

# The heavy chain of neonatal Fc receptor for IgG is sequestered in endoplasmic reticulum by forming oligomers in the absence of $\beta_2$ -microglobulin association

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The heavy chain (HC) of the neonatal Fc receptor (FcRn) for IgG is non-covalently associated with  $\beta_2$ -microglobulin ( $\beta_2$ m). In  $\beta_2$ m<sup>-/-</sup> mice, FcRn functions are greatly impaired. We sought to determine how FcRn HC, particularly its structure and biogenesis, is affected by the absence of  $\beta_2$ m. Human FcRn HC, expressed from the  $\beta_2$ m-null cell line FO-1<sup>FcRn</sup>, was present as a monomeric 45-kDa protein under reducing conditions but primarily as a 92-kDa oligomeric protein under non-reducing conditions. Two-dimensional electrophoresis and MS analysis showed that the 92-kDa protein was a dimer of the 45-kDa HC. Immunostaining showed that FcRn HC in FO-1<sup>FcRn</sup> was co-localized with the endoplasmic reticulum (ER) protein Bip/GRP78 but not with an endosome protein, EEA1. In contrast, FcRn HC in FO-1<sup>FcRn+ $\beta_2$ m</sup> was detected in both the ER and endosome. The dimeric HC in FcRn oligomers was free of  $\beta_2$ m association in FO-1<sup>FcRn+ $\beta_2$ m</sup>. Mutation of non-paired cysteine residues at positions 48 and 251 within the human FcRn cDNA

failed to eliminate the oligomers. The FcRn HC oligomers could be reduced by reconstitution of FO-1<sup>FcRn</sup> with  $\beta_2$ m or by balanced expression of FcRn HC with  $\beta_2$ m, or  $\beta_2$ m fused with a KDEL retention sequence. Similarly, the majority of FcRn HC isolated from neonatal  $\beta_2$ m<sup>-/-</sup> mice was in a dimeric form under non-reducing conditions. The amount of FcRn HC was significantly decreased in  $\beta_2$ m<sup>-/-</sup> mice and FO-1<sup>FcRn</sup>. Furthermore,  $\beta_2$ m-free FcRn HC was sensitive to endoglycosidase digestion. These results indicate that FcRn HC alone can form disulphide-bonded oligomers in the ER, which may represent a misfolded protein. The  $\beta_2$ m association with FcRn HC is critical for correct folding of FcRn and exiting the ER for routing to endosomes and the cell surface.

Key words: dimers, disulphide bond, folding, human, IgG catabolism, mouse.

## INTRODUCTION

The neonatal Fc receptor (FcRn) transports maternal IgG across polarized epithelial cells from the placenta to the fetus in humans, or from ingested milk in the intestine to the bloodstream of newborns in rodents after birth [1–5]. FcRn therefore plays a major role in the passive acquisition of neonatal immunity in newborns. In addition to its role in transporting IgG, FcRn protects IgG from catabolism in adult life [6–9]. The proposed model is that IgG is taken up into cells by pinocytosis or endocytosis from the surrounding tissue fluid or blood. FcRn in acidic compartments, such as the endosome, binds and recycles IgG and carries it out of the cell in order to avoid IgG degradation in the lysosome [6,10]. FcRn has been identified in a variety of human, rodent and bovine adult cells and tissues [11–15]. All forms of FcRn display pH-dependent binding of IgG. Specifically, FcRn preferentially binds IgG at acidic pH (6–6.5) and releases IgG at neutral pH (7–7.4) [14,16,17].

Several molecules, encoded by genes within and outside the major histocompatibility complex (MHC), assemble with  $\beta_2$ -microglobulin ( $\beta_2$ m), i.e. HLA-A, -B, -C, -E, -F, -G, -H and

CD1 [18]. FcRn is the only IgG Fc receptor with a structure similar to that of the MHC class I molecule. FcRn is composed of heavy chain (HC; 45 kDa in humans and 50 kDa in rodents) non-covalently attached to a light chain  $\beta_2$ m (12 kDa) [19–22]. Similar to MHC class I, FcRn HC is composed of three external domains (designated  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$ ) that are anchored to the cell surface by a short transmembrane segment and a carboxyl-terminal cytoplasmic tail [20]. Unlike MHC class I, FcRn is non-polymorphic and lacks a functional peptide-binding groove [20–22]. The absence of an antigen binding groove in FcRn suggests that FcRn folding and biogenesis is different from MHC class I because MHC class I loaded with peptide is only able to reach the cell surface [18]. The X-ray crystallographic structure of FcRn demonstrates that the  $\alpha_1$  and  $\alpha_2$  domains are paired, as are the  $\alpha_3$  domain and  $\beta_2$ m, and  $\beta_2$ m interacts with all three  $\alpha$ -chain domains [21–23]. The co-crystal structure of the FcRn and Fc portion of IgG reveals that the Fc fragment of IgG contacts the sides of all three  $\alpha$ -chain domains and  $\beta_2$ m [23].

Most MHC class I and class I-related molecules require co-expression of  $\beta_2$ m for their stable surface expression and proper function. Several functions of FcRn *in vivo* have been examined

Abbreviations used:  $\beta_2$ m,  $\beta_2$ -microglobulin; CMV, cytomegalovirus; ECL, enhanced chemiluminescence; endo H, endo- $\beta$ -N-acetylglucosaminidase H; ER, endoplasmic reticulum; FcRn, neonatal Fc receptor; GST, glutathione-S-transferase; HC, heavy chain; HRP, horseradish peroxidase; IEC, intestinal epithelial cells; mAb, monoclonal antibody; 2-ME, 2-mercaptoethanol; MHC, major histocompatibility complex; PBST, PBS containing 0.05% Tween 20; PNGase F, N-glycosidase F.

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in  $\beta_2m^{-/-}$  mice, including studies of IgG transport across the intestinal epithelium of suckling mice [2], IgG catabolism [7,8,9] and hypergammaglobulinemia or autoimmunity mediated by autoantibodies [24]. These studies show that transport of IgG from mother to newborns is abolished [2], and that catabolism of IgG is increased in  $\beta_2m^{-/-}$  mice [7,9] compared with that in wild-type mice. The latter FcRn function is further supported by evidence that  $\beta_2m^{-/-}$  mice are resistant to bullous pemphigoid, an autoimmune skin disorder mediated by autoantibodies against hemidesmosomes [25]. Overall, these studies demonstrate that the association of  $\beta_2m$  with FcRn HC is critical for FcRn function *in vivo*.

With the functional disability of FcRn in  $\beta_2m^{-/-}$  mice, it remains to be resolved how the structural features and biogenesis of FcRn HC are exhibited in the absence of  $\beta_2m$  association. In the present biochemical studies, we provide evidence that most FcRn HC expressed in a  $\beta_2m$ -null cell line form oligomers, prominently dimers, under non-reducing conditions. This observation raises the question of whether the oligomeric form of FcRn HC represents a non-functional and misfolded intermediate metabolite, caused by the lack of  $\beta_2m$  association, or whether it represents a functional molecule, if associated with  $\beta_2m$ , that results in a dimer of heterodimers. Several studies have suggested that FcRn forms a dimer [19,23,26]. To better interpret these results, we sought to define further the features of FcRn HC in the presence and absence of  $\beta_2m$  expression. Our results indicate that oligomeric FcRn HC, formed in the absence of  $\beta_2m$  association, may represent a misfolded version of FcRn HC in the ER due to the absence of association with  $\beta_2m$ . Alternatively, the formation of the FcRn HC oligomers in the ER may represent the first step in a degradation pathway for controlling the expression levels of the FcRn HC in cells.

## MATERIALS AND METHODS

### Animals, cell lines and antibodies

Five-month-old and ten-day-old wild-type C57/B6 mice or C57/B6 mice homozygous for the  $\beta_2m^{-/-}$  were purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). The melanoma cell line FO-1 (kindly provided by Dr Soldano Ferrone, New York Medical College, Valhalla, NY, U.S.A.) and FO-1 transfectants were grown in RPMI 1640 complete medium (Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10 mM HEPES, 10% (v/v) fetal calf serum (Sigma, St. Louis, MO, U.S.A.), 1% (v/v) L-glutamine, non-essential amino acids, and 1% (v/v) penicillin/streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> incubator at 37 °C. In serum-free conditions, FO-1 cells and FO-1 transfectants were also grown in Dulbecco's Modified Eagle's Medium/Ham's/F12 medium (Life Technologies).

Human  $\beta_2m$  was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA, U.S.A.). Affinity-purified rabbit anti-(human  $\beta_2m$ ) was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and from Sigma. Goat anti-(mouse  $\beta_2m$ ) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Affinity-purified rabbit anti-actin, rabbit anti-(FLAG epitope) (DYKDDDDK, using single letter code for amino acids), monoclonal antibodies (mAbs) anti-(FLAG M1) and anti-(FLAG M2) were purchased from Sigma. Mouse anti-EEA1 and mouse anti-Bip/GRP78 were from Transduction Laboratories (Lexington, KY, U.S.A.). Cy<sup>TM</sup>3-conjugated AffiniPure goat anti-mouse IgG and Cy<sup>TM</sup>2-conjugated AffiniPure goat anti-rabbit IgG were from Jackson Immuno-Research Laboratories (West Grove, PA, U.S.A.) and horse-

radish peroxidase (HRP)-conjugated rabbit anti-mouse or donkey anti-rabbit antibody were from Pierce (Rockford, IL, U.S.A.).

