

# Calnexin and ERp57 Facilitate the Assembly of the Neonatal Fc Receptor for IgG with $\beta_2$ -Microglobulin in the Endoplasmic Reticulum<sup>1</sup>

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The neonatal FcR (FcRn) consists of an MHC class I-like H chain in noncovalent association with  $\beta_2$ -microglobulin ( $\beta_2$ m). The proper folding of FcRn in the endoplasmic reticulum is essential for FcRn function. Using a low stringency immunoprecipitation of human FcRn, we observed the coprecipitation of an 88-kDa band. Mass spectrometry analysis revealed that this band was identical with calnexin (CNX). This association was verified by Western blotting the CNX or FcRn immunoprecipitates with either an anti-FcRn or anti-CN X Ab. In the  $\beta_2$ m-null FO-1 cell transfected with FcRn H chain alone or both FcRn H chain and  $\beta_2$ m, CNX bound to the FcRn H chain before the FcRn H chain association with  $\beta_2$ m. However, calreticulin only bound to the FcRn H chain- $\beta_2$ m complex. Furthermore, the thiol oxidoreductase ERp57 was detected in FcRn-CN X complexes, suggesting its role in disulfide bond formation of the FcRn H chain. Removal of the N-linked glycosylation site from the FcRn H chain resulted in a decreased association of the FcRn H chain for  $\beta_2$ m. However, the absence of CNX did not significantly affect FcRn assembly as defined by the ability of FcRn to bind IgG and exit to the cell surface. This suggests that other chaperones compensate for the function of CNX in FcRn assembly. In addition, we found that tapasin and TAP were not involved in FcRn assembly, as shown by coimmunoprecipitation in THP-1 cells and IgG-binding assays in 721.220 (tapasin-deficient) and 721.174 (TAP-deficient) cells transfected with FcRn. These findings show the importance of chaperones in FcRn assembly. *The Journal of Immunology*, 2005, 175: 967–976.

The neonatal FcR (FcRn)<sup>3</sup> plays an important role in the passive acquisition of maternal IgG by the transport of circulating IgG across the placenta to the fetus in humans or from ingested milk in the intestine to the bloodstream of newborn rodents (1). In addition to its role in establishing neonatal immunity, FcRn maintains IgG homeostasis in adult life by protecting IgG from degradation (2). The interaction between FcRn and IgG exhibits a remarkable pH dependence, i.e., binding IgG at acidic pH (6–6.5) and releasing IgG at neutral pH (7–7.4) (3). FcRn is composed of an H chain (45 kDa in humans and 50 kDa in rodents) that is noncovalently attached to an L chain  $\beta_2$ -microglobulin ( $\beta_2$ m) (12 kDa). The FcRn H chain is composed of  $\alpha_1$ ,

$\alpha_2$ , and  $\alpha_3$  external domains that are anchored to the cell surface by a short transmembrane domain and a cytoplasmic tail (4, 5). The human FcRn H chain contains one N-linked glycosylation site (6) in comparison with the rodent FcRn H chain that possesses four sites (7).

The functions of FcRn in vivo have been examined in  $\beta_2$ m-deficient mice in several studies, including IgG transport across the intestinal epithelium of suckling mice, IgG catabolism, and hypergammaglobulinemia or autoimmunity mediated by autoantibodies. These studies show that transport of IgG from mother to newborns is impeded (8) and catabolism of IgG is increased in  $\beta_2$ m<sup>-/-</sup> mice compared with wide-type mice (9–11). The transfection of FcRn cDNA into a  $\beta_2$ m-deficient cell line shows that the association of  $\beta_2$ m with the human FcRn H chain is critical for FcRn egress from the endoplasmic reticulum (ER) and proper IgG binding at acidic pH (12, 13). Overall, these studies demonstrate the proper folding of the FcRn H chain with  $\beta_2$ m is critical for the functions and biogenesis of FcRn. However, it remains unclear how the FcRn H chain assembles with  $\beta_2$ m molecule or what types of chaperones are involved in such an assembly in the ER.

FcRn is the only IgG FcR with a structure similar to that of MHC class I or its related molecules, i.e., HLA-A, -B, -C, -F, -G, -H, MR1, and CD1. MHC class I molecules are polymorphic molecules that are found on the surface of nearly every mammalian cell and serve as the ligand for CD8<sup>+</sup> T cells. Human mature MHC class I molecules consist of three subunits: an H chain,  $\beta_2$ m, and an antigenic peptide. Assembly of such a ternary complex occurs in the ER. In the absence of peptides, the heterodimers of human MHC class I H chain- $\beta_2$ m are retained in the ER and are unstable (14). In order for MHC class I ternary complexes to fold correctly, a set of chaperones is sequentially involved in different stages of folding. Together, calnexin (CNX), calreticulin (CRT), ERp57,

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<sup>3</sup> Abbreviations used in this paper: FcRn, neonatal FcR;  $\beta_2$ m,  $\beta_2$ -microglobulin; CNX, calnexin; CRT, calreticulin; DSP, dithiobis(succinimidyl propionate); endo-H, endo-N-acetylglucosaminidase; ER, endoplasmic reticulum; UGGGT, UDP-glucose: glycoprotein glucosyltransferase.

