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Intracellular traffic of the MHC class I-like IgG Fc receptor, FcRn, expressed in epithelial MDCK cells

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SUMMARY

Transfer of passive immunity from 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 MHC class I antigens. We have now expressed the human FcRn in polarized MDCK cells and analyzed the intracellular routing of the receptor. FcRn showed a predominant intracellular localization at steady state. Newly synthesized FcRn was delivered in a non-vectorial fashion to both the apical and basolateral surfaces of MDCK cell monolayers. Following internalization from the apical or basolateral domain, the receptor transcytosed to the opposite surface. These findings provide direct evidence for the transepithelial transport function of FcRn and indicate that the receptor undergoes multiple rounds of transcytosis.

Key words: Fc receptor, Immunoglobulin G, Intestine, Major histocompatibility complex, Neonatal immunity, Placenta, Receptor-mediated endocytosis

INTRODUCTION

Simple epithelia form barriers which allow the selective exchange of macromolecules between the lumen of an organ and the underlying tissue. One mechanism by which molecules can cross the epithelial barrier involves vesicular transepithelial transport or transcytosis. Our current knowledge about transcytosis is mainly based on studies of the polymeric immunoglobulin receptor (pIgR), which mediates the translocation of serosal polymeric IgA and IgM into mucosal and glandular secretions (for a recent review see Hunziker and Kraehenbuhl, 1998). The transcytotic pathway of the pIgR is faithfully reproduced in polarized kidney MDCK cells transfected with the receptor cDNA (Mostov and Deitcher, 1986). Briefly, the pIgR is transported from the TGN to the basolateral surface where it binds ligand and is internalized. Transcytosis occurs via basolateral early endosomes and apically located recycling endosomes (Apodaca et al., 1994b; Barroso and Sztul, 1994) associated with rab17 (Hunziker and Peters, 1998) and requires microtubules (Breitfeld et al., 1990; Hunziker et al., 1990) and probably BFA-sensitive coat proteins (Barroso and Sztul, 1994; Hunziker et al., 1991b; Whitney et al., 1995). Although the pIgR efficiently transcytoses in the absence of ligand, binding of dIgA transduces a signal (Cardone et al., 1996) which stimulates the rate of transcytosis (Song et al., 1994). It is therefore not surprising that transcytosis is regulated by a number of signaling molecules, including calmodulin (Apodaca et al., 1994a; Hunziker, 1994).

In contrast to basolateral to apical transcytosis, little is known about the mechanisms and organelles involved in transport in the opposite direction and the scant information available is derived from proteins that are not physiologically involved in this process (Hunziker and Mellman, 1989; Matter et al., 1993; Sandvig and Vandeurs, 1996). Apical to basolateral transcytosis may be independent of microtubules (Hunziker et al., 1990; Stefaner et al., 1999) and regulated differently from transport in the opposite direction (Matter et al., 1993; Stefaner et al., 1999). One of the few receptors implicated in a physiologically relevant apical to basolateral transcellular transport is FcRn. FcRn, an IgG Fc receptor (FcR) related to MHC-class I molecules (Simister and Mostov, 1989), was initially identified in the neonatal small intestine where it is thought to mediate the uptake of maternal IgG present in colostrum and milk (Brambell, 1970; Brambell et al., 1954; Brambell and Halliday, 1956; Jones and Waldmann, 1972; Morris and Morris, 1974; Rodewald, 1973, 1976, 1980; Simister and Rees, 1985). FcRn may also transport IgG from the maternal circulation across the placental syncytiotrophoblast (Kristoffersen and Matre, 1996; Leach et al., 1996; Simister et al., 1996; Story et al., 1994) and the yolk sac splanchnopleur (Roberts et al., 1990) into the fetal circulation. Similar to the intestine, net transplacental transport of IgG across these epithelia occurs in an apical to basolateral direction.

Binding of IgG to FcRn requires a mildly acidic pH of ~6.0 (for a review see Raghavan and Bjorkman, 1996). FcRn present on the lumenal surface of enterocytes is exposed to the acidic environment of the gut where it can bind IgG (Rodewald, 1976, 1980). In placental syncytiotrophoblast or yolk sac, however, the apical domain is exposed to the neutral pH of the maternal blood. Here, IgG may first have to be taken up in the fluid phase before it can bind to FcRn in acidic endosomes (Ellinger et al., 1999; Roberts et al., 1990). Following transcytosis across intestinal, syncytiotrophoblast or yolk sac cells, exposure of the receptor-ligand complex to the physiological serosal pH at the basolateral surface may lead to the dissociation of IgG. In addition to transcytosis, FcRn may play a more general role in maintaining IgG homeostasis: IgG internalized in the fluid phase may bind to FcRn in endosomes, from where the receptor may recycle the IgG back into the circulation, thus preventing its lysosomal degradation (Ghetie et al., 1996; Israel et al., 1996; Junghans and Anderson, 1996).

The postulated involvement of FcRn in IgG transcytosis and homeostasis so far relies on indirect evidence obtained from β2m knock-out mice and mutant IgG (for a review see Hunziker and Kraehenbuhl, 1998). The transfer of IgG from the mother to the fetus or newborn is decreased in β2m-null animals and these mice also have lower serum IgG levels (Israel et al., 1995; Spriggs et al., 1992; Zijlstra et al., 1990). In addition, normal mice transport mutant IgG defective in binding to FcRn less efficiently to the offspring and this IgG is also turned over more rapidly.

To obtain direct evidence for the postulated role of FcRn in transcytosis, we expressed an epitope-tagged human FcRn in polarized MDCK cells and characterized its intracellular routing. Our experiments support an involvement of FcRn in transepithelial transport and indicate that in contrast to the pIgR, FcRn is reutilized for several rounds of transcytosis.

