β_2-microglobulin is important for cell surface expression and pH-dependent IgG binding of human FcRn

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Summary

FcRn is a heterodimer of an α-chain and β_2-microglobulin (β_2m) and differs from other IgG Fc receptors in that it is structurally related to MHC class I molecules. Several functions attributed to FcRn are affected in β_2-microglobulin (β_2m)-deficient mice, suggesting that the α-chain needs to assemble with β_2m to form a functional receptor. However, the precise role of β_2m in FcRn function is not known. Here we expressed the human FcRn α-chain alone or in combination with β_2m in human melanoma FO-1 cells. We show that β_2m is important for cell surface expression of FcRn and that, in the absence of β_2m, the receptor is retained in the endoplasmic reticulum. Furthermore, in the absence of β_2m, IgG binding is decreased compared with that of native FcRn. Thus, assembly of the FcRn α-chain with β_2m is important for both transport of FcRn from the ER to the cell surface and efficient pH-dependent IgG binding.

Key words: Human immunoglobulin G, Major histocompatibility complex, Endoplasmic reticulum, Oligomerization.

Introduction

FcRn is an IgG Fc receptor involved in the transfer of passive immunity from the mother to the fetus or newborn (reviewed by Ghetie and Ward, 2000; Hunziker and Kraehenbuhl, 1998). The receptor is thought to transport IgG from the maternal circulation across the placental syncytiotrophoblast (primates), and to transcytose IgG in colostrum and milk across the small intestine of the suckling neonate (rodents, ruminants). In addition, FcRn is implicated in the regulation of the IgG serum concentration by binding IgG internalized in the fluid phase and recycling it back into the circulation, thus preventing lysosomal degradation of internalized IgG.

The transcytotic and protective functions of FcRn are intimately linked to its pH-dependent IgG-binding properties. FcRn binds IgG at a mildly acidic pH, as found in the intestinal lumen or in endosomes, but not at neutral pH. In the gut, FcRn is therefore thought to bind IgG on the lumenal surface and, following transcytosis, to release the IgG into the circulation upon exposure to the neutral serosal pH. In the absence of a pH gradient, IgG may be internalized in the fluid phase and bind FcRn in the acidic milieu of endosomes, from where it could either be transcytosed or recycled.

FcRn, like MHC class I, consists of an α-chain and β_2-microglobulin (β_2m). Indications that an association of the FcRn α-chain with β_2m is important for the assembly of a functional receptor come from β_2m-knockout mice (Zijlstra et al., 1990), which show defects in several functions associated with FcRn. Newborn β_2m-deficient pups show lower IgG serum levels at birth and accumulate less IgG before weaning than normal littermates (Israel et al., 1995; Zijlstra et al., 1990). Furthermore, adult mice lacking β_2m have a higher IgG turnover, resulting in lower serum IgG levels (Ghetie et al., 1996; Israel et al., 1996; Junghans and Anderson, 1996).

While the importance of β_2m for a functional FcRn is well recognized, the precise role of β_2m in FcRn function is not known. IgG transport in mice lacking β_2m could reflect a failure of FcRn to either reach the cell surface or to bind IgG. In the case of major histocompatibility complex (MHC) class I molecules, assembly of the α-chain with β_2m and loading of antigenic peptide in the endoplasmic reticulum (ER) are required for cell surface transport of functional class I molecules (Pamer and Cresswell, 1998). However, CD1d, an MHC class I-like CD1 molecule, is efficiently expressed on the cell surface even in the absence of β_2m (Hyun et al., 1999). Thus, assembly with β_2m does not appear to be a general requirement for surface transport of MHC class I-like proteins. The absence of β_2m could also interfere with other intracellular transport steps such as recycling from endosomes to the cell surface. Alternatively, binding of IgG by FcRn may depend on the presence of β_2m.

To determine whether β_2m is required for cell surface expression of FcRn, IgG binding or both, we characterized intracellular transport and ligand-binding properties of human FcRn (hFcRn) expressed in β_2m-deficient FO-1 cells (FO-1) or in FO-1 cells stably expressing human β_2m (FO-1β2m) (D'Urso et al., 1991). We show that in cells lacking β_2m, the FcRn α-chain fails to reach the cell surface and is retained in the ER. Furthermore, binding of human IgG (hIgG) to the FcRn α-chain is reduced at acidic pH in the absence of β_2m. Thus, assembly of the FcRn α-chain with β_2m is important for transport of the receptor from the ER to the cell surface as well as for efficient pH-dependent binding of IgG.

Materials and Methods

Reagents

Protease inhibitor cocktail CLAP [10 μg/ml chymostatin, leupeptin, antipain and pepstatin A (Sigma) in DMSO] was diluted 1:1000 and supplemented with PMSF (Sigma) to a final concentration of 0.57 mM. Immunopure sulfo-NHS-Biotin (Pierce) was prepared as a 200 mg/ml stock solution in DMSO. EndoH and glycosidase F (Roche) were used at 165 mU/ml and 33 U/ml, respectively. Protein A-negative *Staphylococcus aureus* (Wood 46 strain), human IgG and steptavidin-HRP were from Sigma and Streptavidin-agarose was purchased from Upstate Biotechnologies. The Bradford Assay was obtained from Research Biolabs, PVDF membranes (0.2 μm) were from Amersham and the SuperSignal chemiluminescence system from Pierce. Mowiol 4-88 (Calbiochem-Novabiochem) was used at 0.1 g/ml and supplemented with 0.2% (w/v) DABCO (Sigma). Tissue culture media was from Sigma, FCS was from Hyclone, media supplements were from Gibco and G-418 and hygromycin were purchased from Calbiochem-Novabiochem.

Antibodies

For western blotting, hybridoma supernatants containing 9E10 anti-Myc antibodies (kindly provided by R. Iggo, Epalinges, Switzerland), polyclonal anti-β_2m antibodies (Sigma) and a polyclonal rabbit anti-FcRn peptide serum (Praetor et al., 1999) were used. Polyclonal anti-Myc (Santa Cruz Biotechnology) and polyclonal anti-β_2m antibodies (Abcam) were used for co-immunoprecipitation experiments. For immunofluorescence, polyclonal anti-Myc (Upstate Biotechnology), polyclonal anti-β_2m (Abcam), monoclonal anti-EEA1 (Transduction Laboratories) and monoclonal anti-ribophorin II (kindly provided by D. Meyer, Heidelberg, via A. Helenius, Zurich) antibodies were used. HRP-coupled secondary antibodies were purchased from Research Biolabs. Affinity purified fluorescently labeled secondary antibodies were from Molecular Probes.