TAP, and tapasin (TAP-associated glycoprotein) have been described as the MHC class I peptide-loading complexes (14, 15). Polymorphic differences in MHC class I H chain can result in quantitative as well as qualitative differences in how they interact with the ER chaperone proteins. Either individually or in combination, CNX, CRT, and ERp57 have been suggested to perform the general chaperone functions of promoting folding and assembly of MHC class I H chain- $\beta_2m$  complexes, whereas tapasin and TAP molecules are important for loading antigenic peptides. Therefore, these chaperones are essential for assembly of the ternary complex of MHC class I H chain- $\beta_2m$ -peptide as well as for efficient transport of functional and stable class I molecules to the cell surface.

Unlike MHC class I, FcRn is nonpolymorphic and lacks functional Ag-presenting capabilities because its putative peptide-binding groove is nearly closed (5, 16). Furthermore, the interacting site between IgG and FcRn is located outside the narrowed peptide-binding groove (1). The absence of a suitable Ag-binding groove in FcRn suggests that the FcRn H chain- $\beta_2m$  complex does not need to load antigenic peptides to exit the ER. Given the similarity in sharing an H chain- $\beta_2m$  complex and the lack of an Ag-binding groove, the folding and assembly of FcRn H chain- $\beta_2m$  may be distinct from that of MHC class I. Therefore, it is informative to compare the chaperone requirements for FcRn export with that of other MHC class I-related molecules. To better understand this folding process of FcRn, we sought to define the features of FcRn H chain- $\beta_2m$  assembly by identifying the specific intermediate chaperone proteins that interact with the FcRn H chain or the FcRn- $\beta_2m$  complex, and the role of these proteins during assembly of FcRn H chain and  $\beta_2m$ . Using low stringency immunoprecipitation and mass spectrometry analysis, we find that CNX and ERp57 associate with the FcRn H chain before it associates noncovalently with  $\beta_2m$ . However, CRT could only interact with an FcRn H chain- $\beta_2m$  complex. In addition, tapasin and TAP are most likely not required for the assembly of FcRn H chain and  $\beta_2m$  in the ER, consistent with the absence of a peptide-binding groove in FcRn. These studies suggest that FcRn assembly in the ER may be much simpler than that of MHC class I.

## Materials and Methods

### Cell lines and Abs

HeLa, HT-29, and T84 cells were purchased from American Type Culture Collection and maintained in DMEM complete medium supplemented with 10 mM HEPES, 10% FCS (Sigma-Aldrich), 1% L-glutamine, nonessential amino acids, and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> incubator at 37°C. FO-1 (melanoma cell line, kindly provided by S. Ferrone, New York Medical College, Valhalla, NY), CEM (human T-lymphoblastoid leukemia cell line), and CEM-NKR (an NK-resistant variant of CEM, gifts from H. Ploegh, Harvard Medical School, Boston, MA), 721.221 (human B lymphoblastoid cell line), 721.220 (a mutant variant of 721.221), 721.174 (mutant human B lymphoblastoid cell line), and Daudi (a human Burkitt lymphoma) were grown in RPMI 1640 complete medium (Invitrogen Life Technologies). The 721.174, 721.220, and 721.221 cells are gifts from J. Yewdell (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and L. Van Kaer (Vanderbilt University School of Medicine, Nashville, TN).

Human  $\beta_2m$  was purchased from Calbiochem-Novabiochem. Mouse mAb IgG1 anti-ERp57 was purchased from StressGen Biotechnologies. Goat anti-CN<sub>X</sub>, rabbit anti-CRT, and goat anti-mouse  $\beta_2m$  Abs were purchased from Santa Cruz Biotechnology. Affinity-purified rabbit anti-human  $\beta_2m$  was purchased from Boehringer Mannheim or Sigma-Aldrich; rabbit anti-FLAG epitope (DYKDDDDK, a single letter for amino acid) and mAb anti-FLAG M2 were purchased from Sigma-Aldrich. HRP-conjugated rabbit anti-mouse or donkey anti-rabbit Ab were purchased from Pierce. Dithiobis (succinimidylpropionate) (DSP) was purchased from Pierce, and castanospermine was purchased from Calbiochem and was used at 100  $\mu$ g/ml. A mouse mAb HC10 is from H. Ploegh, and mAb anti-ERp57 is from P. Cresswell (Yale University, New Haven, CT).

### Construction of plasmid and site-directed mutagenesis of human FcRn H chain

The constructions of the human  $\beta_2m$  and FcRn expression plasmids, pCDNA $\beta_2m$  and pFLAGCMVhFcRn, have been described previously (17). The plasmid pCDNAFLAGFcRn was constructed by subcloning Nhe-XbaI fragments from pFLAGCMVhFcRn into pCDNA3. The pCDNAFLAGFcRn construct fused a preprotrypsin signal sequence and FLAG epitope into the N terminus of the human FcRn gene (aa 1–342) (6).

The N-glycosylation mutant of human FcRn was generated by replacing the consensus glycosylation sequence, Asn-X-(Ser/Thr) using transformer site-directed mutagenesis kit (BD Clontech). The human FcRn cDNA in the pCDNAFLAG expression vector (Invitrogen Life Technologies) was used as template. The oligonucleotide 5'-GGGCCCTGACAGCACCTCG GTGCC-3' was used for change of asparagine 102 (17) to a serine residue (base substitutions are underlined). The mutant FcRn cDNAs were sequenced to verify fidelity of the mutations. The resultant plasmids were designed for pCDNAFLAGFcRnN/S.