MATERIALS AND METHODS

Materials

Protease inhibitor cocktail contained 10 μg/ml chymostatin, antipain, leupeptin and pepstatin A (all from Sigma Chem. Corp., Buchs, Switzerland) in DMSO and was used at a 1:1000 dilution. EasyTag Express protein labeling mix was from NEN (Du Pont de Nemours, Switzerland). Immunopure sulfo-NHS-Biotin was purchased from Pierce Europe (Oud Beijerland, The Netherlands) and was prepared as a stock of 200 mg/ml DMSO. Fixed *Staphylococcus aureus* cells and Protein A-negative *Staphylococcus aureus* strain (Wood 46 strain) were obtained from Zymed (San Francisco, CA) and used as a 10% suspension in PBS + 0.5% TX-100. Streptavidin-agarose (Sigma Chem Corp., Buchs, Switzerland) and Protein G-Sepharose (Amersham Pharmacia Biotech) were washed with PBS + 0.5% TX-100 before use. Mowiol 4-88 was from Calbiochem-Novabiochem Corp. (La Jolla, CA) and used at 0.1 g/ml supplemented with 0.2% (w/v) DABCO (Sigma Chem. Corp., Buchs, Switzerland).

Antibodies and ligands

Anti-Flag antibodies and anti-Flag antibodies conjugated to Sepharose (Kodak Inc.) were used to detect epitope-tagged hFcRn. AC17 anti-dog lysosomal membrane antibodies were kindly provided by Andre Le Bivic (Marseille, France). Human IgG-biotin and human IgG was purchased from Sigma Chem. Corp. (Buchs, Switzerland), IgG was labeled with Cy3 (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Affinity purified labeled secondary antibodies were from Jackson ImmunoResarch Lab., Inc. (West Grove, PA) or Molecular Probes Inc. (Leiden, The Netherlands).

Cell culture and transfection of MDCK cells

MDCK strain II cells were cultured on plastic or to obtain polarized cell monolayers, on Transwell polycarbonate filter units (Costar Corp., Cambridge, MA) as described (Hunziker and Mellman, 1989). Units of 12 or 24 mm diameter and 0.4 μm pores were used. Cells were transfected with the epitope-tagged FcRn cloned into the pLNCX expression vector (Clonetech) using the calcium-phosphate method and selected in medium supplemented with G418 as detailed (Hunziker and Mellman, 1989). Resistant clones were analyzed for expression by immunofluorescence and two clones were used for further analysis. Hygromycin resistant MDCK cells expressing the pIgR (Hunziker, 1994; Hunziker and Peters, 1998) were transfected with the plasmid encoding Flag-hFcRn to obtain clones stably expressing the two receptors.

Construction of epitope-tagged human FcRn

A human FcRn carrying the Igκ leader sequence and a N-terminal Flag-epitope was constructed using recombinant DNA techniques carried out according to standard procedures. Briefly, the Igκ signal sequence was amplified from pSecTag (Invitrogen Inc.) using a sense primer encoding a *Hpa*I site and an antisense primer overlapping with the Flag sequence. In a second PCR, a sense primer encoding the Flag sequence and overlapping with FcRn just following the cleavage site of the endogenous signal sequence (Simister and Mostov, 1989) and an antisense primer covering the stop codon and carrying a *Cla*I site were used to amplify the hFcRn cDNA (kindly provided by N. Simister, Waltham, MA) in pGEM7 (Promega). The two PCR products were combined and reamplified using the sense and antisense primers from the first and second PCR, respectively. The resulting fragment encoding a hFcRn with the Igk leader sequence and an N-terminal Flag epitope was cloned into the *Hpa*I and *Cla*I site of pLNCX. All PCR products were verified by dideoxy-sequencing. The sequence of the different primers is available upon request.

IgG-agarose precipitation and western blot analysis

Cells were lysed in 5 mg/ml CHAPS in 50 mM phosphate buffer adjusted either to pH 6 or 7.4 (lysis buffer). 0.1 mg of total protein was incubated with 25 μl of IgG-agarose (~10 mg IgG/ml agarose slurry) in lysis buffer pH 6 or 7.4 at 4°C for 12 hours. The IgG-agarose was washed 3 time with lysis buffer, pH 6 or 7.4, and bound proteins were eluted by boiling in sample buffer and analyzed by SDS-PAGE (10% non-reducing gels). For western blot analysis, proteins were transferred to nitrocellulose and probed with the anti-Flag monoclonal antibody M2 (1 μg/ml) or a 1:200 dilution of polyclonal rabbit anti-FcRn peptide serum.

Immunofluorescence

For the identification of clones expressing hFcRn, cells grown on coverslips were washed with PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS+) and fixed for 30 minutes with 2% paraformaldehyde (PFA) in PBS+. After quenching with 50 mM NH_4Cl in PBS (10 minutes) and permeabilization of cells with saponin (0.05% in PBS), unspecific binding sites were blocked for 30 minutes with 10% goat serum in PBS+. Cells were incubated with a mouse anti Flag antibody (5 μg/ml) followed by a labeled goat anti-mouse secondary antibody. To analyze the surface expression of transfected proteins in cells grown on coverslips or Transwell filters, cells were washed with PBS+ and incubated at 4°C with the anti-Flag antibody (10 μg/ml), added from either the apical or basolateral compartment in the case of cells grown on Transwell filters. Cells were then washed, fixed and stained with the labeled secondary antibody.

To monitor internalization, M2 antibodies were prebound for 60 minutes on ice in Leibowitz 15 medium (L-15) supplemented with 0.5% BSA and unbound M2 antibodies were removed by washing with

ice-cold PBS+. The cells were incubated at 37°C for different periods of time in L-15, 0.5% BSA to allow for endocytosis to occur and then returned on ice and washed with PBS+. Internalization of IgG was monitored by incubating cells for 60 minutes at 37°C in the presence of 10 μg/ml of Cy3- or biotin-labeled IgG in L-15, 0.5% BSA adjusted either to pH 6 or 7.4. In some experiments, 1 μg/ml dIgA-biotin was present. Cells were then transferred on ice, washed with PBS+ and fixed. Internalized M2 was detected with a labeled secondary antibody, biotinylated dIgA or IgG were visualized with labeled streptavidin and lysosomes were stained with the monoclonal antibody AC17 (Nabi et al., 1991) as described (Höning and Hunziker, 1995).

