin receptor is translocated to the apical surface (14, 15, 39).

Signals for polarized sorting are not only decoded at the level of the trans-Golgi network but are also recognized in endosomes. Thus, the nonvectorial surface transport of FeRn in the biosynthetic pathway correlates well with the observation that receptors internalized from one or the other cell surface could both recycle and transcytose and is consistent with FeRn encoding a “weak” basolateral signal. Indeed, routing of FeRn/FcRn is very similar to that of low density lipoprotein receptor mutants, which still encode the weak membrane-proximal basolateral sorting signal but lack the strong distal determinant (15). Because serine phosphorylation is implicated in transcytosis of the pIgR (58, 59), phosphorylation of the CKII site may also regulate transcytosis of the rat FeRn, either by acting as an independent signal or by activating or inactivating a basolateral sorting activity. Because the human FeRn lacks the CKII site but still undergoes bidirectional transcytosis, a putative regulatory role of phosphorylation on receptor traffic may be restricted to the rodent receptor. Internalized Fab fragments were not transferred to lysosomes, consistent with a second function of FeRn in addition to transcytosis, namely recycling IgG internalized in the fluid phase, thereby preventing lysosomal degradation of endocytosed IgG (2, 46).

BFA-sensitive coat proteins, microtubules, and the activity of calmodulin have been implicated in transcytosis. BFA inhibits basolateral to apical transcytosis of ligand-loaded but not of empty pIgR (8, 60). Although BFA did not affect FeRn/FcRn mediated transcytosis of Fab fragments, it will be interesting to empty pIgR (8, 60). Although BFA did not affect FcRII/FcRn its basolateral to apical transcytosis of ligand-loaded but not of recycling IgG internalized in the fluid phase, thereby preventing second function of FcRn in addition to transcytosis, namely ferrin receptor is translocated to the apical surface (14, 15, 39).

Functional role, if any, of FeRn phosphorylation and whether specific stimulation of apical to basolateral transcytosis by ligand may ensure efficient transfer of IgG across the enterocyte and syncytiotrophoblast. The observed basolateral to apical transport may then serve to recycle empty FeRn molecules for reuse. The presence of a CKII phosphorylation site in the cytosolic tail of the human FcRn is very similar to that of low density lipoprotein receptor, FcRIIb2, or the transcytosis of Fab fragments, it will be interesting to determine whether IgG binding stimulates the transcytotic activity we found associated with the FeRn tail. Specific stimulation of apical to basolateral transcytosis by ligand may ensure efficient transfer of IgG across the enterocyte and syncytiotrophoblast.

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