Plasmids

In analogy to a Flag-tagged hFcRn (Praetor et al., 1999), a cDNA carrying the Igκ leader sequence and an N-terminal Myc-epitope tag (Myc-hFcRn) was generated and cloned into a modified pLNCX expression vector (Clontech) carrying a hygromycin resistance gene (Reichert et al., 2000). Details on the construction of the G418-resistant plasmid carrying the human β_2m cDNA can be found elsewhere (D'Urso et al., 1991).

Cell culture and transfection

FO-1 and G418-resistant FO-1 cells stably expressing human β_2m (FO-1β2m) (D'Urso et al., 1991) were generously provided by Patrizio Giacomini (Rome, Italy). Cells were cultured in DMEM (low glucose) supplemented with 10% FCS, 50 μg/ml penicillin and 50 μg/ml streptomycin and glutamine. Cells were transfected with a Myc-hFcRn plasmid using the Transfast transfection kit (Promega) and selected in 0.5 mg/ml hygromycin. Resistant clones were analyzed for expression by immunofluorescence and immunoblotting and two clones for each transfection were used in further experiments. Stably transfected cells were maintained in G-418 (0.25 mg/ml) and/or hygromycin (0.5 mg/ml).

Western blot analysis

To detect FcRn and β_2m, cells were lysed in 0.5% Triton X-100 in PBS containing protease inhibitors. Equal amounts of total protein, as determined by Bradford, were separated on 10% Tris-tricine gels. Proteins were transferred onto PVDF membranes by wet blotting at 200 mA for 5 hours. After blocking the membranes with 5% milk in PBS, they were probed with mouse monoclonal 9E10 antibodies (1:1000), polyclonal anti-FcRn serum (1:500) or polyclonal anti-β_2m (1:500), followed by secondary HRP-labeled anti-mouse or anti-rabbit antibodies (1 μg/ml) and visualized by chemiluminescence.

Co-immunoprecipitation

Cells expressing the Myc epitope-tagged human FcRn alone or in combination with β_2m were lysed in 5 mg/ml CHAPS in 50 mM phosphate buffer pH 7.4 containing protease inhibitors. Cell lysates (equal amounts of total protein) were precleared and incubated for 2 hours at 4°C with either 9E10 prebound to protein G sepharose (1 μl/100 μg lysate) or polyclonal anti-β_2m prebound to protein G sepharose (0.5 μg/100 μg lysate). Immune complexes were washed three times with CHAPS lysis buffer and bound proteins were eluted by heating in unreducing sample buffer for 30 minutes at 40°C. SDS-PAGE and blotting was carried out as described above. Membranes were probed with polyclonal anti-Myc (2.5 μg/ml) or polyclonal anti-β_2m (0.2 μg/ml) antibodies.

Immunofluorescence

Cells grown on coverslips were processed for immunofluorescence as described (Stefaner et al., 1999). Briefly, cells were fixed and labeled with polyclonal anti-Myc antibodies (5 μg/ml). To monitor cell surface expression or internalization, cells were incubated in the presence of polyclonal anti-Myc antibodies (5 μg/ml in L-15 medium, pH 7.4) at 4°C for 45 minutes or at 37°C for 60 minutes, respectively. For co-localization experiments, cells grown on coverslips were fixed and stained with polyclonal anti-Myc (5 μg/ml), polyclonal anti-β_2m (5 μg/ml), monoclonal anti-EEA1 (12 μg/ml) or monoclonal anti-ribophorin II (1:100) antibodies. Fluorescently labeled anti-mouse (Alexa 488) or anti-rabbit (Alexa 568) secondary antibodies were used at 2 μg/ml. IgG internalization was performed as described (Praetor et al., 1999). Briefly, cells were allowed to internalize hIgG (1 μg/ml) for 30 minutes at 37°C and then washed on ice with PBS. Internalized IgG was visualized with fluorescently labeled goat anti-human (Alexa 488; 2 μg/ml) secondary antibodies.

Surface biotinylation

Cell surface biotinylation was carried out as described (Praetor et al., 1999). Briefly, precleared cell lysates were incubated with streptavidin-agarose (5 μl/100 μg lysate) or 9E10 prebound to protein G sepharose (1 μl/100 μg lysate) for 2 hours at 4°C. The precipitates were washed three times with RIPA buffer, proteins eluted either by boiling for 10 minutes (streptavidin precipitate) or heating for 30 minutes to 40°C (9E10 precipitate) in reducing sample buffer and analyzed by SDS-PAGE as described above. Streptavidin precipitates were probed with 9E10 antibodies as described. In the case of 9E10 precipitates, membranes were blocked with 1% BSA in PBS and probed with streptavidin-HRP (0.1 μg/ml).

EndoH and glycosidase F digestion

30 μl cell lysate was incubated with EndoH (5 mU) or glycosidase F (1 U) at 37°C for 3.5 hours in the presence of PMSF. Cell lysates were adjusted to pH 5-6 by the addition of 0.5 μl 50 mM sodium acetate pH 5.2 for the EndoH digest. Control lysates were incubated at 37°C for 3.5 hours in the absence of the enzyme. The reaction was terminated by the addition of reducing SDS-PAGE sample buffer.

IgG precipitation

Binding and precipitation of FcRn from cell lysates using IgG-agarose was carried out as outlined (Praetor et al., 1999).

Results

Characterization of FO-1 cells expressing the FcRn α-chain alone or in combination with β2m

Human melanoma FO-1 cells do not express endogenous β2m (D'Urso et al., 1991) and thus provide an excellent system for analyzing the relevance of β2m in FcRn function. Parental FO-1 cells or FO-1 cells stably expressing human β2m (FO-1β2m) (D'Urso et al., 1991) were transfected with a Myc-tagged human FcRn α-chain cDNA (Myc-hFcRn) and clones were screened by immunoblotting and immunofluorescence. Two independent cell clones stably expressing comparable levels of hFcRn were selected for further analysis.

As shown in Fig. 1A, a 47 kDa protein corresponding to hFcRn was detected on blots probed with either anti-Myc (α-Myc) or anti-FcRn (α-FcRn) antibodies in transfected (lanes 3,4) but not in control (lanes 1,2) FO-1 cells. As expected, β2m was not present in FO-1 cells (α-β2m; lanes 1,3) but readily detected in FO-1β2m cells stably expressing human β2m (α-β2m; lanes 2,4).