To measure transcytosis and reinternalization of transcytosed receptor-anti-Flag antibody complexes, cells grown on Transwell filters were incubated in the presence of anti-Flag antibodies (10 μg/ml in L-15) in the apical or basolateral chamber for 60 minutes at 37°C. Cells were then cooled to 4°C and washed with PBS+. The labeled secondary antibody (2 μg/ml) was allowed to bind to transcytosed M2 for 1 hour on ice. Thereafter, cells were either fixed or incubated at 37°C for another 15 minutes to allow reinternalization of bound fluorescent secondary antibodies. Following internalization, cells were washed with L-15, pH 2.5 (3 times 10 minutes each) on ice to remove surface bound antibodies, fixed and mounted.

Fixed cells were mounted in Mowiol and viewed in a Zeiss Axiophot fluorescence microscope using a ×63 oil immersion lens. Pictures were taken with 400 ASA Kodak T-MAX films exposed at 1600 ASA or acquired using a Color Cool View camera (Photonic Science) and Image Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD, USA) and processed with Photoshop version 5.02 software (Adobe Systems, Inc.). Identical parameters for image acquisition and processing were used for apical and basolateral antibody addition or to compare transfected and control cells.

Polarized membrane insertion

Cells grown on 24 mm Transwell filters were washed with PBS+ and incubated for 30 minutes in cysteine-methionine free DMEM containing 10 mM Hepes, pH 7.4. Cells were pulse labeled by placing the filters on a 85 μl drop of ^{35}S-Easytag protein labeling mix (3 mCi/ml, NEN) in cysteine-methionine free DMEM for 15 minutes. After washing the cells once with prewarmed L-15, they were incubated for the time indicated to chase labeled proteins to the cell surface. Cells were cooled on ice, washed 3× with PBS+ and sulfo-NHS biotin (1.5 mg/ml in PBS+) was added to the apical (250 μl) or basolateral (100 μl) compartment for 30 minutes on ice. Cells were then washed 5 times with PBS+, 0.2 M glycine, filters were removed from the holders and cells lysed in 1 ml PBS, 0.5% TX-100 containing protease inhibitors. Nuclei were pelleted and the receptors were immunoprecipitated from the supernatant using immobilized M2 antibodies. One sixth of the immunoprecipitate was used to estimate total labeled protein, whereas surface biotinylated receptors were precipitated from the remainder using streptavidin-agarose as outlined (Hunziker et al., 1991a; Hunziker and Mellman, 1989). In some experiments, anti-Flag antibodies (5 μg/ml) were present in the medium during the chase. After cooling the cells on ice for 60 minutes, they were lysed as described above and lysates were precleared with fixed, Protein A-negative *Staphylococcus A.* Labeled protein that had appeared on the cell surface and thus bound antibody was then precipitated by the addition of fixed *Staphylococcus A.* An aliquot of the cell lysate was supplemented with additional antibody to precipitate total labeled receptors. Precipitates were analyzed by reducing SDS-PAGE (10% reducing gels), autoradiography and densitometry.

RESULTS

Expression of human FcRn in MDCK cells

To characterize the intracellular transport of hFcRn in polarized epithelial cells, we transfected MDCK cells with a hFcRn cDNA encoding a small amino-terminal Flag-epitope tag (see Materials and Methods). MDCK clones stably expressing hFcRn were identified by immunofluorescence staining using the anti-Flag monoclonal antibody M2 and analyzed by western blot analysis. M2 detected a ~47 kDa band in cell lysates from transfected MDCK cells (Fig. 1, lane 2), whereas no M2-reactive proteins were present in untransfected control cells (lane 1). The ~47 kDa band was also selectively detected in transfected cells using a polyclonal rabbit-anti peptide antibody raised against amino acids 176-190 of FcRn (lanes 3 and 4). Two clones stably expressing hFcRn were selected and further analyzed.

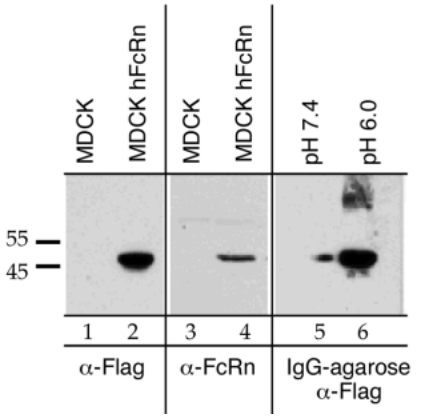

Fig. 1. Expression of hFcRn in transfected MDCK cells and pH-dependent binding of IgG. Lysates from control cells (lanes 1 and 3) or cells expressing FcRn (lanes 2 and 4) were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with the anti-Flag antibody M2 (lanes 1 and 2) or with an anti-FcRn peptide antibody (lanes 3 and 4). In lanes 5 and 6, cell lysates from MDCK cells expressing hFcRn were incubated with IgG-agarose either at pH 7.4 (lane 5) or at pH 6 (lane 6) and bound proteins fractionated by SDS-PAGE, transferred to nitrocellulose and probed with M2.

To determine if the Flag-tagged FcRn was able to bind IgG in a pH-dependent manner, cell lysates from transfected MDCK cells were incubated with IgG-agarose, either at pH 7.4 or 6. Proteins bound to IgG agarose were then analyzed by SDS-PAGE and western blot using the M2 antibody. As shown in Fig. 1, lanes 5 and 6, hFcRn preferentially bound to IgG-agarose at pH 6 (lane 6) and binding was significantly reduced if lysates were incubated with IgG-agarose at neutral pH (lane 5).