### Transfection and protein expression

Stable cell lines, HeLa<sup>FcRn+ $\beta_2m$</sup> , FO-1<sup>mock</sup>, FO-1<sup>FcRn</sup>, and FO-1<sup>FcRn+ $\beta_2m$</sup> , have been described previously (12, 17). FO-1 cells were transfected with pFLAGCMVFcRnN/S and/or pCDNA $\beta_2m$  with Effectene transfection reagent (Qiagen), according to instructions from the manufacturer. The stable cell lines were designated for FO-1<sup>FcRnN/S+ $\beta_2m$</sup>  or FO-1<sup>FcRnN/S</sup>. Positive transfectants were tested for protein expression through Western blot using anti-FLAG or FcRn  $\alpha_2$  domain-specific Abs. Transfectants were maintained in medium containing G418 and blasticidin at a concentration of 0.5–1 mg/ml and 3  $\mu$ g/ml, respectively. Stable transfectants were selected by either G418 or G418 plus blasticidin treatment for single and double transfectants, respectively.

### In-gel digestion and mass spectrometry

HeLa<sup>FcRn</sup> lysates were immunoprecipitated by M2 FLAG Ab. The immunoprecipitates were separated by SDS-PAGE under reducing conditions and stained with Coomassie blue. Protein bands were excised and subjected to in-gel tryptic digestion. Extracted peptides were analyzed using microcapillary reverse-phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) at the Taplin Biological Mass Spectrometry Facility (Emory University School of Medicine). MS/MS spectra were searched against the *nrr.fasta* and *human.nci* databases by Sequest software. The proteins listed meet the requirement of having two or more strong scoring peptides matched to it.

### Cross-linking of proteins in cell lysates

Cross-linking was performed in cell lysates, as previously described (18). Cross-linking in cell lysates was performed by including a 2 mM final concentration of DSP in the lysis buffer that consists of PBS containing 0.5% CHAPS. Cells were incubated in the presence of lysis buffer for 30 min on ice, and the excess cross-linker was quenched with 50 mM glycine. An equal amount of DMSO without DSP was added to uncross-linked controls. Lysates were spun at 1500  $\times$  g to obtain a postnuclear supernatant, which was used for immunoprecipitation.

### Immunoprecipitation, gel electrophoresis, and Western blotting

Protein concentrations were determined by the Bradford method. The lysates were resolved on a 4–20% NuPAGE gel (Invitrogen Life Technologies) under reducing or nonreducing condition. Proteins were electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell Microscience). The membranes were blocked with 5% nonfat milk, probed separately with affinity-purified FLAG Ab, anti-FcRn  $\alpha_2$  domain-specific Ab, or rabbit anti- $\beta_2m$  Ab for 1 h, followed by an incubation with HRP-conjugated rabbit anti-mouse or donkey anti-rabbit Abs. All blocking, incubation, and washing were performed in PBST solution (PBS and 0.05% Tween 20). Proteins were visualized by an ECL (Pierce or NEN Life Science Products) method, according to the instructions of the manufacturer. For reprobing, the blots were first incubated in stripping buffer (100 mM 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.

Immunoprecipitations were performed, as described previously (12). Protein was precipitated with an anti-FLAG mAb. The immunoreactive products were eluted from the protein G complex with gel-loading buffer with or without 2-ME at 100°C. For sequential immunoprecipitations, primary immunoprecipitations made in 1% digitonin or 0.5% CHAPS/PBS were washed three times in 0.1% digitonin or 0.1% CHAPS/PBS. The beads were boiled in 100  $\mu$ l of 1% SDS in water. The beads were removed

by centrifugation and the supernatant was reprecipitated with the relevant Ab. The immunoprecipitates were diluted to 1 ml with 1% Triton X-100/immunoprecipitation buffer with/without iodoacetamide (5–10 mM) and divided into aliquots, followed by immunoprecipitation with subunit-specific Abs and washing with 0.1% Triton X-100/immunoprecipitation buffer.

#### Endo-N-acetylglucosaminidase (endo-H) treatment

Endo-H (New England Biolabs) digestions were performed, as described previously (12). For FcRn digestion in immunoprecipitates, beads bound to Ag-Ab complexes were resuspended in 0.5 ml of endo-H digestion buffer (100 mM sodium acetate, pH 5, 150 mM NaCl, 1% Triton X-100, 0.2% SDS, 0.5 mM PMSF). Beads containing Ab-Ag complexes were pelleted and then eluted with reducing sample buffer. For FcRn digestion in cell lysates, the 50- $\mu$ g lysates were diluted in digestion buffer and digested with endo-H or with peptide N-glycosylase F in 50 mM sodium phosphate (pH 7.5) and 1% (v/v) Nonidet P-40. A mock digestion without enzymes was performed for each digestion. All digestions were performed for 18 h at 37°C. Proteins were analyzed on a 12% SDS-PAGE gel under reducing conditions and immunoblotted, as previously described.

#### IgG-binding assay

IgG-binding assays were performed, as previously described (17), with the following modifications. Cells were lysed by shaking in PBS (pH 6.0 or 7.5) with 0.5% CHAPS (Sigma-Aldrich) and protease inhibitor mixture on ice for 1 h. Postnuclear supernatants containing 0.5–1 mg of soluble proteins were incubated with human IgG-Sepharose (Amersham Biosciences) at 4°C overnight. The unbound proteins were removed with PBS (pH 6.0 or 7.5) containing 0.1% CHAPS. The adsorbed proteins were boiled with reducing electrophoresis sample buffer at 100°C for 5 min. The eluted fractions were subjected to 4–12% SDS-PAGE analysis. Proteins were visualized by Western blotting using anti-FLAG and anti- $\beta_2$ m Abs.