### Production of mouse FcRn domain-specific antibodies

Standard procedures were used for plasmid purification, *Escherichia coli* transformation, restriction endonuclease digestion and ligation, as recommended by New England BioLab (Beverly, MA, U.S.A.) and Qiagen (Valencia, CA, U.S.A.). All PCRs were performed with *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.). The production of human FcRn  $\alpha_2$  domain-specific antibody has been described previously [15]. The mouse FcRn coding region [27], corresponding to  $\alpha_2$  domain (90–179), was amplified by PCR and subcloned at *Bam*HI- and *Eco*RI-digested sites of the pGEX4T-1 expression vector (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The primer pair 5'-GCTGGATCCTACACTGCAGGGCCTGCT-3' and 5'-CGCGAATTCTCACTTCCACTCCAGGTT-3' was used for  $\alpha_2$  amplification. The *Bam*HI and *Eco*RI sites (underlined) were used for cloning. The plasmid encoding the full-length mFcRn, kindly provided by Dr Neil Simister (Department of Biology, Brandeis University, Waltham, MA, U.S.A.), was used as a template. All subclones were verified by sequencing. Recombinant proteins were produced by a modification of the method described previously [28]. Rabbits were immunized with purified fusion protein glutathione-S-transferase (GST)- $\alpha_2$  at Charles River Laboratories (SouthBridge, MA, U.S.A.). The affinity purification of antibody to the  $\alpha_2$  domain was carried out using a method described previously [29]. The non-specific reactivity was minimized by pre-clearing at 4 °C with cell lysates from mock-transfected cells. Subsequently, the supernatant was collected after centrifugation and used for analysis.

### Preparation of epithelial cell lysates from mouse small intestine

Lysates from mouse small intestinal epithelium were prepared by the method described previously [18]. Briefly, the small intestine was removed from each 10-day-old mouse and rinsed with 5 mM EDTA, pH 7.4, to detach epithelial cells. Brush borders were washed in PBS (pH 6.5) and resuspended in PBS (pH 6.5) containing 0.5% CHAPS, 10 mM iodoacetamide (Sigma), and a cocktail of protease inhibitors (Sigma). Cell debris was removed by centrifugation at 14000 *g* at 4 °C. Supernatant from either 8 wild-type mice or 8  $\beta_2m^{-/-}$  mice was pooled. The total protein concentrations were determined by the Bradford method (BioRad, Hercules, CA, U.S.A.) with BSA as a standard. Epithelial cell lysates from adult mouse small intestine were prepared by a similar method.

### Construction of plasmids encoding FcRn and $\beta_2m$

The construction of human  $\beta_2m$  and FcRn expression plasmids, pCDNA $\beta_2m$  and pFlagCMVFcRn, has been described previously [15]. The pFlagCMVhFcRn construct fused a pre-protrypsin signal sequence and a FLAG epitope into the amino terminus of the human FcRn gene (amino acid 1–342) [17]. The plasmid pBIFcRn- $\beta_2m$  was constructed by cloning PCR fragments into pBI (Clontech, Palo Alto, CA, U.S.A.), previously digested with *Eco*RV and *Nhe*I or *Pst*I and *Sal*I respectively. The DNA fragments were amplified with primer pairs for human FcRn (5'-CCGCGATATCTGGTCAGGCCGGTGGCT-3', 5'-CCTAGCTAGCAGGTCGTCCTCAGCAT-3') and for human  $\beta_2m$  (5'-AAAACTGCAGGGCGGGCATTCTGAAGCTG-3', 5'-ACGCGTTCGACTGCTGCTTACATGTCTCGAT-3'). The

pBIFcRn- $\beta_2$ m-KDEL is constructed as for pBIFcRn- $\beta_2$ m, except that the KDEL sequence was fused into the C-terminus of  $\beta_2$ m by an antisense primer (5'-ACGCGTCTGACTTACAGTTCA-TCTTTTCATGTCTCGATCCCACTT-3'). The restriction sites in primers are underlined, and human FcRn and  $\beta_2$ m sequences are in italics. The KDEL sequence is in bold. All constructs were sequenced to verify the fidelity of the amplification and cloning.

### Site-directed mutagenesis of FcRn HC

Site-directed mutagenesis was performed with an *in vitro* Transformer Site-directed Mutagenesis kit (Clontech). Human FcRn cDNA in a pCDNA3 expression vector (Invitrogen, Carlsbad, CA, U.S.A.) was used as the template. The oligonucleotide 5'-GAGGCGGAGCCGCTGGAGCTTGG-3' was used for mutation of cysteine residue 48 [17] to serine, and the oligonucleotide 5'-GAGCACTACTCCTGCATTGTGCAG-3' was used for mutation of cysteine 251 [17] to serine (base substitutions are underlined). The mutant FcRn cDNAs were sequenced to verify the mutations. The resultant plasmids were designed for pCDNAFlagFcRnC48/S and pCDNAFlagFcRnC251/S respectively.

### Transfection and protein expression

FO-1 cells were transfected with pCDNA $\beta_2$ m and/or pFlagCMVFcRn with Effectene transfection reagent (Qiagen) or Electroporator II (Invitrogen), according to instructions from the manufacturer. G418 marker was used to select positive colonies, which were tested for protein expression by Western blotting using anti-FLAG antibody or FcRn  $\alpha_2$  domain-specific antibodies. Transfectants were maintained in medium containing G418 at a concentration of 500  $\mu$ g/ml. The established cell lines were designated FO-1<sup>FcRn</sup> and FO-1<sup>FcRn+ $\beta_2$ m</sup> respectively. For transient transfections, FO-1 cells were transfected by the calcium precipitation method with 2  $\mu$ g of either plasmid pCDNAFLAGFcRnC48/S or pCDNAFLAGFcRnC251/S. FO-1 cells also were transiently transfected by Effectene with 2  $\mu$ g of plasmids pBI, pBIFcRn, pBIFcRn+ $\beta_2$ m, pBIFcRn+ $\beta_2$ mKDEL or 8  $\mu$ g of plasmid encoding a tetracycline-controlled transactivator. Proteins were induced for expression with 1  $\mu$ g/ml doxycycline (Clontech) 48 h after transfection.

### Western blotting, immunoprecipitation and gel electrophoresis

Cell lines and transfectants were lysed in 0.5% CHAPS or 0.5% NP-40 in PBS with or without iodoacetamide (Sigma), and a cocktail of protease inhibitors. Protein concentrations were determined as described previously [15]. The lysates were resolved on a 4–20% NuPAGE gel (Invitrogen) under reducing or non-reducing conditions. Proteins were electrotransferred on to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.). The membranes were blocked with 5% non-fat milk, probed separately with affinity-purified FLAG antibody, anti-(FcRn  $\alpha_2$  domain-specific) antibody, or rabbit anti- $\beta_2$ m antibody for 1 h, following incubation with HRP-conjugated rabbit anti-mouse or donkey anti-rabbit antibody. All blocking, incubation and washing steps were performed in PBS containing 0.05% Tween 20 (PBST). Proteins were visualized by the enhanced chemiluminescence (ECL<sup>®</sup>, Amersham) detection method (Pierce or NEN Life Science Products, Boston, MA, U.S.A.) according to the manufacturer's instructions. For reprobing, the blots were first incubated in stripping buffer [100 mM 2-mercaptoethanol (2-ME), 2% SDS, and 62.5 mM Tris/HCl] for 30 min at 55 °C and then washed fully to remove residual reducing agent. Immunoprecipitation was performed as described

previously [15]. Protein was precipitated with anti-FLAG mAb. The immunoreactive products were eluted from the Protein G complex using gel loading buffer with or without 2-ME at 100 °C.

### Two-dimensional gel electrophoresis and MS

For two-dimensional gel analysis, the cell lysates from FO-1<sup>FcRn+ $\beta_2$ m</sup> were boiled in non-reducing sample buffer. The first-dimensional gel was resolved on SDS/12%-PAGE tube gels under non-reducing conditions. The extruded gel tubes were equilibrated with an SDS/PAGE gel running buffer. The gel tubes were loaded on to the second dimensional gel (SDS/12% PAGE slab gels; Hoffer Scientific Instruments, San Francisco, CA, U.S.A.) under reducing or non-reducing conditions. The proteins were electrotransferred onto nitrocellulose and immunoblotted as described above. For MS analysis, FO-1<sup>FcRn</sup> lysates were immunoprecipitated by M2 Flag antibody. The immunoprecipitates were separated by SDS/PAGE under non-reducing conditions and stained with Coomassie Blue. Protein bands were excised and subjected to in-gel tryptic digestion. Extracted peptides were analysed using microcapillary reverse-phase liquid chromatography coupled with tandem MS ('LC-MS/MS') at Taplin Biological Mass Spectrometry Facility at Harvard University, Boston, MA, U.S.A. MS/MS spectra were analysed using the nrp.fasta and human.nci databases by Sequest software.