These results show that a Flag-tagged hFcRn can be expressed in MDCK cells and that the N-terminal addition of the Flag-epitope tag does not affect the ability of the receptor to bind IgG in a pH-dependent fashion.

FcRn mediates endocytosis

To visualize the intracellular distribution of hFcRn, cells grown on coverslips were fixed, permeabilized and stained with the anti-Flag antibody M2. As shown in Fig. 2a, hFcRn was enriched in intracellular vesicles whereas untransfected cells did not stain (Fig. 2b). Under conditions where visualization of the strong vesicular staining was optimal (i.e. Fig. 2a), only a faint membrane staining was apparent if compared to untransfected cells (Fig. 2b).

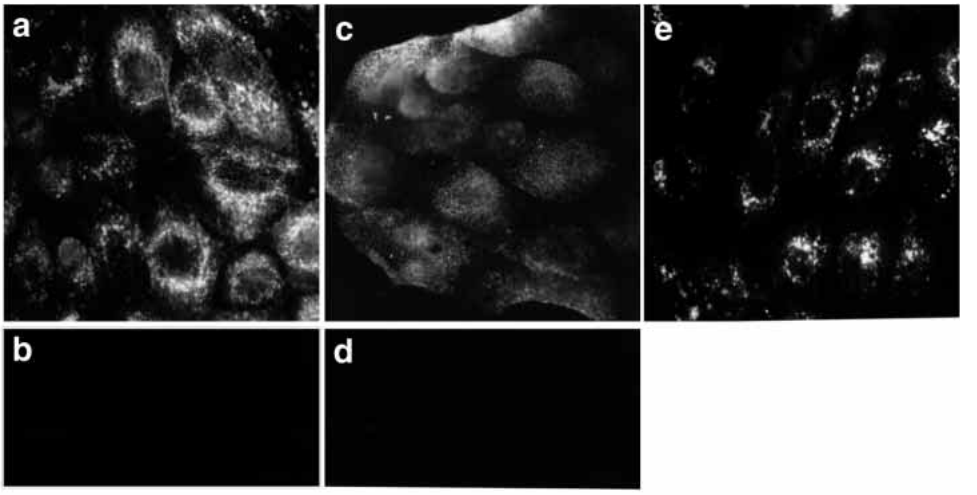

Fig. 2. Steady state distribution, surface localization and internalization of hFcRn. Cells expressing hFcRn (a) or untransfected cells (b) were fixed, permeabilized and stained with anti-Flag monoclonal antibody followed by a labeled secondary reagent. In c-e, anti-Flag antibodies were bound to cells expressing (c and e) or not (d) hFcRn at 4°C and the cells were then washed and either fixed (c and d) or incubated at 37°C for 30 minutes to allow for the internalization of bound antibody (e). After internalization, anti-Flag antibody remaining on the cell surface was removed by washing with acid (pH 2.5) and the cells were fixed, permeabilized and stained with a labeled secondary antibody.

To determine if a small fraction of FcRn was present on the cell surface and if these molecules were able to mediate endocytosis, cells were allowed to bind M2 on ice (Fig. 2c,d). The cells were then incubated for 30 minutes at 37°C to allow bound M2 to internalize (Fig. 2e). Cells were then fixed, permeabilized and M2 was detected with a labeled secondary antibody. Surface bound M2 (Fig. 2c) was internalized into an endosomal compartment concentrated in the perinuclear region of the cell (Fig. 2e). Internalization of bound M2 occurred via hFcRn since untransfected control cells did not bind the antibody (Fig. 2d).

Since FcRn binds IgG in a pH-dependent manner, we next analyzed the ability of FcRn to bind and internalize human IgG. Interestingly, we were unable to detect significant binding of IgG to cells expressing hFcRn, either at pH 6 or pH 7.4 (data not shown, see Discussion). However, if cells were incubated for 60 minutes at 37°C in the presence of low IgG concentrations (i.e. 10 μg/ml) at pH 6, the IgG was selectively internalized by cells expressing hFcRn (Fig. 3c) but not by untransfected control cells (Fig. 3d). In contrast, neither control cells nor cells expressing hFcRn were able to internalize IgG at neutral pH (Fig. 3a and b). Thus, IgG internalization at pH 6 occurred via FcRn and the lack of IgG binding to cells expressing hFcRn at 4°C was not due to the inability of the receptors to bind IgG.

To determine if IgG internalized via FcRn was delivered to endosomes or transferred to lysosomes, MDCK cells stably expressing the pIgR and Flag-hFcRn were allowed to internalize dIgA and labeled IgG in medium at pH 6 for 60 minutes at 37°C. Cells were then fixed, permeabilized and the internalized IgG and dIgA, or lysosomes, were visualized. IgG (Fig. 3e) and dIgA (Fig. 3f) were internalized into similar