Immunofluorescence experiments confirmed the co-localization of β2m with the FcRn α-chain in FO-1β2m cells. As shown in Fig. 1B, the FcRn α-chain (α-Myc) and β2m (α-β2m) were detected by indirect immunofluorescence in transfected FO-1β2m cells (Fig. 1Bb,d), but not in control FO-1 cells (Fig. 1Ba,c). Merging the staining for the α-chain (green) and β2m (red) showed extensive co-localization of the two proteins (Fig. 1Be, yellow).

To demonstrate directly that β2m associates with the FcRn

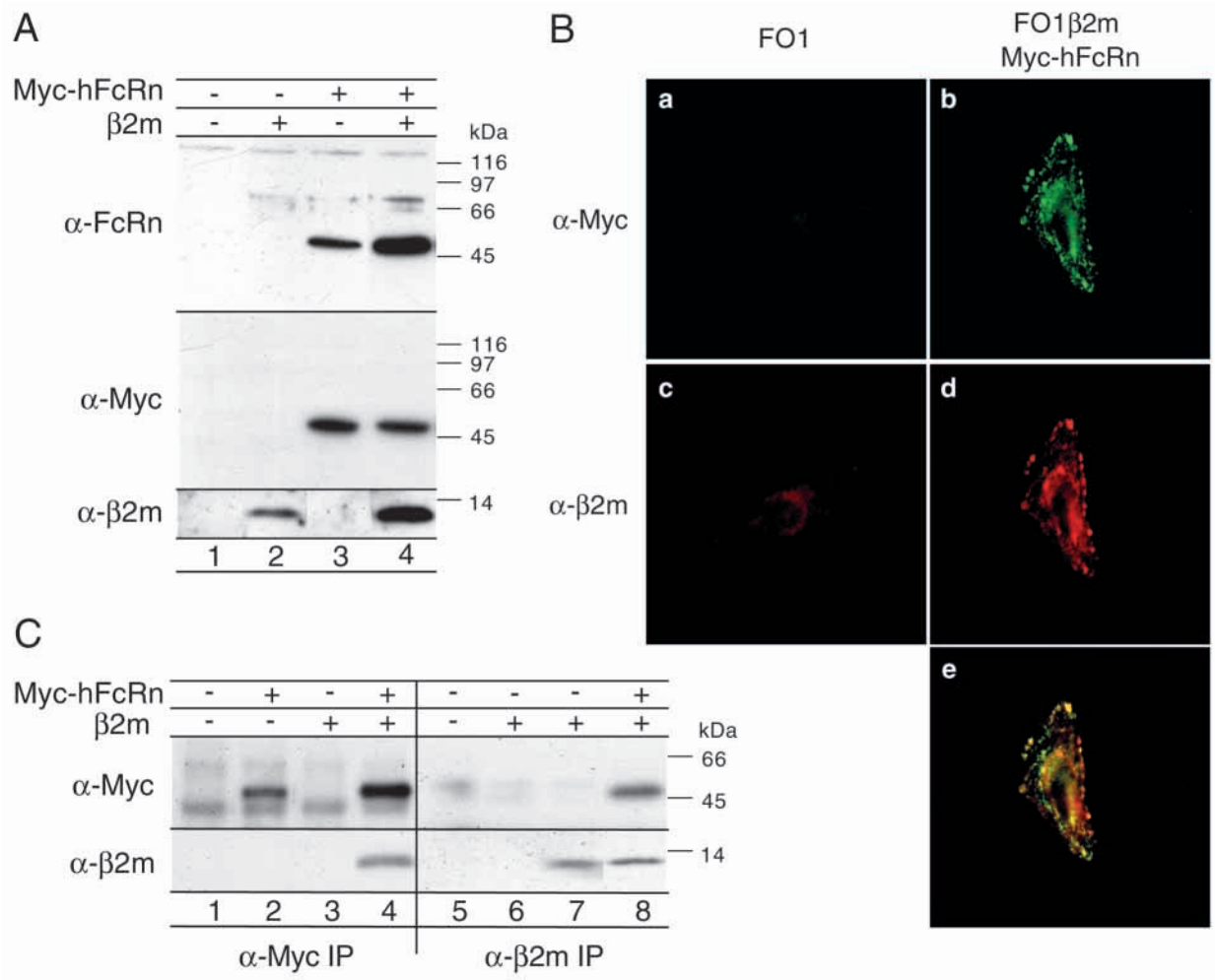

Fig. 1. Characterization of FO-1 cells expressing the FcRn α-chain alone or in combination with β2m. (A) Western blot analysis. Lysates of FO-1 cells (lane 1), FO-1β2m cells (lane 2), FO-1 cells expressing Myc-hFcRn (lane 3), and FO-1β2m cells expressing Myc-hFcRn (lane 4) were analyzed by SDS-PAGE and immunoblotting using polyclonal anti-FcRn peptide antibodies (α-FcRn; upper panel), monoclonal anti-Myc antibodies (α-Myc; middle panel), and polyclonal anti-β2m antibodies (α-β2m; lower panel). For reasons unknown, the anti-FcRn serum detected the α-chain less well in lysates from FO-1β2m than in FO-1 cells. (B) Co-localization of the FcRn α-chain and β2m. FO-1 cells (a,c) and FO-1β2m cells expressing Myc-hFcRn (c,d,e) were fixed, permeabilized and stained with monoclonal anti-Myc (α-Myc; a,b) and polyclonal anti-β2m antibodies (α-β2-m; c,d). Panel e shows the merged staining for Myc-hFcRn (green) and β2m (red). (C) Co-immunoprecipitation of the FcRn α-chain and β2m. FO-1 cells (lanes 1,5), FO-1 cells expressing Myc-hFcRn (lanes 2,6), FO-1β2m cells (lanes 3,7) and FO-1β2m cells expressing Myc-hFcRn (lanes 4,8) were lyzed and immunoprecipitated with monoclonal anti-Myc antibodies (α-Myc IP; lanes 1-4) or polyclonal anti-β2m antibodies (α-β2m IP; lanes 5-8). Precipitated proteins were immunoblotted with polyclonal anti-Myc antibodies (α-Myc; top panel) and polyclonal anti-β2m antibodies (α-β2m; bottom panel). The data in Fig. 1 is representative of at least three independent experiments carried out using two different cell clones.