### Analysis of N-linked glycosylation

N-linked glycosylation was analysed as described previously [28]. In brief, native FcRn in cell lysates was digested with endo- $\beta$ -N-acetylglucosaminidase H (endo H; New England Biolabs) in a digestion buffer [100 mM sodium acetate, pH 5, 150 mM NaCl, 1% (v/v) Triton X-100, 0.2% (w/v) SDS, 0.5 mM PMSF] or with peptide:N-glycosidase F (PNGase F; New England Biolabs) in 50 mM sodium phosphate (pH 7.5) and 1% (v/v) NP-40. A mock digestion without enzymes was performed. All digestions were performed for 18 h at 37 °C. Proteins were analysed on a 4–20% NuPage gel and immunoblotted as previously described above.

### Flow cytometry analysis

FO-1 transfectants ( $5 \times 10^5$  cells/ml) from fresh cultures were washed twice in suspension buffer (1% fetal bovine serum in calcium-free PBS). The cell suspension was incubated with 22.8  $\mu$ g anti-(FLAG M1) mAb for 1 h, washed twice with suspension buffer, and stained with fluorescein-conjugated rabbit anti-mouse antibody (a gift from Dr Yuansheng Wang, Brigham and Women's Hospital, Boston, MA, U.S.A.). The cells were washed twice with suspension medium before fixation with 0.5% (w/v) paraformaldehyde in PBS. Control staining with isotype-matched IgG2b was performed for each analysis. Analysis was performed using a FACS Sort and CellQuest software (Becton Dickinson, San Diego, CA, U.S.A.).

### Confocal fluorescence microscopy

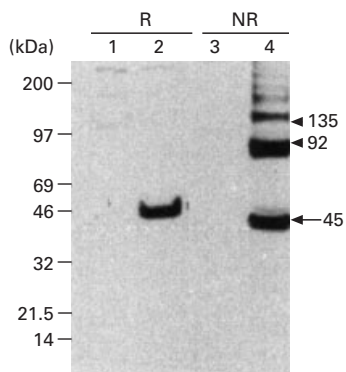
FO-1 cells and transfectants were cultivated on coverslips and maintained in serum-free medium for 48 h before intracellular staining. The coverslips were rinsed in PBS and cells were cold-fixed in 4% (v/v) paraformaldehyde in PBS for 30 min at 4 °C. Subsequent manipulations were performed at 25 °C. After two washes with PBS, the coverslips were immersed and permeabilized in PBS containing 3% (w/v) BSA and 0.2% (v/v) Triton X-100 for 30 min. Cells were incubated with affinity-purified rabbit anti-FLAG or rabbit anti- $\beta_2$ m and mAb anti-

Bip/GRP78 or EEA1 in PBST with 3% (w/v) BSA for 1 h. Cells were then incubated with Cy<sup>TM</sup>3-conjugated affiniPure goat anti-(mouse IgG) and Cy<sup>TM</sup>2-conjugated AffiniPure goat anti-rabbit IgG in PBST containing 3% (w/v) BSA. Cell nuclei were stained with 4,6-diamidino-2-phenylindole ('DAPI'; Molecular Probes, Eugene, OR, U.S.A.) in PBS. After each step, cells were washed at least three times with 0.1% Tween-20 in PBS. To mount coverslips, the ProLong<sup>TM</sup> antifade kit was used (Molecular Probes). Images were captured using a 100 $\times$  oil-immersion objective on a Nikon inverted microscope linked to a Delta Vision deconvolution imaging system (Applied Precision, Seattle, WA, U.S.A.). The images were colourized and processed using the ImagePro Plus System from Micro Video Instruments (Avon, MA, U.S.A.).

## RESULTS

### Human FcRn HC forms a major 92-kDa protein in FO-1<sup>FcRn</sup> under non-reducing conditions

The melanoma cell line FO-1 lacks  $\beta_2m$  gene transcription and protein synthesis [30]. To define the features of FcRn HC in the absence of  $\beta_2m$  expression, we transfected human FcRn HC cDNA into FO-1 cell line and analysed protein expression under reducing and non-reducing conditions. In addition to the 45-kDa protein (Figure 1, lanes 2 and 4), which is the expected size of FcRn HC monomer, a prominent 92-kDa band (Figure 1, lane 4) and less prominent high-molecular-mass bands (above 135 kDa) were present under non-reducing conditions (Figure 1, lane 4). We also stained the blots with affinity-purified rabbit anti-(FcRn HC  $\alpha_2$  domain) antibody or a rabbit anti-FcRn antibody. All these antibodies strongly or weakly recognized the 92-kDa protein, suggesting that these high-molecular-mass bands represent a species of FcRn HC. In addition, the antibodies failed to recognize the corresponding protein bands in mock-transfected FO-1 cells under both conditions (Figure 1, lanes 1 and 3). In the presence of 2-ME or dithiothreitol, these high-molecular-mass bands were diminished in the Western blot, suggesting that their formation was mediated by a disulphide bond. An alternative possibility for the formation of these bands is that the disulphide bond formed intermolecularly during the



**Figure 1** The expression of FcRn heavy chain in  $\beta_2m$ -negative FO-1 cells

FO-1 cells stably expressing FLAG-tagged FcRn heavy chain (lanes 2 and 4) and transfected with vector alone (lanes 1 and 3) were lysed and 40  $\mu$ g of cellular proteins were resolved on a 4–12% NuPAGE gel under reducing (R, lanes 1 and 2) and non-reducing (NR, lanes 3 and 4) conditions. Immunoblotting was performed with affinity-purified rabbit anti-FLAG antibody and an HRP-conjugated donkey anti-rabbit IgG and detected by ECL. Molecular mass (kDa) is marked on the left. The FcRn monomer (45 kDa) is indicated with an arrow. The 92- and 135-kDa high-molecular-mass bands are marked with an arrowhead.

cell lysis procedure rather than naturally inside cells. To exclude this possibility, we lysed cells in the presence of 10–80 mM iodoacetamide (Sigma). The iodoacetamide treatment failed to eliminate this 92-kDa molecule during isolation of membrane proteins (results not shown). The 92-kDa protein was also visualized in Western blot when cell lysates were prepared by directly boiling FO-1<sup>FcRn</sup> cells in gel-loading buffer. All these results confirm that this 92-kDa product was not the product of aberrant disulphide bond formation occurring during or after homogenization. In addition, the 92-kDa protein was also detected in a HeLa cell line transfected with FcRn cDNA, suggesting that the appearance of the 92-kDa band is not FO-1 cell-specific (results not shown). Since the 135-kDa protein and other minor molecules represent only a small proportion of the high-molecular-mass species, the 92-kDa band was the focus for future studies and discussions. In addition, we noticed that the ratio of protein band densities and the migration of the 45-kDa form of the HC, as observed by ECL, exhibited a difference between reducing and non-reducing conditions, therefore preventing a comparison of the band densities. These differences are probably caused by the changes in conformational structure imposed upon the FcRn HC, which contains intrachain disulphide bonds, by reducing versus non-reducing conditions. The artificial crosslinking during the radiolabelling steps have also been seen in other studies of protein dimeric and multimeric formation. Therefore, we focused on the unlabelled and crude lysates for our analysis of FcRn HC.

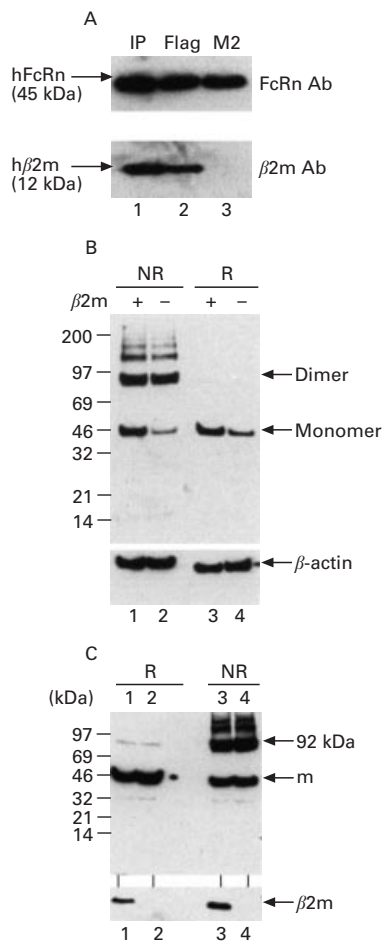
### The 92-kDa protein of human FcRn HC is formed less efficiently in FO-1<sup>FcRn + $\beta_2m$</sup> cells

FcRn HC is associated non-covalently with  $\beta_2m$ . The presence of the 92-kDa FcRn HC in FO-1<sup>FcRn</sup> may be due to the lack of  $\beta_2m$  expression. To address this question, we reconstituted FO-1<sup>FcRn</sup> cells with expression of the  $\beta_2m$  molecule. Expression and association of  $\beta_2m$  with FcRn HC in FO-1<sup>FcRn +  $\beta_2m$</sup>  was confirmed by immunoprecipitation with mAb anti-FLAG M2 and Western blotting, followed by blotting with affinity-purified rabbit anti- $\beta_2m$  (Figure 2A, lanes 1 and 2). FO-1<sup>FcRn</sup> was used as a negative control (Figure 2A, lane 3). In FO-1<sup>FcRn +  $\beta_2m$</sup> , approx. half of the HC was monomeric under non-reducing conditions and the remainder formed a 92-kDa product (Figure 2B, lane 1). In contrast, the majority of FcRn HC was formed as a 92-kDa product in FO-1<sup>FcRn</sup> (Figure 2B, lane 2). The amount of FcRn HC in FO-1<sup>FcRn +  $\beta_2m$</sup>  was greater than that of FcRn HC in FO-1<sup>FcRn</sup>. This experiment was done at least three times and similar results obtained.