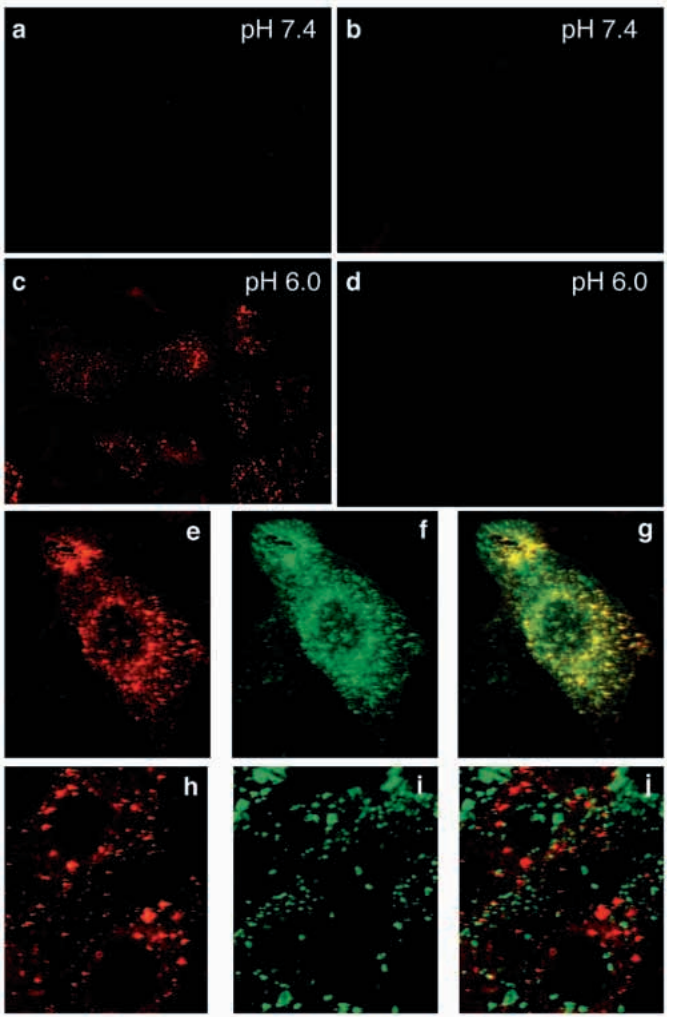

Fig. 3. Cells expressing hFcRn internalize IgG. (a-d) IgG internalization is pH-dependent. Control cells (b and d) or cells expressing hFcRn (a and c) were incubated in medium of pH 7.4 (a and b) or pH 6 (c and d) in the presence of biotin-labeled human IgG (10 μg/ml) for 60 minutes at 37°C. The cells were cooled on ice and unbound IgG was removed by washing. The cells were fixed and biotinylated IgG was visualized with labeled streptavidin. (E-J) Internalized IgG is not transferred to lysosomes. Cells expressing hFcRn and the pIgR were allowed to internalize Cy3-IgG (10 μg/ml) and dIgA-biotin (5 μg/ml) at pH 6 for 60 minutes at 37°C. Cells were then fixed, permeabilized and internalized IgG (e and h), dIgA-biotin (f) or an endogenous lysosomal marker (AC17; i) visualized. Merging e and f shows extensive endosomal colocalization of IgG and dIgA internalized via FcRn or the pIgR (g). In contrast, merging h and i shows little if any delivery of internalized IgG to lysosomes (j).

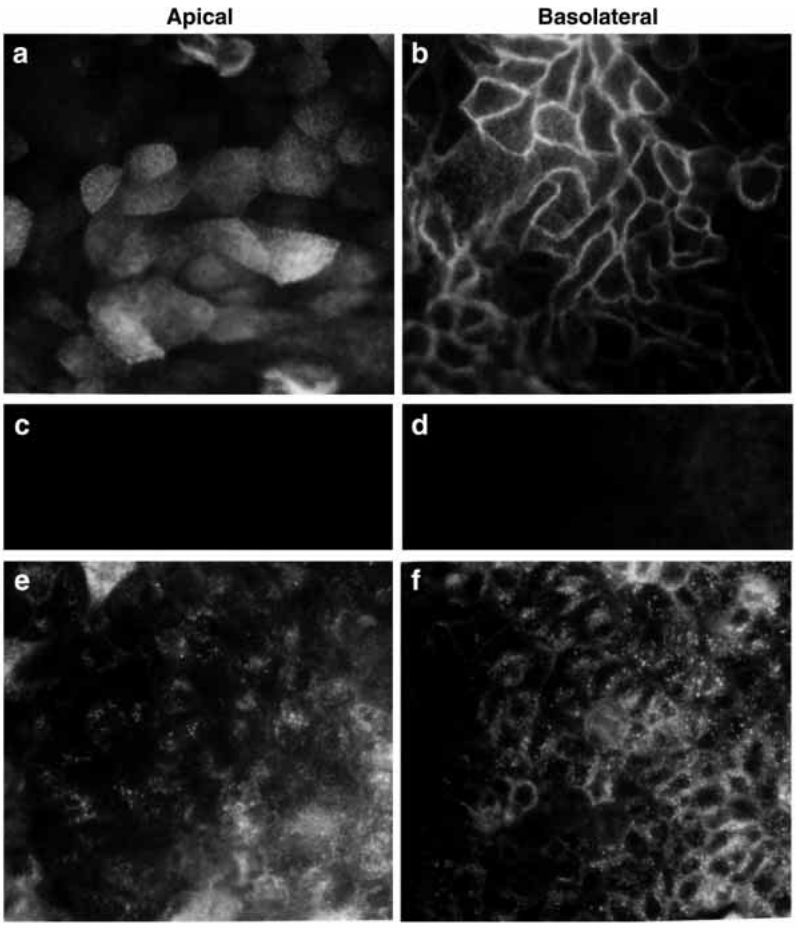

Fig. 4. hFcRn is expressed on the apical and basolateral cell surface. Cells grown on Transwell filters and expressing hFcRn (a,b,e,f) or not (c and d) were incubated with the M2 antibody added either from the apical or the basolateral compartment on ice. Cells were then washed and either directly fixed (a-d) or transferred to 37°C for 30 minutes to allow bound antibody to internalize (e and f) before fixation. Cells were then permeabilized and M2 was visualized using a labeled secondary antibody.

endosomal structures and merging of the two panels showed extensive colocalization of the two internalized ligands (Fig. 3g). In contrast, the distribution of internalized IgG (Fig. 3h) was distinct from the lysosomal staining obtained with AC17 (Fig. 3i), with little of the internalized IgG colocalizing with a lysosomal marker (Fig. 3j).

In conclusion, these results show that hFcRn is enriched in an intracellular compartment at steady state but that a smaller fraction of receptors is present on the cell surface. Receptors on the cell surface are able to internalize and internalization of IgG via hFcRn depends on an acidic extracellular pH. Furthermore, IgG internalized via FcRn is present in early endosomal compartments and does not reach lysosomes.