α-chain in FO-1β2m cells, cell lysates were immunoprecipitated with anti-Myc antibodies and immunoprecipitates blotted with anti-β_2m antibodies (Fig. 1C, lanes 1-4). Alternatively, β_2m immunoprecipitates were blotted with anti-Myc antibodies to detect the FcRn α-chain (Fig. 1C, lanes 5-8). β_2m was specifically co-precipitated with the α-chain from cells expressing both proteins (Fig. 1C, lane 4) but not from control FO-1 cells (Fig. 1C, lane 1) or from cells expressing the α-chain alone (Fig. 1C, lane 2) or β_2m alone (Fig. 1C, lane 3). Similarly, FcRn was co-precipitated with β_2m only from FO-1β2m cells expressing the receptor α-chain (Fig. 1C, lane 8).

Thus, FO-1 cells expressing the FcRn α-chain either alone or in combination with β_2m were obtained and, in cells co-expressing the two proteins, the α-chain and β_2m assembled with each other.

β_2m is important for efficient pH-dependent binding of IgG by FcRn

We next determined whether FO-1 and FO-1β2m cells expressing the FcRn α-chain were able to bind and internalize IgG. Cells were allowed to endocytose hIgG (1 μg/ml) at 37°C, either at pH 6.5 or pH 7.4, and internalized IgG was detected with a labeled secondary antibody. As shown in Fig. 2A, FO-1β2m cells expressing the FcRn α-chain internalized IgG at pH 6.5 (Fig. 2Ad) but not at pH 7.4 (Fig. 2Ah), consistent with the known pH-dependence of ligand binding by FcRn. FO-1 cells expressing the α-chain alone failed to internalize hIgG at either pH (Fig. 2Ac,g). Similarly, control FO-1 and FO-1β2m cells not expressing the α-chain did not internalize IgG (Fig. 2Aa,b,e,f), showing that, where detected (Fig. 2A,d), internalization was receptor mediated and not due to fluid phase endocytosis.

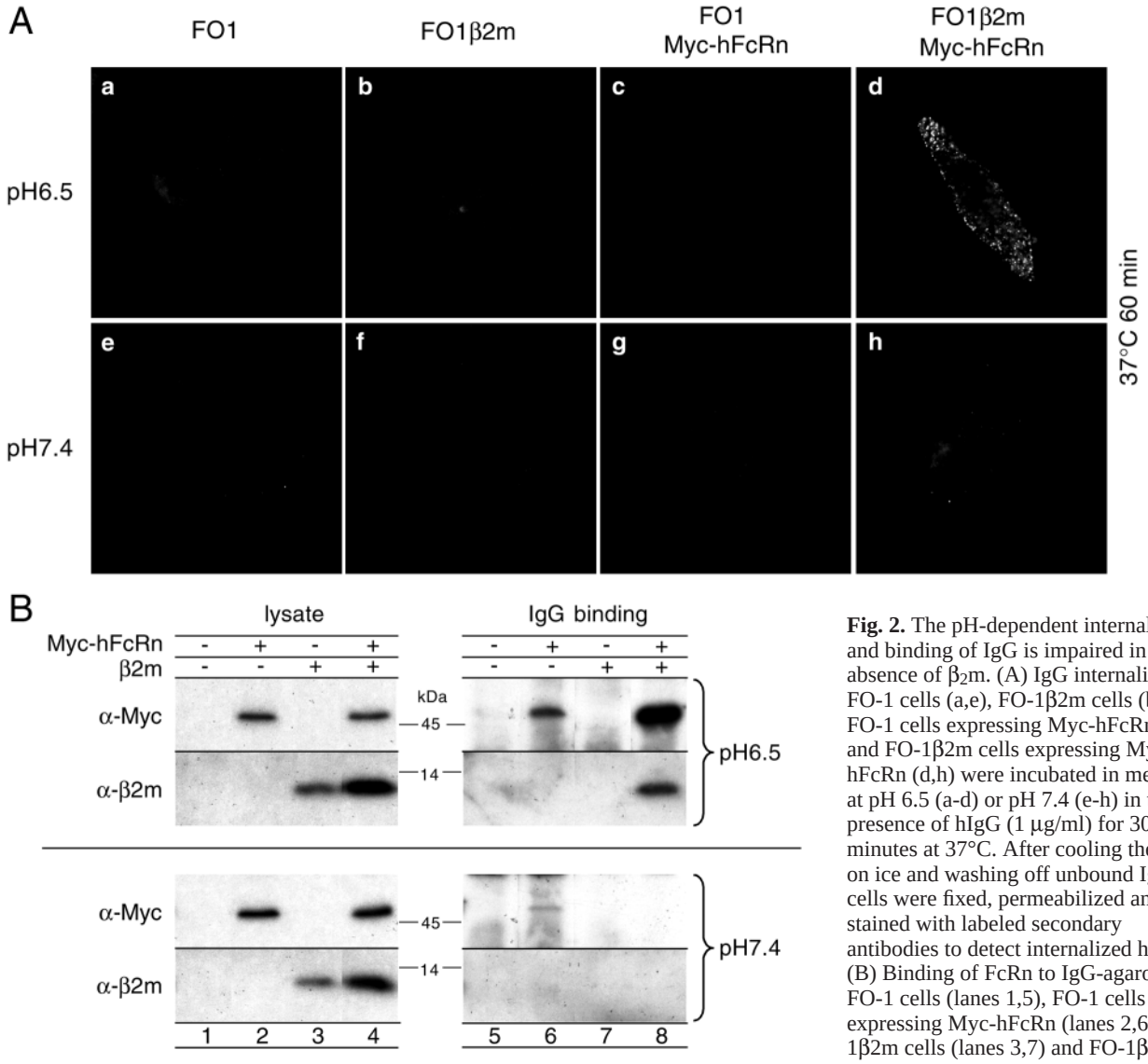

Fig. 2. The pH-dependent internalization and binding of IgG is impaired in the absence of β_2m. (A) IgG internalization. FO-1 cells (a,e), FO-1β2m cells (b,f), FO-1 cells expressing Myc-hFcRn (c,g), and FO-1β2m cells expressing Myc-hFcRn (d,h) were incubated in medium at pH 6.5 (a-d) or pH 7.4 (e-h) in the presence of hIgG (1 μg/ml) for 30 minutes at 37°C. After cooling the cells on ice and washing off unbound IgG, cells were fixed, permeabilized and stained with labeled secondary antibodies to detect internalized hIgG. (B) Binding of FcRn to IgG-agarose. FO-1 cells (lanes 1,5), FO-1 cells expressing Myc-hFcRn (lanes 2,6), FO-1β2m cells (lanes 3,7) and FO-1β2m cells expressing Myc-hFcRn (lanes 4,8) were lyzed in CHAPS buffer at pH 6.5 (top panels; pH 6.5) or pH 7.4 (bottom panels; pH 7.4). Cell lysates were blotted using monoclonal anti-Myc (α-Myc) or polyclonal anti-β_2m (α-β2m) antibodies (lanes 1-4; lysate). Alternatively, cell lysates were incubated with IgG-agarose and bound proteins then eluted and blotted (lanes 5-8; IgG binding). The data in Fig. 2 is representative for at least three independent experiments carried out using two different cell clones.