We examined further whether exogenous  $\beta_2m$  can prevent or reduce the formation of the 92-kDa product, since  $\beta_2m$  exists in different tissue fluids.  $\beta_2m$  may enter cells following endocytosis. Human  $\beta_2m$ , at a concentration of 100  $\mu$ g/ml added into FO-1<sup>FcRn</sup> medium under serum-free conditions and incubated for 48 h, failed to remove or eliminate the 92-kDa protein (Figure 2C). Since bovine  $\beta_2m$  in FBS can exchange with human  $\beta_2m$  on MHC class I on cells cultured *in vitro* [31], we eliminated the possible interference from bovine  $\beta_2m$  by performing this experiment for FO-1 transfectants cultivated in a serum-free medium.

### The 92-kDa protein is a homodimer of FcRn

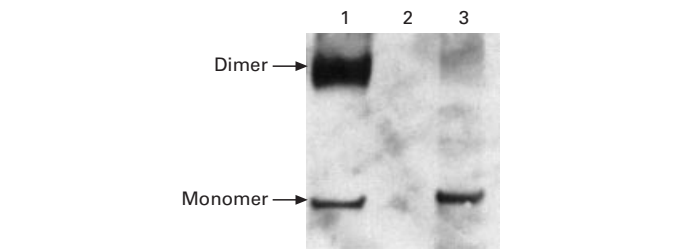
The 92- and 135-kDa products may represent dimeric and trimeric forms of the 45-kDa monomer of FcRn HC based on the molecular masses respectively. To confirm this, we subjected cell lysates from FO-1<sup>FcRn +  $\beta_2m$</sup>  to two-dimensional electrophoresis



**Figure 2**  $\beta_2$ m-negative FO-1 cells forms a 92-kDa band more efficiently than  $\beta_2$ m-positive FO-1 cells

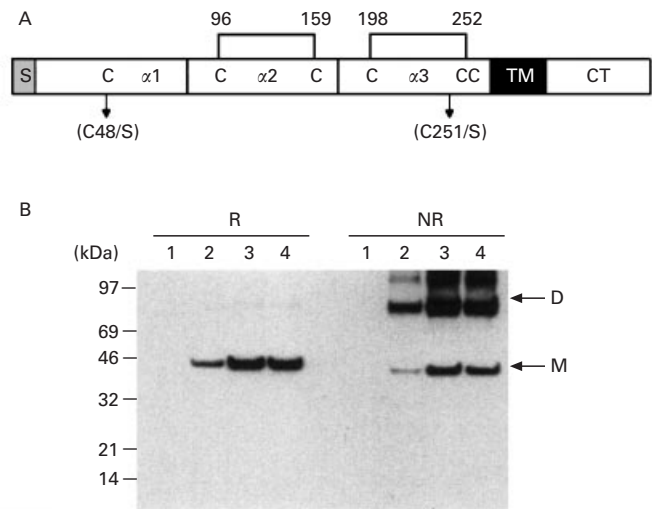
(A) FcRn HC was associated with the  $\beta_2$ m molecule in FO-1<sup>FcRn+/ $\beta_2$ m</sup> cells. The cell lysate from FO-1<sup>FcRn+/ $\beta_2$ m</sup> (lanes 1 and 2) or FO-1<sup>FcRn-</sup> (lane 3) was immunoprecipitated by mAb anti-FLAG M2. Immunoprecipitates were subjected to 4–12% NuPAGE under reducing conditions and transferred to a nitrocellulose membrane. Immunoblotting was performed with an affinity-purified rabbit anti-(human FcRn  $\alpha_2$  domain) (upper panel) or anti- $\beta_2$ m (lower panel) antibody. Blots were developed with ECL. (B) Total cell lysates (30  $\mu$ g) from FO-1<sup>FcRn+/ $\beta_2$ m</sup> (lanes 1 and 3) or FO-1<sup>FcRn-</sup> (lanes 2 and 4) stable cell lines were resolved on a 4–12% NuPAGE gel under non-reducing (NR, lanes 1 and 2) and reducing (R, lanes 3 and 4) conditions. Immunoblotting was performed with affinity-purified rabbit anti-FLAG mAb and HRP-conjugated donkey anti-(rabbit IgG) and development using ECL. Molecular mass (kDa) is indicated on the left. FcRn monomer (m) is indicated with an arrow. The 92-kDa high-molecular-mass band is marked with an arrowhead. The  $\beta$ -actin (42 kDa) was blotted with rabbit anti- $\beta$ -actin as an internal control. The experiment was performed three times with similar results. (C) Addition of exogenous  $\beta_2$ m in the medium did not prevent the formation of the 92-kDa protein in FO-1<sup>FcRn-</sup> cells. The  $\beta_2$ m (100  $\mu$ g/ml) was added to FO-1<sup>FcRn-</sup> cells cultivated in serum-free medium in a 2 ml volume. The FO-1<sup>FcRn-</sup> cells were harvested 24 h later and washed three times with cold PBS. The total cell lysates (60  $\mu$ g) were resolved on a 4–12% NuPAGE gel under reducing (R, lanes 1 and 2) and non-reducing (NR, lanes 3 and 4) conditions. The immunoblotting was performed as described above with affinity-purified rabbit anti-FLAG or anti- $\beta_2$ m antibody. The positions of the 92-kDa protein, 45-kDa monomer (m), and  $\beta_2$ m are indicated with arrows. Molecular mass is marked on the left.

under reducing and non-reducing conditions. When cell lysates were examined in first- and second-dimensional electrophoresis under non-reducing conditions, the protein spots were clearly in the 92 kDa and 45 kDa positions when probed with affinity-purified anti-FLAG antibody (results not shown). However, when cell lysates were subjected to first-dimensional electrophoresis under non-reducing conditions and second-dimensional



**Figure 3** The 92-kDa dimer of FcRn HC does not associate with  $\beta_2$ m molecule

Total cell lysates (500  $\mu$ g, pH 7.5) from FO-1<sup>FcRn-</sup> (lane 2) and FO-1<sup>FcRn+/ $\beta_2$ m</sup> (lane 3) were incubated with Protein G–Sepharose beads after being previously incubated with BBM1, an anti- $\beta_2$ m mAb. Lane 1 represents cell lysate from FO-1<sup>FcRn+/ $\beta_2$ m</sup>. The immunoprecipitates or cell lysates were resolved under non-reducing conditions in 4–12% NuPAGE and transferred on to nitrocellulose membrane. The immunoblotting was probed with affinity-purified rabbit anti-FLAG antibody, HRP-conjugated donkey anti-rabbit antibodies, and visualized by ECL. The positions of monomer and dimer are indicated.



**Figure 4** Effect of cysteine (Cys<sup>48</sup> or Cys<sup>251</sup>) to serine point mutations on the formation of FcRn HC oligomerization

(A) A schematic illustration of the distribution of cysteine residues within the external domain of human FcRn HC. Cysteine residues (C) are found at positions 48, 96, 159, 198, 251 and 252 of the mature human FcRn HC [17]. The putative paired cysteines (Cys<sup>96</sup>–Cys<sup>159</sup> and Cys<sup>198</sup>–Cys<sup>252</sup>) are defined by alignment of different species of FcRn HC. Horizontal lines indicate disulphide bonds inferred from homology to the immunoglobulin superfamily domain. The non-paired cysteine, Cys<sup>48</sup> or Cys<sup>251</sup>, was replaced with a serine residue. S, signal sequence; TM, transmembrane domain; CT, cytoplasmic domain. (B) The mutant of FcRn heavy chain forms a dimer. FO-1 cells ( $1 \times 10^6$ ) in a 6-well plate was transiently transfected with 2  $\mu$ g of pFlagCMVFcRnC48/S or pFlagCMVFcRnC251/S by the calcium phosphate precipitation method. The soluble lysates (30  $\mu$ g), 72 h after transfection, were subjected to NuPAGE under reducing (R) and non-reducing (NR) conditions, transferred on to a nitrocellulose membrane, and blotted with affinity-purified rabbit anti-FLAG, HRP-conjugated donkey anti-rabbit antibodies and developed with ECL. Lane 1: FO-1<sup>mock</sup>, Lane 2: FO-1<sup>FcRn</sup> stable cell line; Lane 3: FO-1<sup>FcRnC48/S</sup>; Lane 4: FO-1<sup>FcRnC251/S</sup>. The molecular mass (kDa) is indicated on the left. Dimer (D) and monomer (M) are indicated with arrows.

electrophoresis under reducing conditions, the 92-kDa protein spot changed into a spot with an electrophoretic mobility identical to that of the 45-kDa FcRn HC spot (results not shown). These results suggest that the 92-kDa product might be a dimer of the 45-kDa product.

Alternatively, the appearance of the 92-kDa form could be explained by the covalent interaction of an unknown 45-kDa

protein with FcRn HC, generating a 92-kDa product under non-reducing conditions. We may have failed to show this putative protein in Western blots because of the specificity of the antibody. To exclude this possibility, the 92-kDa position in immunoprecipitates was excised for MS analysis. Searches of protein databases showed that the peptide sequences (results not shown) were matched with the amino acid sequence of human FcRn [17]. Therefore, the results from an analysis by two different approaches showed that the 92-kDa protein was a dimer of FcRn.