FcRn is present on the apical and basolateral surface of MDCK cell monolayers

If hFcRn mediates transcytosis, the receptor is expected to be exposed on the apical as well as the basolateral surface of epithelial cells. We therefore visualized by indirect immunofluorescence the steady state distribution of hFcRn to the apical and basolateral domain of MDCK cells grown on Transwell™ filters. Cells were allowed to bind anti-Flag antibody from the apical or basolateral compartment on ice, fixed and bound M2 was visualized using a labeled secondary antibody (Fig. 4a-d). Alternatively, cells carrying prebound M2 were incubated at 37°C to allow for internalization of bound anti-Flag antibody prior to fixation (Fig. 4e and f). Internalized anti-Flag was then detected following permeabilization of the cells.

Cells expressing hFcRn bound antibody from the apical compartment, resulting in a punctate staining pattern characteristic of the apical plasma membrane with its numerous microvilli (Fig. 4a). The cells also bound basolaterally added antibody as evidenced by the typical cobweb like staining (Fig. 4b). In contrast, untransfected control cells showed no binding (Fig. 4c and d). The presence of FcRn on the apical and basolateral cell surface was not due to a lack of cell polarization since gp80 was predominantly secreted into the apical medium (Urban et al., 1987) (data not shown).

If cells carrying M2 bound to the apical or basolateral domain were incubated for 15 minutes at 37°C, the anti-Flag antibody was internalized into an endosomal compartment (Fig. 4e and f), indicating that FcRn can internalize from both plasma membrane domains.

In conclusion, these data show that hFcRn localizes to the apical and basolateral surface of MDCK cells and that the receptor can be internalized from both domains.

Newly synthesized FcRn is transported to the cell surface in a non-vectorial fashion

Since FcRn is implicated in transcytosis, the presence of the receptor on the apical and basolateral surface of polarized MDCK cells may result from the transport of newly synthesized receptors to one domain followed by their relocation to the opposite surface by transcytosis. We therefore analyzed the appearance of a cohort of newly synthesized

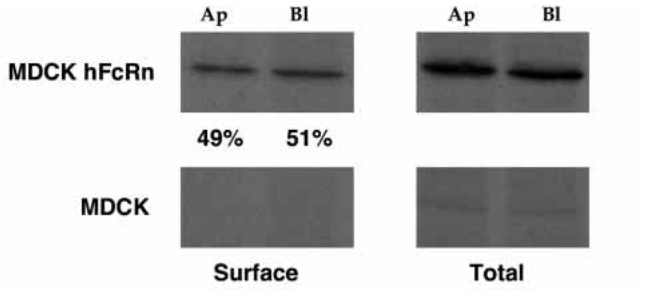

Fig. 5. Newly synthesized hFcRn is delivered to the apical and basolateral cell surface. MDCK cells expressing or not hFcRn were metabolically labeled for 15 minutes. Labeled proteins were chased for 30 minutes and the apical (Ap) or basolateral (Bl) cell surface was then biotinylated. Cells were lysed and total receptors were immunoprecipitated with the anti-Flag antibody. 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-PAGE and autoradiography. The bands on the autoradiograph were quantitated by densitometry and the amount of surface receptor was normalized to total labeled receptor. 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 minutes in the continuous presence of the anti-Flag antibody to detect labeled receptors that would only transiently be exposed on the cell surface (data not shown).

receptors on the apical and basolateral surface. Cells grown on Transwell units were pulse labeled for 15 minutes with [^{35}S]methionine and cysteine and labeled proteins were chased to the cell surface for 30 minutes. After cooling the cells on ice, the apical or basolateral surfaces were biotinylated on separate filters. Following cell lysis, total receptors were immunoprecipitated and an aliquot was used to determine the total amount of receptor labeled. FcRn that had appeared on the cell surface was precipitated from the rest of the lysate using streptavidin-agarose. The samples were analyzed by SDS-PAGE and autoradiography and the fraction of biotinylated surface receptors was normalized to the amount of total labeled receptors for each filter.

Surprisingly, an equal fraction of pulse-labeled hFcRn was biotinylated from the apical and basolateral compartment (Fig. 5a), indicating that newly synthesized hFcRn were transported to the apical as well as to the basolateral domain. Similar results were obtained if cells were chased for 30 minutes in the continuous presence of the anti-Flag antibody (data not shown) to detect labeled receptors only transiently exposed on the cell surface (Hunziker et al., 1991a).

This experiment shows that after synthesis, hFcRn is transported in a non-polarized manner to the apical and basolateral domains of MDCK cells.

FcRn transcytoses in both directions

Next, we analyzed the ability of hFcRn to transcytose in the apical to basolateral or in the opposite direction using an immunofluorescence-based assay to visualize transcellular transport of hFcRn. Cells were incubated for 60 minutes at 37°C in the presence of anti-Flag antibodies present in the apical or basolateral compartment and then transferred to 4°C. Anti-Flag antibodies that had transcytosed at 37°C were then visualized by binding a labeled goat anti-mouse antibody for 60 minutes at 4°C from the opposite chamber. As outlined in Fig. 6a and b, if anti-Flag antibodies are transcytosed via hFcRn, then they should be able to bind the labeled secondary antibody on the opposite cell surface.

Following the incubation of cells expressing hFcRn with M2 in the apical chamber, addition of the secondary antibody from the basolateral compartment resulted in the typical staining of the basolateral cell surface (Fig. 6c), indicating that M2 had been transferred from the apical chamber across the cells to the to the basolateral surface. Similarly, if M2 was added to the basolateral compartment, the secondary antibody was able to detect anti-Flag antibody transcytosed to the apical cell surface (Fig. 5d). Transfer of M2 from one compartment to the opposite did not occur by paracellular diffusion and was receptor specific since no labeling was observed if the incubation with the anti-Flag antibody occurred at 4°C (Fig. 6g and h). No staining was also observed if the M2 was omitted (Fig. 6i and j), indicating that binding and internalization of the labeled secondary antibody required the presence of transcytosed M2. Furthermore, electrical resistance measurements before and after each experiment confirmed the intactness of the cell monolayers (data not shown).