#### Flow cytometry analysis

CEM, CEM-NKR, and .174, .220 transfectants ( $5 \times 10^5$  cells/ml) from fresh cultures were washed twice in suspension buffer (1% FBS in calcium-free PBS). The cell suspension was incubated with 10  $\mu$ g of anti-FLAG M2 mAb for 1 h, washed twice with suspension buffer, and stained with a fluorescein-conjugated rabbit anti-mouse Ab. The cells were washed twice with suspension medium before fixation with 0.5% paraformaldehyde in PBS. Control staining with mock transfectants was performed for each analysis. Analysis was performed using a FACSort and CellQuest software (BD Biosciences).

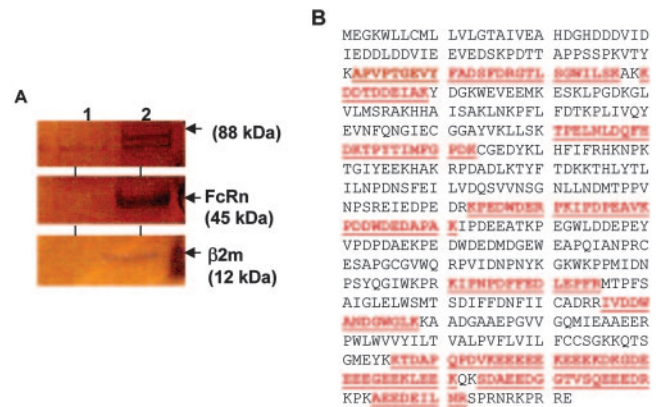
#### RT-PCR

Cells were pelleted and resuspended at  $10^6$  cells/ml in Tri-Reagent (Molecular Research Center). Total RNA was extracted according to the method recommended by the manufacturer. First-strand cDNA was synthesized, and the human tapasin gene was amplified by primers (5'-CGCG GATCCAGGAGGTCGACGCCATGA-3', 5'-CTAGTCTAGATTACTCTGCTTCTCTTT G-3') with a one-step RT-PCR kit (Qiagen). The mRNA was also amplified by GAPDH-specific primers (5'-GAGAAG GCTGGGGCTCAT-3', 5'-TGCTGATGATCTTGAGGCTG-3') as an internal control to monitor the quality of the RNA purification and cDNA synthesis. The PCR fragment was analyzed by agarose gel electrophoresis and stained with ethidium bromide.

## Results

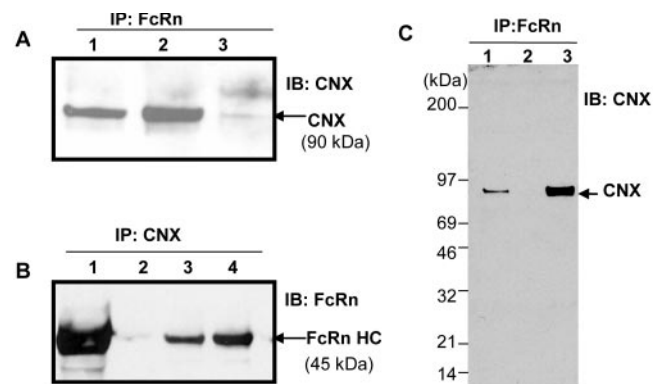
#### Identification of CNX interaction with human FcRn

Proteins that are associated with one another are generally able to be coimmunoprecipitated provided appropriate lysis conditions and Abs are used. To explore the molecules associated with human FcRn, we performed an immunoprecipitation with FLAG Ab from the cell lysates of HeLa<sup>FcRn</sup> or HeLa<sup>mock</sup> cell lines. This approach detected an 88-kDa band in the silver staining of the immunoprecipitates from the lysates of HeLa<sup>FcRn</sup>, but not from that of HeLa<sup>mock</sup> (Fig. 1). The 45- and 12-kDa bands that were coimmunoprecipitated from the HeLa<sup>FcRn</sup> transfectant represent the FcRn H chain and  $\beta_2$ m, respectively (Fig. 1A). To identify the origin of this 88-kDa band, the immunoprecipitate was excised for mass spectrometry analysis. The search of protein databases showed that the peptide sequences matched with the amino acid sequence of human CNX (Fig. 1B). To further confirm the interaction between CNX and FcRn, we next



**FIGURE 1.** Identification of CNX in FcRn immunoprecipitates. *A*, The cell lysates of HeLa<sup>FcRn+ $\beta_2$ m</sup> (lane 2) or HeLa<sup>mock</sup> (lane 1) were immunoprecipitated with an anti-FLAG M2 mAb. Immunoprecipitates were subjected to 4–12% NuPAGE electrophoresis under reducing condition. The proteins were stained by silver staining. An unidentified 88-kDa band, FcRn, and  $\beta_2$ m were labeled, with arrows. Each experiment was at least performed three times. *B*, The 88-kDa band (top) was sequenced by mass-spectrometry analysis. The peptides (underlined) were matched with the CNX sequence.