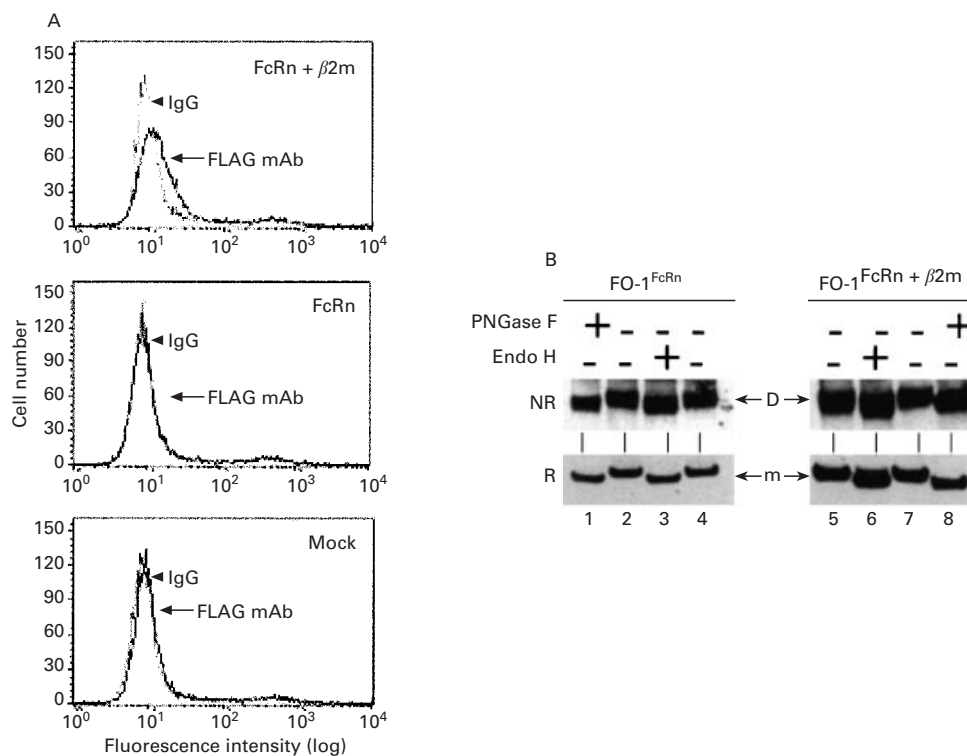
#### Human FcRn dimer is free of $\beta_2m$ association

FcRn is a complex of HC non-covalently associated with  $\beta_2m$ . To determine whether the dimer is associated with  $\beta_2m$  molecule, we conducted an immunoprecipitation experiment with a  $\beta_2m$ -specific mAb IgG1, BBM1 [32]. The immunoprecipitates were subjected to electrophoresis under non-reducing conditions and blotted with an anti-FLAG antibody. The results showed that the FcRn monomer, but not the dimer (Figure 3, lane 3), was visualized in BBM1 precipitates. These results indicate that the dimer was not associated with  $\beta_2m$ . Therefore, FO-1<sup>FcRn+ $\beta_2m$</sup>  cells contain FcRn HC- $\beta_2m$  complexes,  $\beta_2m$ -free HC, and an HC-HC

dimer. The mixture of FcRn HC structures may be due to an unbalanced expression of FcRn and  $\beta_2m$  in transfected cells, an instability of the HC oligomer during cell lysis, conformational differences in  $\beta_2m$ -free HC that do not permit oligomer formation, or dissociation of FcRn HC from  $\beta_2m$ .

#### Mutation of non-paired cysteines, Cys<sup>48</sup> and Cys<sup>251</sup>, within the external domain of FcRn fails to eliminate the formation of an oligomer

Six cysteine residues appear within the external domain of human FcRn HC [17]. The cysteine pairs within the  $\alpha_2$  (Cys<sup>96</sup>-Cys<sup>159</sup>) or  $\alpha_3$  (Cys<sup>198</sup>-Cys<sup>252</sup>) domain are putatively linked with disulphide bonds to form immunoglobulin superfamily domains, based on the alignment with rodent and bovine FcRn HC. Two unpaired cysteines were located in the  $\alpha_1$  (Cys<sup>48</sup>) or  $\alpha_3$  (Cys<sup>251</sup>) domain (Figure 4A). Studies in MHC class I molecules have shown that the formation of the HC dimer can be caused by disulphide bonding between non-paired cysteine residues in the external or cytoplasmic domain [33]. It is highly likely that these non-paired cysteine residues are also responsible for the oligomerization of FcRn HC if it represents a functional molecule. To define this, we replaced the Cys<sup>48</sup> and Cys<sup>251</sup> in the external



**Figure 5** The HC of FcRn is localized to the ER in the absence of  $\beta_2m$  association

(A) The cell-surface expression of FcRn in FO-1<sup>FcRn</sup> and FO-1<sup>FcRn+ $\beta_2m$</sup>  analysed by flow cytometry. FO-1 transfectants ( $5 \times 10^5$  cells/ml) were incubated with mAb anti-(FLAG M1) antibody and with fluorescein-conjugated rabbit anti-mouse antibody. Cells were fixed with 0.5% (v/v) paraformaldehyde in PBS. Results are expressed as histograms of fluorescence intensity (log scale). The solid-lines represent staining of cells with anti-(FLAG M1) mAb (arrow), and dashed lines represent cells stained with isotype-matched IgG2b (arrow head). The staining was conducted twice with similar results. (B) Sensitivity of FcRn HC to endo H digestion in FO-1<sup>FcRn</sup> and FO-1<sup>FcRn+ $\beta_2m$</sup>  cells. Total cell lysates (36  $\mu$ g) were incubated without enzymes (lanes 2, 4, 5 and 7) or in the presence of endo H (lanes 3 and 6) or PNGase F (lanes 1 and 8) for 18 h in native conditions. Lysates were resolved on a 4–20% NuPAGE gel under non-reducing (NR, upper panel) and reducing (R, lower panel) conditions. Proteins were transferred on to nitrocellulose membrane and probed with affinity-purified rabbit anti-FLAG antibody, HRP-conjugated donkey anti-rabbit antibodies, and developed with ECL. It was noted that lane 6 of the lower panel represented a mixture of endo H-sensitive and endo H-resistant proteins. Only the region of interest of the gel is shown. Dimer (D) and monomer (m) are indicated with an arrow. (C, see facing page) Localization of FcRn heavy chain and  $\beta_2m$  in FO-1<sup>FcRn</sup> and FO-1<sup>FcRn+ $\beta_2m$</sup> . Confocal microscopy was performed as described in the Materials and Methods section. Cells grown on glass coverslips were fixed with 3.7% (v/v) paraformaldehyde and permeabilized in 0.2% (v/v) Triton X-100. Subsequently, the cells were incubated with affinity-purified rabbit anti-FLAG-(panels B, D, F, and H) or anti- $\beta_2m$ -(panels J and L) specific antibody and mAb anti-Bip/GRP78 (panels A, E, I) or EEA1 (panels C, G, K), followed by Cy<sup>TM</sup>3-conjugated AffiniPure goat anti-(mouse IgG) and Cy<sup>TM</sup>2-conjugated AffiniPure goat anti-rabbit IgG against IgG of the corresponding species.