Similar results were obtained with 10 μg/ml IgG (data not shown).

The above results indicate that in the absence of β_2m, FcRn does not bind IgG or, alternatively, it is not expressed on the cell surface. To test whether β_2m was required for IgG binding, we analyzed whether FcRn present in cell lysates of transfected FO-1 or FO-1β2m cells was able to bind to IgG agarose. As shown in Fig. 2B, FcRn from FO-1β2m cells efficiently bound to IgG agarose at pH 6.5 (Fig. 2B, top panel, lane 8, α-Myc) but no binding was detected at pH 7.4 (Fig. 2B, bottom panel, lane 8, α-Myc). β_2m was present in the bound fraction (Fig. 2B, top panel, lane 8, α-β_2m), consistent with the binding of an α-chain-β_2m heterodimer to the IgG-agarose. In contrast, binding of the α-chain from lysates of FO-1 cells lacking β_2m, although still detectable, was reduced at pH 6.5 (Fig. 2B, top panel, lane 6, α-Myc). Interestingly, while FcRn in FO-1β2m lysates failed to bind to IgG agarose at pH 7.4, binding of the α-chain was reproducibly observed in the absence of β_2m (Fig. 2B, lower panel, lane 6, α-Myc). Immunoblots of aliquots of the FO-1 and FO-1β2m cell lysates used for the binding experiments confirmed the presence of similar amounts of the FcRn α-chain, ruling out the possibility that differences in binding were due to different expression levels of the α-chain (lanes 1-4).

In conclusion, binding of IgG to FcRn was significantly reduced in the absence of β_2m. However, since IgG binding was not completely abolished in the absence of β_2m, the reduced ligand binding alone is unlikely to account for the lack of IgG internalization in FO-1 cells.

β_2m is important for surface expression of FcRn

We next analyzed the subcellular distribution of the FcRn α-chain in FO-1 and FO-1β2m cells by indirect immunofluorescence to determine whether β_2m was required for cell surface expression of FcRn. Cells were fixed, permeabilized and labeled with anti-Myc antibodies to visualize the steady state distribution of the FcRn α-chain (Fig. 3a-d). As expected, the α-chain was detected only in transfected FO-1 or FO-1β2m cells (Fig. 3c,d) but not in untransfected control cells (Fig. 3a,b). In FO-1β2m cells, the FcRn α-chain showed a discrete punctate distribution (Fig. 3d), whereas the labeling in parental FO-1 cells was rather diffuse and reticular (Fig. 3c). To determine whether the FcRn α-chain

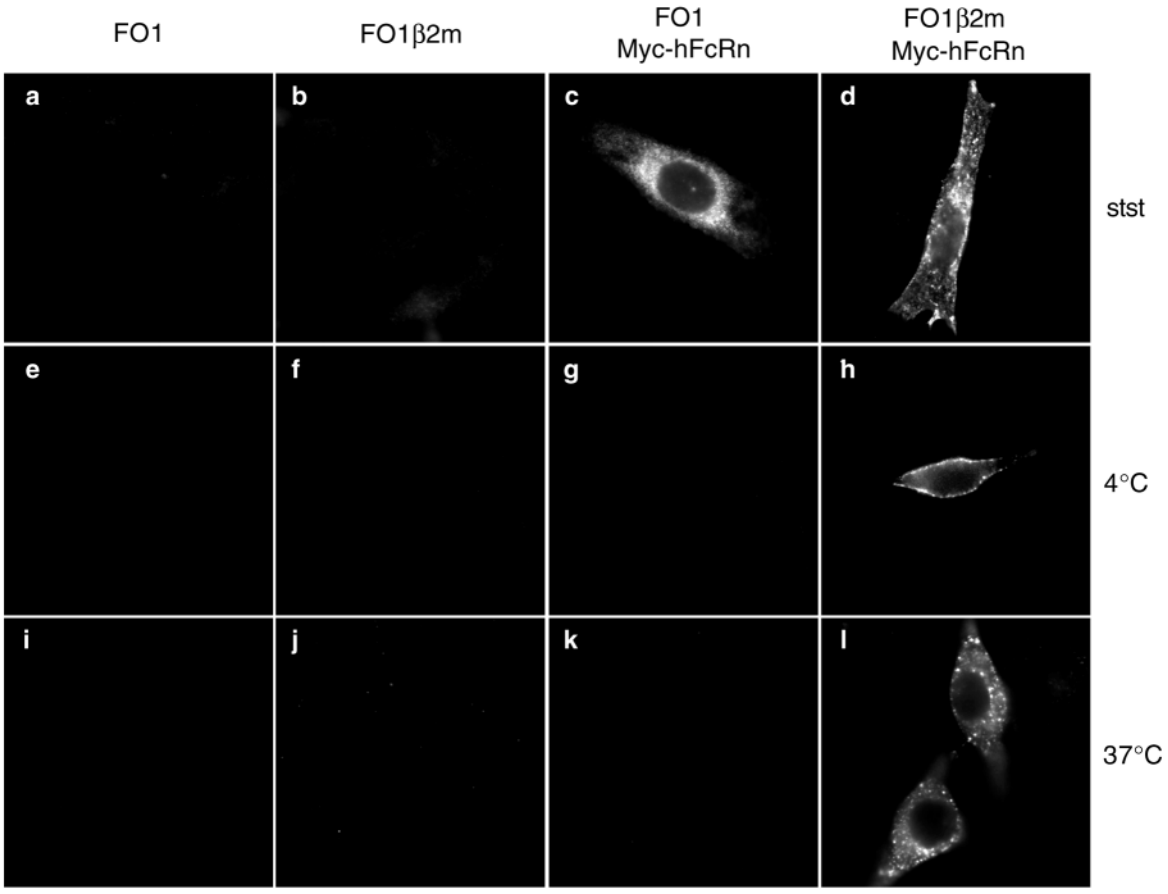

Fig. 3. Subcellular distribution and internalization of FcRn. FO-1 cells (a,e,i), FO-1β2m cells (b,f,j), FO-1 cells expressing Myc-hFcRn (c,g,k) and FO-1β2m cells expressing Myc-hFcRn (d,h,l) were fixed, permeabilized and stained with anti-myc antibodies to visualize the subcellular distribution of Myc-hFcRn (a-d). Alternatively, cells were incubated with anti-Myc antibodies at 4°C, fixed and surface-bound anti-Myc was detected with labeled secondary antibodies to visualize Myc-hFcRn present on the cell surface (e-h). In panels i-l, cells were allowed to internalize anti-Myc antibodies for 60 minutes at 37°C, washed with acid to remove non-internalized antibodies bound to the cell surface and then fixed, permeabilized and stained with labeled secondary antibodies to detect anti-Myc antibodies that had been internalized. Panels show representative data of one of two clones analyzed.