conducted immune-precipitation-immune-blotting experiments from the cell lysates of FO-1 cells transfected with FcRn cDNA (12). The composition of the immunoprecipitates with either an anti-human FcRn (Fig. 2A) or anti-CN X Ab (Fig. 2B) was blotted with an anti-CN X (Fig. 2A) or anti-FLAG Ab (Fig. 2B). As shown in Fig. 2A, anti-FLAG Ab coimmunoprecipitated CNX (Fig. 2A, lanes 1 and 2). Similarly, the CNX Ab coimmunoprecipitated FcRn H chain (Fig. 2B, lanes 3 and 4). Both experimental approaches failed to coimmunoprecipitate CNX (Fig. 2A, lane 3) and FcRn (Fig. 2B, lane 2) from mock-transfected FO-1 cells. It is possible that the association of FcRn and CNX discovered was the result of FcRn overexpression in



**FIGURE 2.** CNX interacts with FcRn. Cell lysates were immunoprecipitated (IP) with Abs, and immunoprecipitates were subjected to 4–12% NuPAGE electrophoresis under reducing conditions and transferred to a nitrocellulose membrane for Western blotting. Immunoblots (IB) were developed with ECL. Each experiment was at least performed two times. *A*, The cell lysates from FO-1<sup>FcRn</sup> (lane 1), FO-1<sup>FcRn+ $\beta_2$ m</sup> (lane 2), and FO-1 mock (lane 3) were immunoprecipitated by anti-FLAG M2 mAb and blotted with anti-CN X Ab. The cell lysates from FO-1 mock (lane 2), FO-1<sup>FcRn</sup> (lane 3), and FO-1<sup>FcRn+ $\beta_2$ m</sup> (lane 4) were immunoprecipitated by anti-CN X Ab and blotted with FLAG M2 mAb. The cell lysate (lane 1) from FO-1<sup>FcRn+ $\beta_2$ m</sup> was used as a positive control. *C*, THP-1 cell lysate was immunoprecipitated by an FcRn  $\alpha_2$  domain-specific rabbit serum (lane 1) and preimmune serum (lane 2), and blotted with anti-CN X Ab. THP-1 cell lysate (lane 3) was used as a positive control for immune blotting.

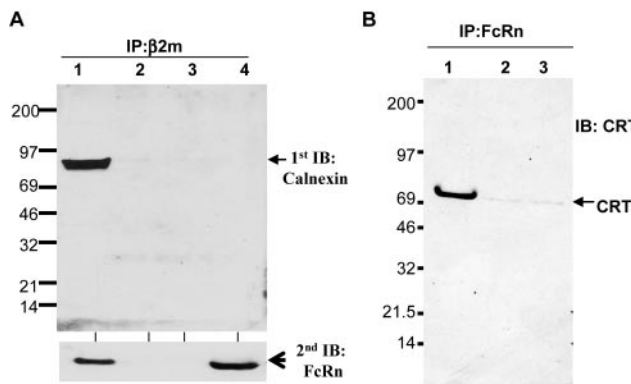
the FO-1 transfectant. To exclude this possibility, the immunoprecipitates from THP-1 cell lysates (17) were blotted with an anti-CN $X$  Ab. The results showed that CN $X$  was coimmunoprecipitated with FcRn by a human FcRn  $\alpha_2$  domain-specific serum Ab (17) (Fig. 2C, lane 1), but not a preimmune serum Ab (Fig. 2C, lane 2). These reciprocal coimmunoprecipitation approaches strongly suggest that FcRn and CN $X$  form a complex.

*CNX interacts with  $\beta_2m$ -free FcRn H chain, but not  $\beta_2m$ -associated FcRn H chain*

The functional FcRn molecule consists of an FcRn H chain and  $\beta_2m$ . FO-1 is a melanoma cell line that lacks  $\beta_2m$  gene transcription and protein synthesis (19). We have previously expressed FcRn alone or both FcRn and  $\beta_2m$  together in FO-1 cells (12). To show whether CN $X$  interacts with FcRn H chain alone or an FcRn- $\beta_2m$  complex, the cell lysates from FO-1<sup>mock</sup>, FO-1<sup>FcRn</sup>, and FO-1<sup>FcRn+ $\beta_2m$</sup>  were immunoprecipitated with a  $\beta_2m$ -specific Ab, BBM1. The resultant immunoprecipitates were sequentially blotted with anti-CN $X$  (Fig. 3A, first blot) and FLAG M2 Ab (Fig. 3A, second blot). The result showed that CN $X$  could not be detected in any BBM1 immunoprecipitates (Fig. 3A, lanes 2–4), although it was detected in the cell lysates (Fig. 3A, lane 1). The BBM1 Ab was, however, able to coimmunoprecipitate the FcRn H chain from the lysates of FO-1<sup>FcRn+ $\beta_2m$</sup>  cells (Fig. 3A, lane 4). In addition, we were able to coimmunoprecipitate the FcRn H chain and CN $X$  from the lysates of FO-1<sup>FcRn</sup> cells (Fig. 2, A, lane 1, and B, lane 3). Therefore, we conclude that CN $X$  interacts preferentially with the FcRn H chain alone.