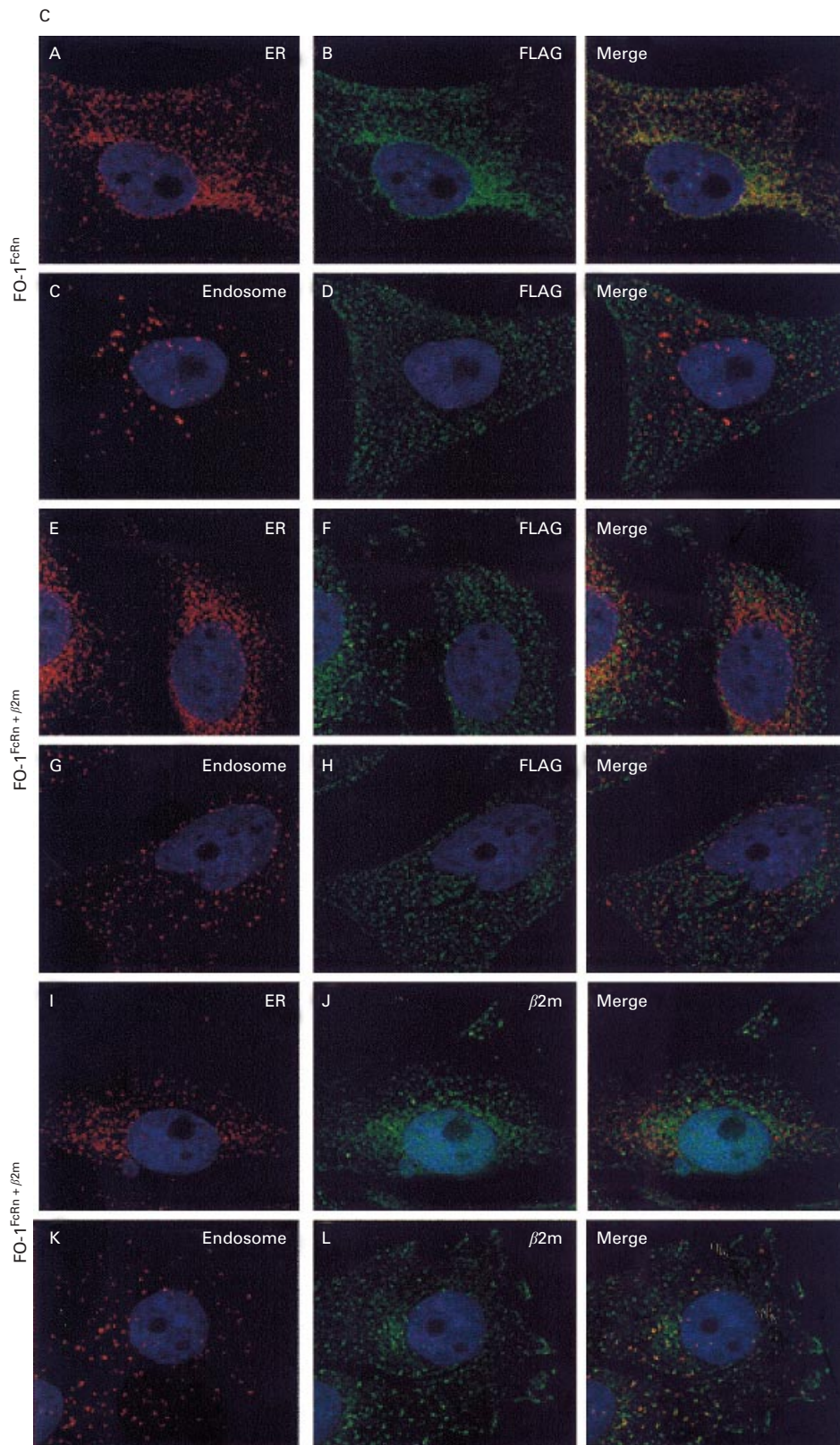


Figure 5 For legend, see facing page.

domain of FcRn HC with serine residues (Figure 4A). When both mutant plasmids were transfected into FO-1 cells, the oligomer was still detectable under non-reducing conditions (Figure 4B, lanes 3 and 4). This demonstrates that the normally paired cysteines within the external domain are probably involved in oligomer formation.

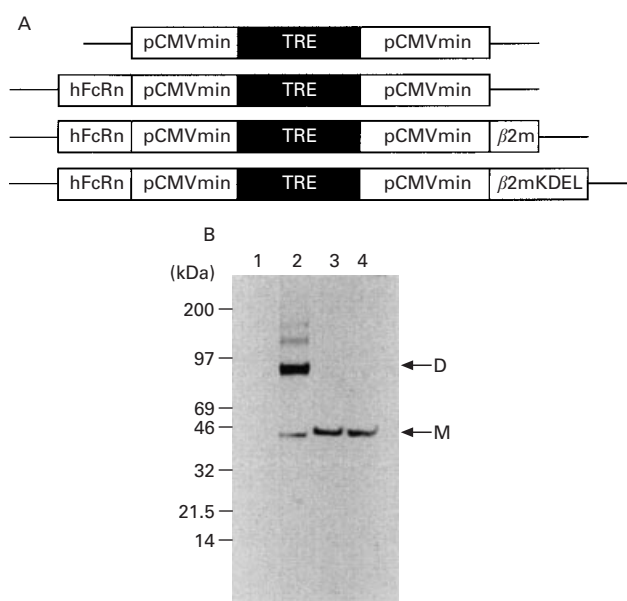
### Human FcRn HC is confined to the endoplasmic reticulum (ER) in the absence of FcRn expression

The location of FcRn HC in FO-1 cells in the presence or absence of  $\beta_2m$  expression was identified by three different experiments. First, flow cytometric analysis of cell-surface staining (Figure 5A) with mAb anti-FLAG (arrow) or a control IgG2b (arrow head) showed that FcRn HC appeared only in the cell surface of FO-1<sup>FcRn+ $\beta_2m$</sup> , but not in that of FO-1<sup>FcRn</sup> and FO-1<sup>mock</sup>, suggesting that FcRn HC appeared on the cell surface only when  $\beta_2m$  was expressed. To examine the location of FcRn HC within cells further, we performed endoglycosidase digestion. Endo H is a glycosidase that cleaves the high mannose oligosaccharide formed only in ER, and PNGaseF is an amidase that cleaves high mannose, hybrid and complex oligosaccharides, formed in both the ER and Golgi complexes. The monomer and 92-kDa dimer of FcRn HC in FO-1<sup>FcRn</sup> were fully sensitive to endo H and PNGase F digestion (Figure 5B). However, the monomer of FcRn HC in FO-1<sup>FcRn+ $\beta_2m$</sup>  was partially resistant to endo H digestion but fully sensitive to PNGase F (Figure 5B). The full sensitivity to endo H digestion confirms that the monomer and oligomer of FcRn HC in FO-1<sup>FcRn</sup> were resident in the ER compartment without their passing into the Golgi stacks to acquire complex oligosaccharides resistant to endo H digestion.

To confirm further that FcRn HC in FO-1<sup>FcRn</sup> is distributed in the ER, we co-localized FcRn HC with organelle markers of the ER and the endosomal compartment. Staining with an affinity-purified anti-FLAG antibody showed prominent punctate staining in the FO-1 transfectants (Figure 5C, middle panels B, D, F, H) but no staining in the mock-transfected cells (results not shown). In the absence of  $\beta_2m$  expression, FcRn HC was co-localized only with the ER marker Bip/GRP78 (Figure 5C, merge of panels A and B) but not with the Golgi marker p230 or GM130 (results not shown) and the early endosome marker EEA1 (Figure 5C, merge of panels C and D). In the presence of  $\beta_2m$  expression, FcRn HC co-localized with the ER (Figure 5C, merge of panels E and F), Golgi (results not shown), and endosome (Figure 5C, merge of panels G and H) markers. The  $\beta_2m$  also co-localized with the ER (Figure 5C, merge of panels I and J) and endosome (Figure 5C, merge of panel K and L) markers in FO-1<sup>FcRn+ $\beta_2m$</sup> . Since bovine and human  $\beta_2m$  share 75.8% amino acid identity, we cultivated FO-1 transfectants in serum-free medium to exclude the possibility that antibody for human  $\beta_2m$  was recognizing bovine  $\beta_2m$  derived from FBS. FcRn HC and  $\beta_2m$  were found in vesicles that co-expressed EEA1, indicating that FcRn HC must have exited the ER and trafficked to the endosomal compartment in FO-1<sup>FcRn+ $\beta_2m$</sup>  cells. On the basis of these observations, we conclude that the co-expression of  $\beta_2m$  is required for FcRn HC to exit the ER.

### Co-regulated expression of FcRn and $\beta_2m$ reduces the formation of FcRn HC dimers

Since the majority of FcRn HC in FO-1<sup>FcRn</sup> formed oligomers (Figures 1 and 2) and FcRn HC was localized in the ER compartment of FO-1<sup>FcRn</sup> (Figure 5), we asked whether a balanced expression of FcRn and  $\beta_2m$  could reduce or inhibit the formation of the oligomer. Therefore, we placed both FcRn and



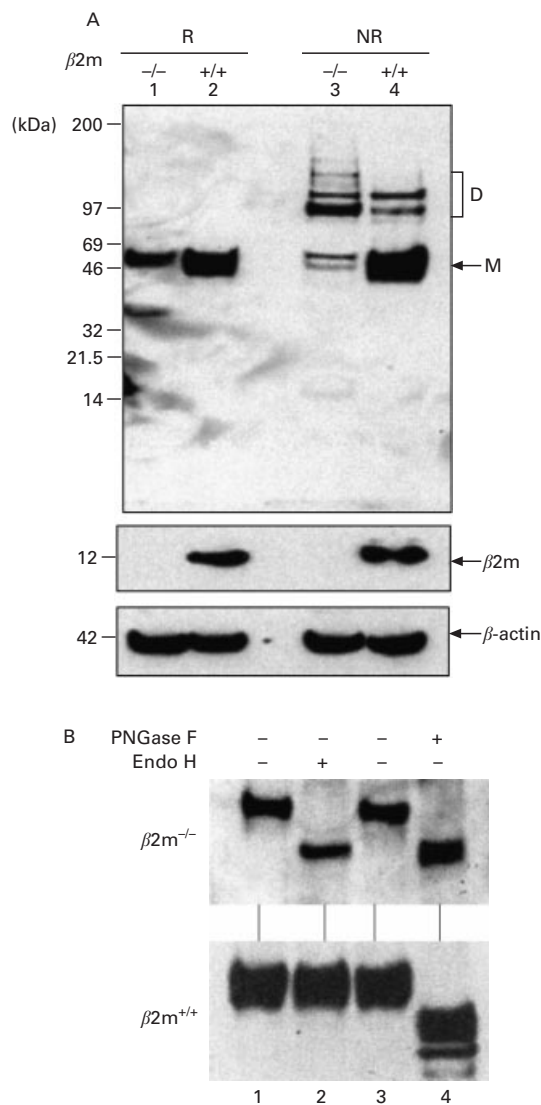
**Figure 6** The co-regulated expression of  $\beta_2m$  and FcRn HC reduces the formation of an FcRn HC dimer

(A) Schematic description of bi-directional Tet expression vectors encoding both human FcRn HC and  $\beta_2m$  or  $\beta_2m$ KDEL under a CMV minimal promoter. A bi-directional promoter contains the tet operator sequences flanked by two identical minimal CMV promoters in opposite orientations. TRE, tetracycline responsive element. (B) The co-regulated expression of  $\beta_2m$  and FcRn HC in FO-1 cells. FO-1 cells ( $1 \times 10^6$ ) were transfected with plasmid pBI (lane 1), pBIFcRn (lane 2), pBIFcRn- $\beta_2m$  (lane 3) and pBI  $\beta_2m$ KDEL and a plasmid encoding tetracycline-controlled transactivator. The protein expression was induced with 1  $\mu$ g/ml doxycycline following transfection for 48 h. Cell lysates (30  $\mu$ g) were electrophoresed in 4–12% NuPAGE under non-reducing conditions and transferred on to a nitrocellulose membrane. Immunoblotting was performed as described in the Materials and methods section. Molecular mass (kDa) is marked on the left. FcRn monomer (m) and dimer (D) are shown with arrows.