was expressed on the cell surface, anti-Myc antibodies were allowed to bind to live cells on ice and visualized by indirect immunoflurescence (Fig. 3e-h). Surface staining for the FcRn α-chain was observed only in transfected FO-1β2m cells (Fig. 3h) but not in FO-1 cells lacking β_2m (Fig. 3g) or in untransfected FO-1 or FO-1β2m cells (Fig. 3e,f).

Since the FcRn cycles between the plasma membrane and endosomes (Praetor et al., 1999), it is conceivable that the FcRn α-chain shows a predominant intracellular equilibrium distribution in FO-1 cells but nevertheless transiently appears on the cell surface. We therefore incubated cells in the presence of anti-Myc antibodies in culture media (pH 7.4) at 37°C for 60 minutes, a sensitive assay to measure the transient surface appearance of a membrane protein since proteins cycling through the plasma membrane will bind and internalize antibodies in the media (Höning and Hunziker, 1995). As shown in Fig. 3, FO-1β2m cells expressing the FcRn α-chain efficiently accumulated anti-Myc antibodies in endocytic vesicles (Fig. 3l) but no antibody uptake was observed in FO-1 cells expressing hFcR alone (Fig. 3k). Antibody uptake in FO-1β2m cells was receptor mediated since control cells showed no internalization (Fig. 3i,j). This data therefore indicates that the FcRn α-chain is not delivered to the cell surface of β_2m-deficient cells and is in agreement with the lack of IgG internalization in these cells (Fig. 2A).

To confirm biochemically that the FcRn α-chain was absent from the surface of FO-1 cells, we carried out surface biotinylation experiments. Following modification of cell surface proteins with a membrane-impermeable biotinylation reagent, cells were lysed, FcRn was immunoprecipitated with anti-Myc antibodies and precipitates were blotted with streptavidin-HRP. Alternatively, biotinylated surface receptors were first precipitated with streptavidin-agarose and blots probed with anti-Myc antibodies. As shown in Fig. 4, biotinylated surface FcRn was readily detected in FO-1β2m cells (Fig. 4, lane 6, SA-HRP and α-Myc) but was absent or strongly reduced in FO-1 cells (Fig. 4, lane 4, SA-HRP and α-Myc). Immunoblotting of cell lysates (Fig. 4, lanes 1,2) confirmed that FO-1 cells expressed similar or larger amounts of the FcRn α-chain than FO-1β2m cells, showing that the absence of the α-chain on the cell surface in these cells was not due to lower expression levels.

Thus, cell surface expression of the FcRn α-chain is greatly reduced in the absence of β_2m.

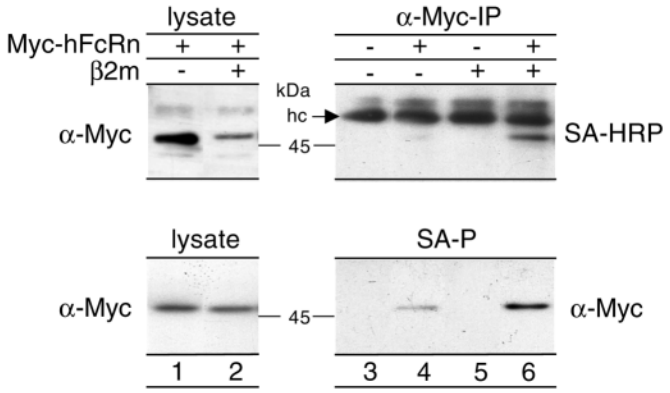

Fig. 4. Detection of cell surface FcRn. The cell surface of FO-1 cells (lane 3), FO-1β2m cells (lane 5), FO-1 cells expressing Myc-hFcRn (lanes 1,4) and FO-1β2m cells expressing Myc-hFcRn (lanes 2,6) were biotinylated on ice with a membrane-impermeable reagent. Cells were lysed and an aliquot of the lysates directly analyzed by immunoblotting (lysate; lanes 1,2) to detect the presence of Myc-hFcRn (α-Myc). The remaining lysate was precipitated with anti-Myc antibodies (α-Myc IP) and biotinylated Myc-hFcRn present in the precipitates detected by blotting with streptavidin-HRP (SA-HRP, lanes 3-6, top panel). Alternatively, biotinylated proteins were first precipitated with streptavidin-agarose (SA-P) and precipitates immunoblotted to detect Myc-hFcRn (α-Myc, lower panel). hc, heavy chain of the antibody used for immunoprecipitation. The data shown is representative of at least three independent experiments, each carried out using two different cell clones.

β_2m is important for FcRn to exit the endoplasmic reticulum

Since the FcRn α-chain was not detected on the surface of cells lacking β_2m, we next determined whether the α-chain is retained in an intracellular compartment in these cells by analyzing whether the FcRn α-chain co-localizes with markers for early endosomes (i.e. EEA1, Fig. 5 a-f), lysosomes (i.e. lamp-1, Fig. 5g-l) and the ER (i.e. ribophorin II, Fig. 5m-r). Antibodies to the endosomal marker EEA1 labeled a vesicular compartment in FO-1 and FO-1β2m cells. As observed above (Fig. 3), the α-chain was present in a vesicular compartment in FO-1β2m cells but showed a more diffuse reticular labeling in FO-1 cells. While the α-chain (red) showed extensive but incomplete co-localization with EEA1 (green) in FO-1β2m cells, no co-localization was observed in FO-1 cells. Thus, a fraction of FcRn was present in early endosomes in FO-1β2m but not in FO-1 cells. The FcRn α-chain (red) did not co-localize with the lysosomal marker lamp-1 (green) in either FO-1 or FO-1β2m cells (Fig. 5g-l), consistent with previous results obtained in MDCK cells (Praetor et al., 1999).