*CRT interacts with  $\beta_2m$ -associated FcRn, but not FcRn H chain alone*

Because the membrane-bound CN $X$  is structurally and functionally related to soluble CRT (20), we assessed whether CRT can also bind the FcRn H chain. To show whether CRT interacts with FcRn H chain alone or an FcRn- $\beta_2m$  complex, the cell lysates

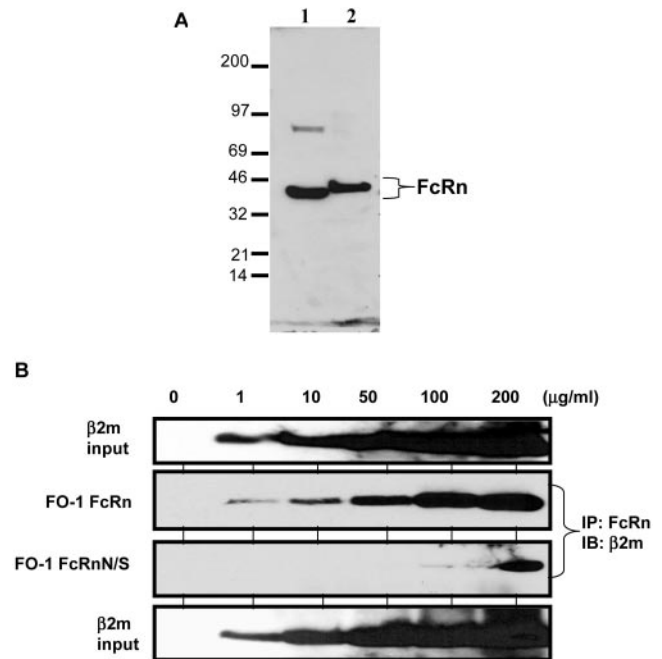


**FIGURE 3.** CN $X$  interacts with FcRn H chain alone, but CRT interacts with FcRn- $\beta_2m$  complex. All immunoprecipitates were subjected to 4–12% NuPAGE electrophoresis under reducing condition. Blots were developed with ECL. The molecular mass markers in kilodaltons are indicated on the left. Each experiment was at least performed two times. A, The cell lysates from FO-1 mock (lane 2), FO-1<sup>FcRn</sup> (lane 3), and FO-1<sup>FcRn+ $\beta_2m$</sup>  (lane 4) were immunoprecipitated by anti- $\beta_2m$  mAb BBM1. The proteins were transferred to a nitrocellulose membrane for Western blotting with anti-CN $X$  (first blot) and anti-FLAG (second blot), respectively. The cell lysate (lane 1) from FO-1<sup>FcRn+ $\beta_2m$</sup>  was used as a positive control for immunoblotting. B, The cell lysates from FO-1<sup>FcRn+ $\beta_2m$</sup>  (lane 1), FO-1<sup>FcRn</sup> (lane 2), and FO-1<sup>mock</sup> (lane 3) were immunoprecipitated by anti-FLAG M2 mAb. The proteins were transferred to a nitrocellulose membrane for Western blotting with an anti-CRT Ab.

from the FO-1<sup>mock</sup>, FO-1<sup>FcRn</sup>, and FO-1<sup>FcRn+ $\beta_2m$</sup>  cell lines were respectively immunoprecipitated with the FLAG M2 mAb. The resultant immunoprecipitates were then blotted with an anti-CRT Ab (Fig. 3B). These studies showed that CRT could not be detected in the FO-1<sup>FcRn</sup> immunoprecipitates (Fig. 3B, lane 2), but was detectable in the immunoprecipitates of the FO-1<sup>FcRn+ $\beta_2m$</sup>  cell line (Fig. 3B, lane 1). These studies indicate that CRT associated with an FcRn- $\beta_2m$  complex, but not the FcRn H chain alone.

*The effect of an N-linked sugar mutant on FcRn H chain and  $\beta_2m$  assembly*

CN $X$  and CRT, as lectins, display a remarkable preference for N-linked oligosaccharides within glycoproteins (20). To test this, an asparagine to serine substitution was introduced into the human FcRn H chain position. This mutation prevents attachment of a core saccharide to asparagine 102 within the  $\alpha_2$  domain, the single site of glycosylation in the human FcRn H chain (6). This mutation allowed us to determine whether removal of N-linked glycans alters the binding affinity between FcRn and  $\beta_2m$ . FO-1 cells were therefore transfected with the FcRn glycan mutant cDNA, FO-1<sup>FcRnN/S</sup>. The fast migration of the band observed was consistent with FcRn from FO-1<sup>FcRnN/S</sup> cell line (Fig. 4A, lane 1) and confirmed that the removal



**FIGURE 4.** Effect of FcRn mutant for N-linked glycans on association of FcRn H chain and  $\beta_2m$ . All immunoprecipitates or cell lysates were subjected to 4–12% NuPAGE electrophoresis under reducing conditions. The proteins were transferred to a nitrocellulose membrane, and immunoblotting was performed with the corresponding Abs and HRP-conjugated Abs. Blots were developed with ECL. The experiment was at least performed two times. A, Expression of FcRn glycan mutant in FO-1 cells. The cell lysates from FO-1<sup>FcRnN/S</sup> (lane 1) and FO-1<sup>FcRn</sup> (lane 2) were blotted with anti-FLAG M2 mAb. The molecular mass in kilodaltons are shown on the left. B, The cell lysates from FO-1<sup>FcRnN/S</sup> and FO-1<sup>FcRn</sup> were mixed with soluble  $\beta_2m$  overnight by shaking at 4°C. The FcRn was immunoprecipitated by anti-FLAG M2 mAb, and the immunoprecipitates were immunoblotted with the BBM1 mAb. FO-1<sup>FcRn</sup> and FO-1<sup>FcRnN/S</sup> were lysed in CHAPS-PBS containing different amounts of human  $\beta_2m$  and protease inhibitors. To allow for assembly, the mixed lysates were incubated overnight at 4°C. FLAG M2 mAb (10  $\mu$ g/ml) was added and incubated for an additional 2 h, and protein G-agarose was used to isolate FcRn or FcRn- $\beta_2m$  complexes. The quantities of  $\beta_2m$  were indicated.