If cells carrying labeled secondary antibody bound to transcytosed M2 (as shown in Fig. 6c and d) were warmed for 15 minutes at 37°C, the labeled antibodies were internalized into a vesicular compartment (Fig. 6e and f), showing that transcytosed hFcRn could reinternalize from the opposite cell surface.

In conclusion, these experiments show that hFcRn is able to transcytose in the apical to basolateral as well as in the basolateral to apical direction and that transcytosed receptors can be reinternalized.

DISCUSSION

Although FcRn is thought to be the receptor mediating the transepithelial transport of IgG across the small intestine, the placental syncytiotrophoblast and the yolk sac, the evidence in support for such a role for FcRn has so far been indirect (Ghetie and Ward, 1997; Hunziker and Kraehenbuhl, 1998) and is mainly based on the following two observations. First, transfer of maternal IgG to the fetus or suckling newborn is reduced in β2m knock-out mice (Israel et al., 1995; Zijlstra et al., 1990) and second, mutant IgG that is defective in binding to FcRn is only poorly transferred from the mother to the fetus or newborn (Kim et al., 1994; Medesan et al., 1996, 1997). Our study now provides direct evidence that FcRn can indeed mediate transcytosis across epithelial cells.

FcRn expressed in MDCK cells was predominantly localized to a vesicular compartment concentrated in a perinuclear region of the cells and spreading to the periphery. Compared to the intracellular staining in permeabilized cells, little FcRn was detected on the cell surface, consistent with the weak surface expression of FcRn in intestine (Berryman and Rodewald, 1995), placenta (Kristoffersen and Matre, 1996) and yolk sac (Roberts et al., 1990). Those receptors that were present on the cell surface, however, were able to endocytose. Cytosolic di-leucine based signals mediate endocytosis of the macrophage IgG Fc receptor FcγRIIb2 (Hunziker and Fumey, 1994) and, based on the analysis of chimera encoding deletion

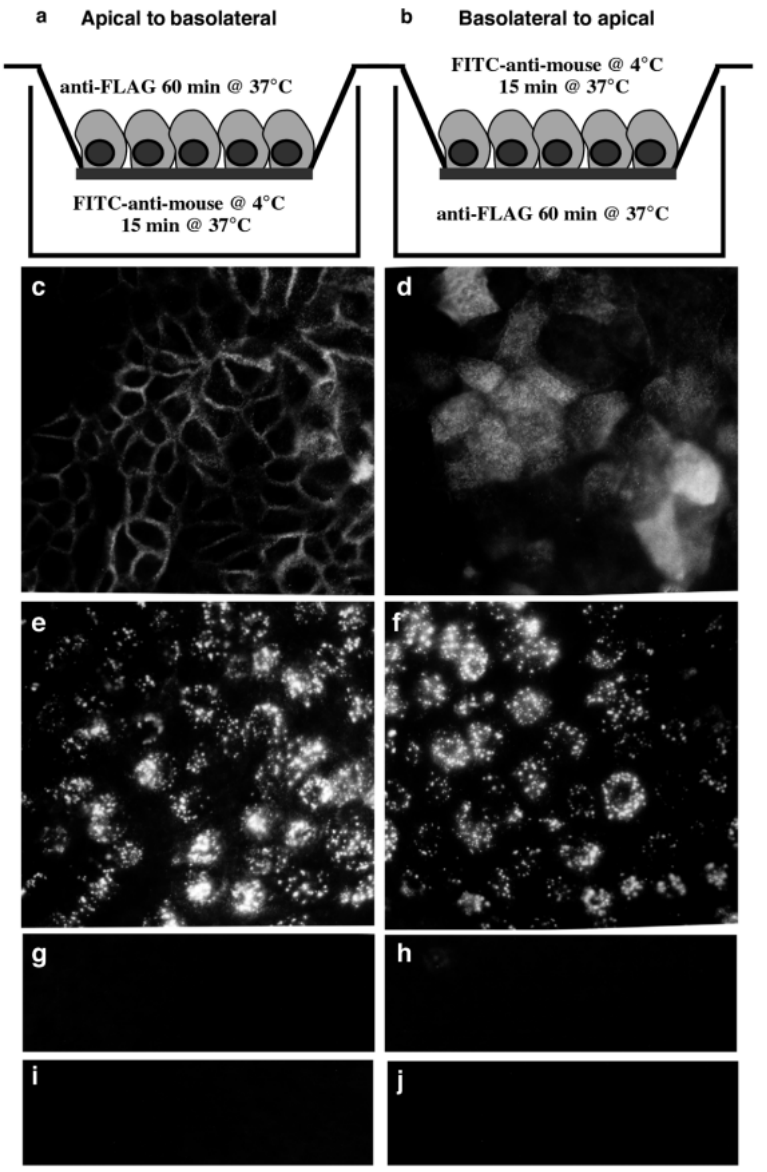

Fig. 6. hFcRn transcytoses in both directions and is reinternalized from the opposite cell surface. MDCK cells expressing hFcRn were incubated for 60 minutes in the presence of anti-Flag monoclonal antibodies added to the apical (c,e,g) or basolateral (d,f,h) compartment at 37°C (c-f) or on ice (g and h). After cooling the cells on ice, unbound antibody was washed. A labeled secondary antibody was allowed to bind from the opposite compartment for 60 minutes on ice to detect anti-Flag antibodies that had transcytosed as depicted in a and b. Cells were then washed and either directly fixed (c,d,g-j) or warmed for 15 minutes at 37°C (e and f) to allow for internalization of bound antibodies prior to fixation. Membrane staining (c and d) indicates transcytosis of anti-Flag and vesicular labeling (e and f) shows that transcytosed anti-Flag can be reinternalized from the opposite domain. No binding of secondary antibody was seen if the cells were left on ice during the presence of the anti-Flag antibody (g and h) or if the anti-Flag antibody was omitted (i and j).

and point mutants of the cytosolic domain of FcRn, a similar motif is critical for endocytosis of FcRn (Stefaner et al., 1999).