$\beta_2m$  cDNA under the control of a minimal cytomegalovirus (CMV) promoter (Figure 6A). This promoter would allow two genes to be transcribed in a co-regulated manner since it contains the tetracycline operator sequences flanked by two identical minimal CMV promoters in opposite orientations. Since  $\beta_2m$  is a secreted protein, its secretory nature may lead to the unbalanced expression of both molecules in the ER. We therefore engineered an ER retention signal sequence, KDEL, in the C-terminus of  $\beta_2m$  (Figure 6A). The mouse  $\beta_2m$  with an ER retention signal sequence has been shown to retain  $\beta_2m$  in the ER [34]. Expression of  $\beta_2m$ -KDEL in the transfectant was shown by reverse transcription-PCR (results not shown), and we found that co-regulated expression of both versions of  $\beta_2m$  can reduce the dimeric formation of FcRn HC (Figure 6B, lanes 3 and 4). This suggests that  $\beta_2m$  may play a role in FcRn HC folding in the ER by reducing the formation of oligomers. In addition, the natural leader sequence of the FcRn in this experiment was used to target FcRn to the secretory pathway. This confirms the results obtained with a preprotrypsin signal sequence, as provided by the expression vector.

### Mouse FcRn HC forms dimers and the amount of protein is significantly reduced in $\beta_2m^{-/-}$ mice

Rodent and human FcRn HC share approx. 60% amino acid identity [17]. Five out of six cysteine residues are conserved between mouse and human FcRn HC. We reasoned that mouse



**Figure 7** Expression pattern of mouse FcRn in the intestinal epithelium of neonatal  $\beta_2m^{-/-}$  and wild-type mice

All immunoblotting was performed with affinity-purified rabbit anti-(mouse FcRn  $\alpha_2$  domain) specific antibody and HRP-conjugated donkey anti-rabbit IgG, and developed with ECL. **(A)** The HC of mouse FcRn formed a dimer under non-reducing conditions. Total cellular proteins (40  $\mu$ g) from neonatal mouse intestinal epithelium were resolved in a 4–12% NuPAGE under reducing (R, lanes 1 and 2) and non-reducing (NR, lanes 3 and 4) conditions. Immunoblotting was performed as described above. Mouse  $\beta_2m$  was blotted to confirm the absence of  $\beta_2m$  expression in  $\beta_2m^{-/-}$  mice (lanes 1 and 3), and  $\beta$ -actin was blotted to show the loading of equal amounts of protein. Molecular mass (kDa) is marked on the left. Mouse FcRn monomer (m) and dimer (D) are indicated by an arrow and a bracket respectively. **(B)** Sensitivity of mouse FcRn HC from  $\beta_2m^{-/-}$  and  $\beta_2m^{+/+}$  mice to endo H digestion. Total cell lysates (40  $\mu$ g) from  $\beta_2m^{-/-}$  (upper panel) and  $\beta_2m^{+/+}$  (lower panel) mice were subjected to mock digestions (lanes 1 and 3), digestion with endo H (lane 2), or digestion with PNGase F (lane 4) for 18 h under native conditions. Lysates were resolved on a 4–20% NuPAGE gel. Immunoblotting was performed as described above. Only the region of interest in the gel is shown.

FcRn HC may also form oligomers in the absence of  $\beta_2m$  association. In neonatal mice, FcRn is highly expressed in the intestinal epithelial cells (IEC) during the first 3 weeks after birth. After the animals are weaned, the expression of FcRn in IEC diminishes rapidly and substantially. We therefore endeavoured to define whether a corresponding dimer in FO-1<sup>FcRn</sup> also appears in neonatal mice, especially in  $\beta_2m^{-/-}$  mice *in vivo*. We generated

a rabbit antibody to the  $\alpha_2$  domain of mouse FcRn HC. The antibody recognized mouse FcRn protein only when it was extracted from neonatal intestinal epithelium but not from adult intestine (results not shown), supporting the specificity of the antibody for FcRn. The cell extracts from the small intestine of neonatal mice were examined under reducing and non-reducing conditions. With 2-ME, as expected, mouse FcRn was present as a 50-kDa band (Figure 7A). Without 2-ME, FcRn was displayed as discrete protein bands ranging from 90 kDa to 100 kDa (Figure 7A). However, the majority of FcRn in  $\beta_2m^{-/-}$  mice was detected as bands of high molecular mass, probably representing a dimeric form (Figure 7A). On the contrary, the majority of FcRn from wild-type mice was seen as a monomer (Figure 7A). The amount of FcRn in  $\beta_2m^{-/-}$  mice was significantly decreased compared with that observed in  $\beta_2m^{+/+}$  mice (Figure 7A). FcRn isolated from mouse intestine displayed multiple discrete bands in Western blots (Figure 7B), suggesting that these bands may represent non-glycosylated, underglycosylated, and fully glycosylated proteins. Mouse FcRn possesses four putative N-linked glycosylation sites [27].

#### Mouse FcRn HC from $\beta_2m^{-/-}$ mice is sensitive to endo H digestion

Human FcRn HC in FO-1<sup>FcRn</sup> localized to the ER and exhibited sensitivity to digestion with endo H (Figures 5B and 5C). We questioned whether FcRn HC from  $\beta_2m^{-/-}$  mice also exhibited a similar sensitivity. The total cell lysates from  $\beta_2m^{-/-}$  and  $\beta_2m^{+/+}$  mice were subjected to endo H and PNGase F digestion. Mouse FcRn HC from  $\beta_2m^{-/-}$  mice was sensitive to both endo H and PNGase F digestion (Figure 7B, lanes 2 and 4) as compared with the mock digestion (Figure 7B, lanes 1 and 3). However, FcRn from  $\beta_2m^{+/+}$  mice was sensitive to PNGase F digestion (Figure 7B, lane 4) but not to endo H digestion (Figure 7B, lane 2), proving that FcRn HC in  $\beta_2m^{-/-}$  mice, like human FcRn HC in FO-1<sup>FcRn</sup>, is probably resident in the ER compartment, but that FcRn HC from  $\beta_2m^{+/+}$  mice leaves the ER compartment. However, endo H-sensitive rat FcRn is detectable on the cell surface when its cDNA is expressed in an inner medullary collecting duct kidney cell line [35]. This discrepancy might be related to differential glycosylation patterns in different tissues and cell types.

#### DISCUSSION

In the present study we show that in the absence of  $\beta_2m$ , FcRn forms oligomers. Moreover, the FcRn HC oligomer may represent a misfolded protein rather than a functional molecule. MHC class I HC has been shown to form dimers [33,36]. HLA-B27 HC in particular can form a homodimer through a disulphide bond formed between non-paired cysteines in the  $\alpha_1$  domain, and this dimer is capable of presenting peptides to T cells [33]. Given the similarity in structure between the MHC class I and FcRn molecules, an FcRn dimer may also exist. This has been shown clearly in neonatal rat FcRn [19] and visualized in crystals of rat FcRn and FcRn/Fc complexes [21,23], although a human FcRn does not form dimers in crystals [22]. However, evidence is lacking on whether, and how, FcRn forms a dimer *in vivo*. Since an FcRn HC dimer represents a major form in our study, the possibility that it mirrors the dimeric structure observed previously by others is very intriguing [19,21,23]. Therefore, we examined whether an FcRn dimer from oligomerization represents a functional and correctly folded protein, or a misfolded protein. Since the FcRn HC dimer appeared only in the absence of reducing agents, we reasoned that the amount of FcRn HC

dimer would be reduced if it represents a correctly folded protein after removal of the non-paired cysteine residues in the external domain (Figure 4A). However, the FcRn HC dimer still appeared in FO-1 cells expressing FcRn with a mutation in either of the non-paired cysteines (Figure 4B). This suggests that the FcRn HC dimer may represent a misfolded protein in which the paired cysteines are involved in disulphide bond formation between two FcRn HC molecules. The dimers may also represent a mixture of dimers formed by non-paired and paired cysteines. Therefore, a mutation analysis following the removal of non-paired cysteines could not exclude this possibility. Alternatively, mutations of cysteine to serine may affect the conformation of the external domain in a manner that promotes dimerization via paired cysteines. Two independent experiments did not support the above possibility. First, an FcRn HC dimer was not associated with  $\beta_2m$  (Figure 3). Secondly, although the FcRn dimer from FO-1<sup>FcRn</sup> and FO-1<sup>FcRn+ $\beta_2m$</sup>  lysates could bind IgG, its binding was pH-independent with detection under both pH 7.5 and pH 6.0 conditions (results not shown), a phenomenon that may be physiologically irrelevant given the known pH dependence of IgG binding of the mature FcRn glycoprotein. The binding of the dimeric form to IgG at both pH 6.0 and 7.5 may be caused by its conformational change and could represent a misfolded version of FcRn HC. This may be different from the dimers with physiological function previously observed by others [19,23,26].