of *N*-linked glycans had occurred in the mutant FcRn H chain in comparison with the wild-type FcRn in the FO-1<sup>FcRn</sup> cell line (Fig. 4A, *lane 2*). Subsequently, we performed an *in vitro* assembly experiment of the human FcRn H chain with  $\beta_2m$  by mixing the cell lysates from FO-1, FO-1<sup>FcRnN/S</sup>, or FO-1<sup>FcRn</sup> with different concentrations of soluble  $\beta_2m$  molecules. The assembly of the FcRn H chain with  $\beta_2m$  was measured by immunoprecipitating the FcRn H chain and then blotting with a  $\beta_2m$ -specific Ab. Fig. 4B shows that wild-type FcRn H chain could assemble with low concentrations of  $\beta_2m$  (1  $\mu\text{g/ml}$ ). In contrast, the glycan-mutant FcRn H chain could only assemble with  $\beta_2m$  at high concentrations of  $\beta_2m$  (100–200  $\mu\text{g/ml}$ ). These studies show the dependence of the FcRn H chain and  $\beta_2m$  interaction on the presence of the *N*-glycan within the  $\alpha_2$  domain of human FcRn.

#### *FcRn H chain can assemble with $\beta_2m$ and bind IgG in the absence of CNX*

As CNX has been shown to associate with the FcRn H chain alone, it seemed possible that CNX might directly promote FcRn H chain binding to  $\beta_2m$ . CEM-NKR cells are deficient in CNX expression at both the mRNA and protein levels (21, 22). CNX can be detected, however, in parental CEM cells. Fig. 5A shows confirmation that the NKR cells do not express CNX in comparison with the CEM or Daudi cell lines. We therefore generated a stable FcRn-expressing CEM-NKR cell line by transfecting the human FcRn cDNA into CEM-NKR (Fig. 5B). As can be seen in Fig. 5C, FcRn and  $\beta_2m$  were able to associate in the absence of CNX in the NKR cell line (*lanes 2 and 4*). In our previous study, in the absence of  $\beta_2m$ , FcRn H chain fails to exit the ER and bind IgG (12). We therefore assessed IgG binding to FcRn at either pH 6.0 or 7.5 using different concentrations of cell lysates from the CEM-NKR-FcRn cell line. These studies showed that the FcRn H chain interacted with  $\beta_2m$  and bound IgG at pH 6.0, but not at pH 7.5, in the absence of CNX expression. To further show that an FcRn- $\beta_2m$  complex can exit the ER in CNX-deficient CEM-NKR cells, FcRn was assessed for the acquisition of resistance to digestion with endo-H (Fig. 5E). These studies showed that FcRn acquired resistance to Endo-H digestion in CEM-NKR cells (Fig. 5E, *lane 4*) similar to FcRn expressed in the CEM transfectants (Fig. 5E, *lane 2*). These studies suggest that the FcRn H chain- $\beta_2m$  complex may exit the ER in the absence of CNX. Furthermore, FcRn could be detected on cell surface in both of the CEM-FcRn (Fig. 5F) and CEM-NK-FcRn (Fig. 5F) transfectants by staining with the FLAG M2 mAb and flow cytometry. The levels detected were relatively high considering previous studies in epithelia (1); therefore, this may not be unexpected given previous high expression levels detected in human monocytes, another hemopoietic cell type (17).

#### *CNX can interact with the polypeptide backbone of human FcRn H chain*

There are several examples of CNX binding to proteins that lack *N*-linked oligosaccharides, suggesting that CNX also recognizes the polypeptide portion of incompletely folded proteins (23–26). As shown in Fig. 4B, an FcRn H chain mutant that lacks a carbohydrate side chain can assemble with  $\beta_2m$  at high concentrations, suggesting the CNX interacts with the polypeptide backbone of the FcRn H chain. Therefore, we examined this possibility. First, we immunoprecipitated the FcRn H chain from lysates of FO-1<sup>mock</sup>, FO-1<sup>FcRnN/S</sup>, and FO-1<sup>FcRn</sup>. The results showed that CNX could be detected in the anti-FcRn immunoprecipitates from both the FO-1<sup>FcRnN/S</sup> (Fig. 6A, *lane 1*) and FO-1<sup>FcRn</sup> (Fig. 6A, *lane 2*) cell lines, but not from the FO-1<sup>mock</sup> transfectant (Fig. 6A, *lane 3*). To further prove that polypeptide interactions mediate this association, we digested the FLAG-mAb immunoprecipitates from the FO-1<sup>FcRn</sup> and FO-1<sup>mock</sup> cell lines with endo-H (Fig. 6B), washed

the beads completely with cold PBS, and subjected these to SDS-PAGE electrophoresis followed by Western blotting with CNX-specific Ab. These results showed that treatment of the immunoprecipitates by endo-H did not change the association between the deglycosylated FcRn H chain from CNX (Fig. 6B, *lane 3*) when compared with the mock-digested immunoprecipitates (Fig. 6B, *lane 1*). Taken together with the studies in Fig. 6, these studies suggest that CNX can associate with FcRn in a carbohydrate-dependent and independent manner.