Interestingly, even though anti-Flag antibodies did bind to the surface of cells expressing FcRn, we were unable to detect significant levels of IgG binding to the cell surface. A likely explanation is that the prolonged incubation of cells on ice leads to the dissociation of β2m, resulting in FcRn molecules defective in IgG binding. This scenario is plausible since endogenous β2m is known to dissociate from MHC class I heavy chains on the surface of cultured cells, resulting in the accumulation of inactive class I molecules on the plasma membrane (Rock et al., 1991). Alternatively, the failure to detect IgG binding to transfected cells may reflect a weak expression of β2m by MDCK cells, leading to the presence of FcRn molecules on the cell surface which are defective in IgG binding. In any case, the lack of IgG binding was not due to the inability of the Flag-tagged to bind IgG per se, since detergent-solubilized receptors bound to IgG-agarose and IgG added to the medium at concentrations as low as 10 μg/ml was efficiently internalized at 37°C. Uptake of IgG under these conditions was only observed in transfected cells and required an acidic extracellular pH, strongly suggesting that IgG was internalized following its binding to receptors cycling between the cell surface and endosomes.

In polarized MDCK cells, FcRn was present on the apical and the basolateral cell surface at equilibrium, a distribution expected for a receptor that transcytoses between the two surfaces. However, in contrast to the pIgR which is efficiently sorted to the basolateral surface in the biosynthetic route, newly synthesized FcRn was delivered in a non-vectorial fashion to both the apical and basolateral plasma membrane. Non-vectorial transport of hFcRn may reflect the presence of a weak basolateral sorting signal in conjunction with a putative recessive apical determinant located in the ecto- and/or transmembrane domain. Interestingly, the cytosolic di-leucine motif required for endocytosis does not play a major role in basolateral sorting (Stefaner et al., 1999) and the FcRn tail contains no other typical basolateral sorting determinant.

FcRn molecules present on the apical or basolateral surface are internalized and transcytosed to the opposite domain.

Transcytosed FcRn molecules were able to reinternalize, indicating that FcRn shuttles between the two plasma membrane domains. The pIgR can only undergo one round of transcytosis since the receptor is unable to transcytose in the apical to basolateral direction and its ectodomain is proteolytically cleaved on the apical surface. Efficient basolateral sorting may ensure that all pIgR molecules are directed to the surface where ligand to be transcytosed has to bind. In the case of FcRn, efficient sorting to one or the other domain during biosynthesis may not be required because the receptor is able to shuttle between the apical and basolateral surface. Signals for polarized sorting are not only decoded at the level of the TGN but are also recognized in endosomes (Aroeti and Mostov, 1994; Matter et al., 1993). Thus, the non-vectorial surface transport of FcRn in the biosynthetic pathway correlates well with the observation that receptors internalized from one or the other cell surface can both recycle and transcytose and is consistent with FcRn encoding a 'weak' basolateral signal. Not surprisingly, routing of FcRn is very similar to that of LDLR mutants which still encode the 'weak' membrane-proximal basolateral sorting signal but lack the 'strong' distal determinant (Matter et al., 1992).

Despite the bidirectional transcytosis of the receptor, net transport of IgG may still be unidirectional. Since binding of IgG to FcRn is pH-dependent, unidirectional transport of IgG across an epithelium may depend on a pH-gradient. Due to the acidic environment of the lumen of the gut, IgG may selectively bind to FcRn present on the apical surface but not to receptors exposed to the neutral serosal pH. In contrast, in the placenta and yolk sac, FcRn is not exposed to an acidic environement. Since these epithelia are in direct contact with the maternal blood, IgG present at mg/ml concentrations in the circulation may be efficiently internalized in the fluid phase and then bind to FcRn in acidic endosomes. Thus, directional IgG transport across the syncytiotrophoblast and the yolk sac could be driven by an IgG concentration gradient between the maternal and the fetal circulation (Ellinger et al., 1999). In both cases, IgG transcytosed to the basolateral surface is likely to dissociate from the receptor after exposure to the neutral serosal pH. An alternative possibility is that routing of FcRn in enterocytes or placental syncytiotrophoblast differs from that in MDCK cells, although at least in transfected Caco-2 cells, preliminary experiments suggest bidirectional transcytosis of FcRn (A. Praetor and W. Hunziker, unpublished observations).

In addition to transcytosis, FcRn has been proposed to play a more general role in regulating IgG homeostasis by binding IgG internalized in the fluid phase in acidic endosomes and recycling it back to the cell surface. Transcytosis may be considered a specialized form of recycling in polarized cells (Mellman, 1996) and also another transcytotic receptor, the pIgR, recycles when expressed in fibroblasts (Deitcher et al., 1986). IgG internalized via FcRn colocalized with dIgA internalized via the pIgR and was not delivered to lysosomes. Thus, the intracellular traffic of FcRn observed in MDCK cells is consistent with the two functions postulated for the receptor, i.e. IgG transcytosis and diverting IgG internalized in the fluid phase from lysosomal degradation by recycling it back to the cell surface (Ghetie and Ward, 1997; Hunziker and Kraehenbuhl, 1998). It will now be interesting to determine if IgG stimulates the transcytotic activity of FcRn. Specific stimulation of transcytosis by ligand may ensure efficient apical to basolateral transfer of IgG across the enterocyte and syncytiotrophoblast, with the observed basolateral to apical transport enabling the recycling of empty FcRn molecules for reuse. MDCK and other cell lines expressing FcRn will provide valuable in vitro systems to study IgG transcytosis and maintenance of IgG homeostasis. In addition, they allow to characterize the organelles and the molecular mechanisms underlying transcytosis in the apical to basolateral direction and vice versa.

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