Localization of FcRn HC oligomers in the ER may represent a first step in the degradation pathway of FcRn HC metabolism. In confocal experiments, the FcRn HC could not be detected in the early endosome of the FO-1<sup>FcRn</sup> but could be detected in that of FO-1<sup>FcRn+ $\beta_2m$</sup>  (Figure 5C). The FcRn HC in FO-1<sup>FcRn</sup> may be mis-sorted without routing to the early endosome, and thus we failed to co-localize FcRn HC with the endosome marker. However, various results countered this conjecture. First, FcRn HC could not be detected in the cell surface of FO-1<sup>FcRn</sup> but could be detected in the case of FO-1<sup>FcRn+ $\beta_2m$</sup>  (Figure 5A). Secondly, FcRn HC did not appear in Golgi in FO-1<sup>FcRn</sup> but was detected in FO-1<sup>FcRn+ $\beta_2m$</sup>  (results not shown). Thirdly, FcRn HC monomers and dimers from FO-1<sup>FcRn</sup> (Figure 5B) or isolated from  $\beta_2m^{-/-}$  mice (Figure 7B) were sensitive to endo H digestion. Therefore, we conclude that most FcRn HC oligomers are retained in the ER. Of greater interest is our finding that the amount of FcRn HC was remarkably lower in  $\beta_2m^{-/-}$  mice and FO-1<sup>FcRn</sup> when compared with the amounts in the wild-type mice and FO-1<sup>FcRn+ $\beta_2m$</sup>  (Figure 7A). It is likely that, in the absence of  $\beta_2m$  association, FcRn HC is misfolded as a major dimeric form and is targeted to the protein degradation pathway because of an ER quality control mechanism. Similarly, MHC class I HC might be retrotranslocated from the ER to the cytoplasm by ubiquitin-dependent and Sec6-mediated pathways [37]. Our preliminary results showed that human FcRn HC could be ubiquitinated when the FO-1<sup>FcRn</sup> cell is transfected with the ubiquitin expression vector in the presence of the proteasome inhibitor (results not shown), although additional experiments are necessary to confirm and dissect this pathway. It is therefore possible that formation of FcRn HC oligomers acts as the first step to ensure that the ER compartment recognizes misfolded FcRn HC and finally targets misfolded FcRn HC to the protein degradation pathway. The FcRn HC oligomerization might ensure that cells do not overprotect IgG and that a balance between protection and degradation is maintained. The inactivation or removal of excess FcRn HC may be significant *in vivo* because it helps to maintain homeostasis of IgG levels and proper functioning of the IgG immune system. Perhaps excessive FcRn leads to hyper-immunoglobulinaemia or accumulation of autoantibody *in vivo* because of its role in IgG protection [25].

On the other hand, the  $\beta_2m$  may play a role in FcRn folding in the ER and the association with  $\beta_2m$  may be necessary to the exiting of FcRn HC from the ER. Our data suggest that reconstitution of  $\beta_2m$  in FO-1<sup>FcRn</sup> reduced the formation of FcRn HC oligomers (Figure 2). The role of  $\beta_2m$  in FcRn folding is further supported by the balanced expression of  $\beta_2m$  or  $\beta_2m$ -KDEL with FcRn HC (Figure 6). In addition, the majority of FcRn HC in  $\beta_2m^{-/-}$  mice appeared in dimeric form. Therefore,  $\beta_2m$  may help FcRn HC achieve or maintain a folded conformation with correctly paired cysteines to form the Ig superfamily domain.  $\beta_2m$  independently promotes the folding and disulphide bond formation in MHC class I HC [38,39] and induces conformational changes of CD1b HC [40]. However, we cannot rule out the possibility of potential roles for other chaperone proteins. Our preliminary data showed that when the N-linked glycosylation site was deleted, human FcRn HC dimer and oligomer were much more easily formed in comparison with the wild-type counterpart, suggesting a possible role for calnexin and/or calreticulin in FcRn folding. Without  $\beta_2m$  expression, most of the MHC class I and CD1b HC pools are retained in the ER by calnexin and/or calreticulin [39–41]. It is likely that the high-molecular-mass species of FcRn HC may, at least in part, be the result of overexpression and the consequential saturation of other chaperone molecules necessary for correct folding. In addition, our confocal data showed that the association with  $\beta_2m$  is critical for the exiting of FcRn from the ER and its appearance in the early endosome (Figure 5C). The HLA B27 HC dimer [33] and CD1d HC [42] are exceptions and can be expressed on the cell surface.

The FcRn HC oligomers may be present under special circumstances of limited  $\beta_2m$  expression. Under physiological conditions,  $\beta_2m$  is expressed as a secretory molecule or by association with MHC class I HC; however,  $\beta_2m$ -free HC may appear under some pathophysiological conditions. First,  $\beta_2m$  is a substrate-limiting molecule because it is shared with MHC class I and its related molecules. The HC of MHC class I and its related molecules can be up-regulated by interferon- $\gamma$  [43], which may tilt the balance between HC and  $\beta_2m$  expression and may result in  $\beta_2m$ -free HC in the ER under inflammatory conditions. Therefore, the formation of the HLA-B27 HC dimer may reflect the up-regulation of HC under inflammatory conditions, such as spontaneous inflammatory arthritis [33]. It can easily be envisioned that simultaneous expression of several MHC HC homologues in a single cell results in the preferential formation of the HC- $\beta_2m$  complex only when an HC has a high affinity for its partner  $\beta_2m$ . It is not yet clear whether there is affinity variation between a variety of MHC HC and  $\beta_2m$ , or whether inflammatory cytokines can up-regulate the expression of FcRn HC. Secondly, tumour cells undergo frequent point mutations or deletions in the  $\beta_2m$  gene. Such lesions have been described in colon, breast, and lung carcinomas and in malignant melanomas [30,44–46]. FcRn expression has been identified in the colon [12] and breast [13]. FcRn is involved in the homeostasis of serum IgG levels *in vivo*. The abnormalities in the  $\beta_2m$  gene raise questions about the function of FcRn in certain malignant cells. Cancer tissues are characterized by the infiltration of a network of blood vessels to supply blood for its aggressive growth and metastasis. However, cancer tissues also feature the leakage of large molecules from blood vessels [47], including IgG. Therefore, an abundance of IgG could be present within the interstitial space of cancer tissues and adsorbed by malignant cells. Some cancer tissues may be important sites for the catabolism of IgG *in vivo*, perhaps resulting in the down-regulation of humoral immunity in cancer patients with an increase in IgG catabolism. Gene therapy may thus be an alternative way to restore the  $\beta_2m$

expression for a functional FcRn molecule in cancer tissues. Our findings may help delineate the impact of  $\beta_2m$  abnormalities on the catabolism of IgG in malignant cells. Thirdly, human and murine CMVs encode UL-18 or m144 gene products that are associated with  $\beta_2m$  [48,49]. The predominant expression of these products in CMV-infected cells may saturate the host  $\beta_2m$ , causing the significant accumulation of  $\beta_2m$ -free FcRn HC in virus-infected cells. Since CMV can infect epithelial, endothelial and macrophage cells, where FcRn is functionally expressed, the UL-18 or m144 gene products may provide an alternative pathway for the down-regulation of the humoral immunity by simply usurping the host  $\beta_2m$  to sequester FcRn HC in the ER.

In conclusion, we have demonstrated that FcRn HC is sequestered in the ER by forming disulphide bond-linked oligomers in the ER. We provide evidence that the association of  $\beta_2m$  with FcRn HC is critical for proper folding and that only  $\beta_2m$ -associated molecules are able to exit the ER and route to appropriate intracellular compartments, such as the endosome, where FcRn presumably binds IgG. Whether this oligomeric form of FcRn represents a degradation pathway of FcRn metabolism remains to be determined.

This work was in part supported by grants from the National Institutes of Health (DK/AI53056, DK51362 and DK44319 to R.S.B., DK/AI53056 to W.I.L. and DK34854 to Harvard Digestive Diseases Center), a Career Development Award from the Crohn's and Colitis Foundation of American (X.Z.), and the Jane Coffin Childs Memorial Fund for Medical Research (J.P.). We gratefully acknowledge Dr Neil Simister for his helpful discussions and for providing human and mouse FcRn cDNA plasmids. We thank Dr E. Sally Ward (Center for Immunology and Cancer Immunobiology Center, University of Texas, Southwestern Medical Centre, TX, U.S.A.) for providing us with rabbit anti-FcRn peptide serum antibody and Dr Xiaotong Li (Department of Medicine, Brigham and Women's Hospital, Boston, MA, U.S.A.) for the gift of the ubiquitin expression plasmid and Lili Miao for technical assistance. We acknowledge the helpful editing of the manuscript by Ms Nancy Voynow from the Editorial Service at Brigham and Women's Hospital.

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Received 30 January 2002/15 July 2002; accepted 6 August 2002

Published as BJ Immediate Publication 6 August 2002, DOI 10.1042/BJ20020200