To determine whether the reticular staining for the FcRn α-chain observed in FO-1 cells corresponds to the ER, we also determined whether the FcRn α-chain co-localizes with ribophorin II (m-r). A diffuse reticular staining characterisitic for the ER was obtained with anti-ribophorin II antibodies. Merging the staining for the FcRn α-chain (red) with that of ribophorin II (green), showed extensive co-localization in FO-1 but not in FO-1β2m cells. The FcRn α-chain in FO-1 cells also co-localized with a second ER marker, protein disulfide isomerase (data not show). Thus, in β_2m-deficient cells the FcRn α-chain was retained in the ER.

To confirm biochemically that the FcRn α-chain did not exit the ER in cells lacking β_2m, we analyzed whether the single N-linked carbohydrate in hFcRn (Israel et al., 1997) remained EndoH sensitive in FO-1 cells. Cell lysates were incubated with buffer, EndoH or, to cleave all N-linked carbohydrates, glycosidase F, and then immunoblotted for detection of the FcRn α-chain or β_2m. As shown in Fig. 6, EndoH (lane 4) and glycosidase F (lane 6) treatment resulted in a reduction of the apparent molecular mass of the FcRn α-chain in lysates from FO-1 cells (lanes 1-6, α-Myc), consistent with the cleavage of an N-linked carbohydrate. In FO-1β2m cells (Fig. 6, lanes 7-12, α-Myc), however, while sensitive to glycosidase F treatment (Fig. 6, lane 12), the FcRn α-chain was resistant to digestion by EndoH (Fig. 6, lane 10). Incubation of lysates with buffer alone (Fig. 6, lanes 3,5,9,11) had no effect, showing that the change in α-chain mobility was due to deglycosylation. As

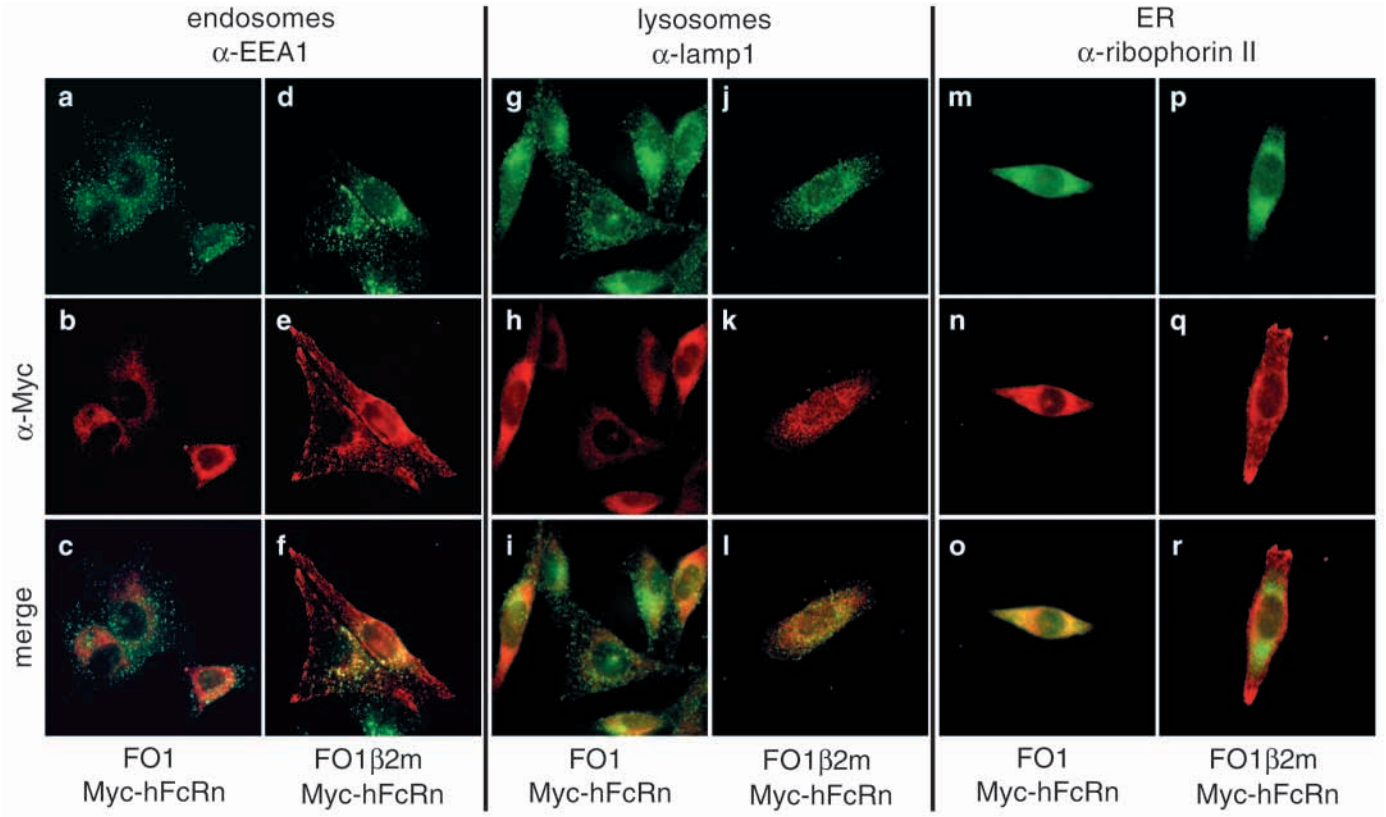

Fig. 5. FcRn colocalizes with an ER marker in the absence of β_2m. FO-1 (a-c,g-i,m-o) or FO-1β2m expressing Myc-hFcRn (panels d-f,j-l,p-r) were fixed, permeabilized and stained with monoclonal anti-EEA1 (a,d), anti-lamp1 (g,j) or anti-ribophorin II (m,p) antibodies to label endosomes, lysosomes or the ER, respectively, and polyclonal anti-Myc antibodies (b,e,h,k,n,q) to detect Myc-hFcRn. In c,f,i,l,o,r, the staining for endosomes, lysosomes or ER (green) was merged with that for Myc-hFcRn (red). The panels show representative data for one of two clones analyzed.

expected from the lack of N-linked carbohydrates, the mobility of β_2m was unaffected by EndoH or glycosidase F treatment (Fig. 6, lanes 7-12, α-β_2m).

In conclusion, morphological and biochemical data show that the FcRn α-chain is retained in the ER in β_2m-deficient cells.

Discussion

A requirement for β_2m for a functional FcRn has been inferred from studies in β_2m-knockout mice in which several of the functions thought to be associated with the receptor, including pre- and postnatal IgG transfer (Israel et al., 1995) and IgG homeostasis (Ghetie et al., 1996; Israel et al., 1996; Junghans and Anderson, 1996), are impaired. Despite the apparent relevance of β_2m, its precise role for FcRn function has not been established. In the case of the homologous MHC class I molecules, β_2m is required but not sufficient for the α-chain to exit the ER and a similar role may seem plausible for FcRn due to its homology to MHC class I. However, the experimental data from β_2m-deficient mice, could also reflect a requirement of β_2m for IgG binding. Furthermore, the less efficient IgG transport in β_2m-null mice may also result from an increased turnover rate or an altered intracellular trafficking of the α-chain in the absence of β_2m. For example, since only a minor fraction of FcRn is on the cell

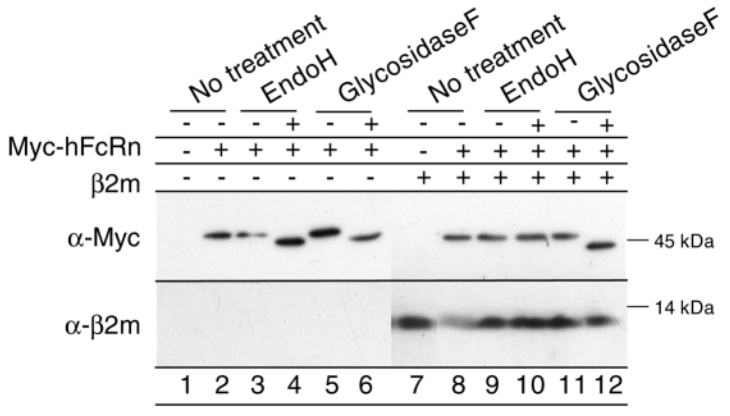

Fig. 6. FcRn remains EndoH sensitive in the absence of β_2m. Lysates of FO-1 cells (lane 1), FO-1β2m cells (lane 7), FO-1 cells expressing Myc-hFcRn (lanes 2-6) and FO-1β2m cells expressing Myc-hFcRn (lanes 8-12) were either directly blotted (lanes 1,2,7,8) or first incubated with enzyme buffer alone (lanes 3,5,9,11) or with EndoH (lanes 4,10) or glycosidase F (lanes 6,12) for 3.5 hours at 37°C. Blots were probed with monoclonal anti-Myc (α-Myc; top panel) or polyclonal anti-β_2m (α-β2m; bottom panel) antibodies. The data is representative for three independent experiments, each carried out with two different cell clones.

surface at equlibrium (Berryman and Rodewald, 1995; Kristoffersen and Matre, 1996; Roberts et al., 1990), a small change in the steady-state distribution in the absence of β_2m may result in a significant decrease in the amount of FcRn present on the cell surface.

To establish experimentally the precise role of β_2m in FcRn function, we expressed the α-chain in β_2m-deficient FO-1 cells, either alone or in combination with β_2m. Although the FcRn α-chain could be expressed in cells lacking β_2m, it failed to efficiently appear on the cell surface. Based on its co-localization with ER markers and the retention of EndoH-sensitive N-linked carbohydrates in FO-1 cells, the α-chain was not exported from the ER in β_2m-deficient cells. Thus, as for MHC class I antigens, β_2m is important to satisfy ER quality control that allows FcRn to exit the ER.

In the case of MHC class I antigens, folding and assembly is a multi-step process and involves several chaperones (Pamer and Cresswell, 1998). Assembly with β_2m is essential but not sufficient for surface transport of MHC class I, which also requires binding of the antigenic peptide. In contrast, H-2 class I alleles may differ from HLA alleles in this respect since they can be transported to the cell surface in the absence of bound peptide (Pamer and Cresswell, 1998). Since FcRn does not bind peptide, it may rather resemble H-2 molecules in that binding of β_2m may be sufficient to conform to ER quality control. However, an additional level of ER quality control for FcRn may involve the assembly of FcRn homodimers from α-chain-β_2m heterodimers (A.P. and W.H., unpublished). Since FcRn dimerization does not require ligand (A.P. and W.H., unpublished), dimerization is likely to occur in the ER and to be integral to the assembly of a transport competent receptor.

In addition to the importance of β_2m for exit of the α-chain from the ER, β_2m was also required for efficient pH-dependent binding of IgG by FcRn. In the absence of β_2m, binding of the α-chain to IgG-agarose was significantly reduced at pH 6.5. The co-crystal structure of FcRn and Fc shows contact points between the Fc domain of IgG and the N-terminal Ile of β_2m (Burmeister et al., 1994; Martin et al., 2001) and in vitro mutagenesis studies corroborate a role for Ile(1) in IgG binding (Vaughn et al., 1997). Ile(1), widely conserved in β_2m from different species (e.g. human, mouse and rat), may mediate a hydrophobic interaction with IgG near residues 309-311 on the Fc domain (Vaughn et al., 1997). Alternatively, Ile(1) may play an indirect role in IgG binding since the presumably protonated α-NH_2 [pK_a ~8 (Fersht, 1985)] is positioned to form a hydrogen bond with the backbone carbonyl group of residue 115 in the α-chain as well as a pH-dependent salt bridge with Glu(117) in the heavy chain (Vaughn et al., 1997). Thus, the protonated α-NH_2 could help to align Glu(117) on the α-chain to form an anionic binding site for His(310) on Fc (Vaughn et al., 1997).

Although the critical role of β_2m in FcRn function was established in non-polarized FO-1 melanoma cells, β_2m is probably equally important for FcRn surface expression and ligand binding in other cell types in which the receptor plays a physiological role in IgG transport. The importance of β_2m for FcRn function suggests that expression of a transport competent receptor could be regulated indirectly via β_2m expression levels. Little is known concerning the developmental regulation of FcRn and β_2m expression in different organs and tissues. In neonatal rats, α-chain mRNA is present in the intestine at birth and declines within 10 days (Simister and Mostov, 1989), indicating that FcRn expression is regulated at the level of α-chain transcription or mRNA stability. However, in mammary glands of possum and bovine, FcRn α-chain mRNA levels remain constant throughout lactation, but expression of β_2m mRNA increases at the time of active IgG transfer into milk (Adamski et al., 2000). Thus, depending on the tissue, species or developmental stage, expression of functional FcRn may be controlled by the mRNA levels for either the α-chain or β_2m. Furthermore, since most cells express MHC class I antigens and newly synthesized MHC class I and FcRn are likely to compete for the available β_2m in the ER, the expression of the MHC class I and FcRn α-chains and β_2m must be coordinately regulated.

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