#### *Erp57 interacts in combination with an FcRn-CN X complex*

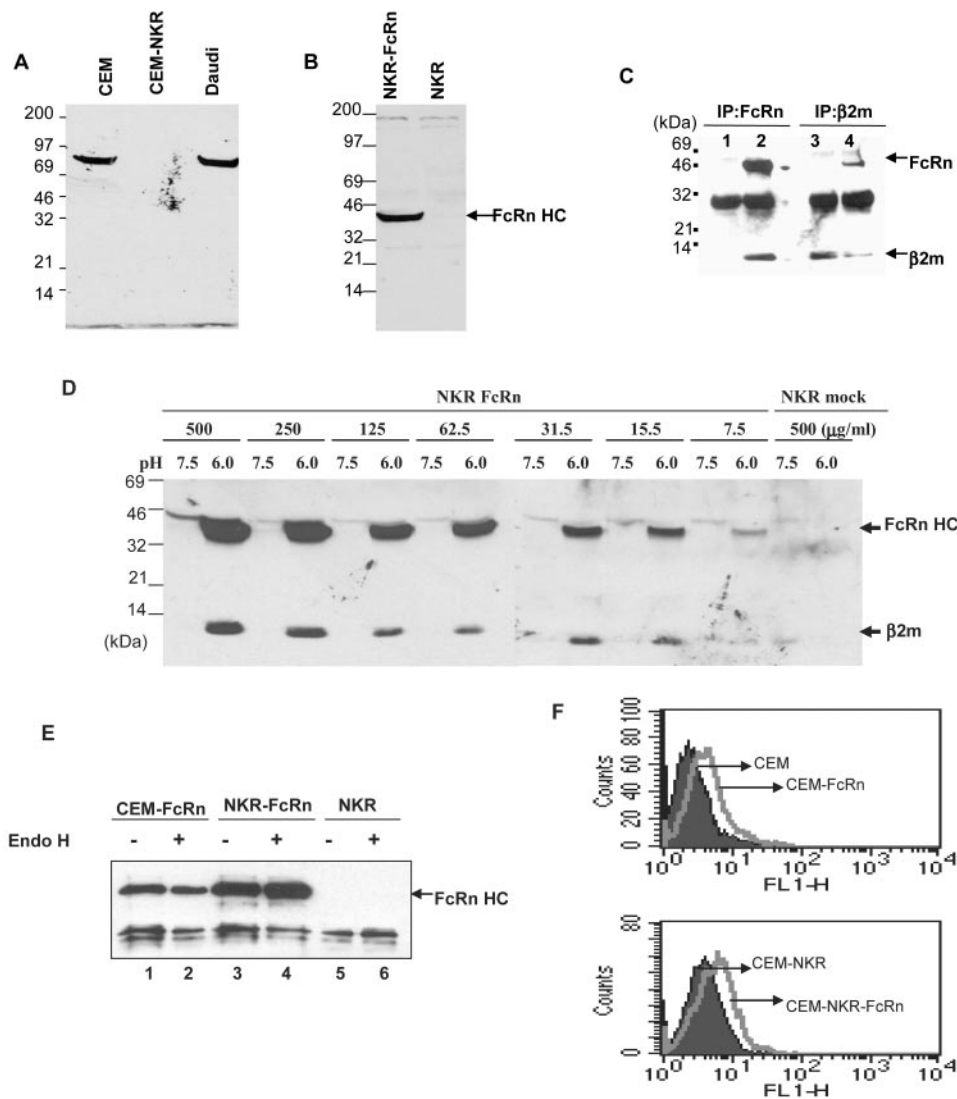
CNX can interact with the ER resident thiol oxidoreductase, ERp57 (27). Therefore, we performed an immunoprecipitation with anti-FLAG Ab under non-denaturing conditions. The immunoprecipitates were then blotted with an ERp57-specific Ab. As shown in Fig. 7A, the anti-FLAG Ab was able to immunoprecipitate ERp57 (*lane 2*). In another set of studies as shown in Fig. 7B, the cell lysates from FO-1<sup>mock</sup> (*lane 1*), FO-1<sup>FcRn</sup> (*lane 2*), and FO-1<sup>FcRn+ $\beta_2m$</sup>  (*lane 3*) were immunoprecipitated with an anti-ERp57- or anti-CN X-specific Ab and immune blotted with the opposite Ab. These studies revealed that CNX interacted with ERp57 in all cell lines. The coimmunoprecipitation of FcRn and ERp57 could also be visualized from [<sup>35</sup>S]methionine- and [<sup>35</sup>S]cysteine-labeled cells in sequential immunoprecipitation experiments (data not shown). Hence, ERp57 interacts with FcRn perhaps in combination with CNX.

#### *FcRn H chain can assemble with $\beta_2m$ and bind IgG in the absence of tapasin and TAP*

Tapasin and TAP are critical for the complete assembly of the MHC class I H chain- $\beta_2m$ -peptide complex (28–31). Given the structural similarities between MHC class I and FcRn H chain and their association with CNX or CRT, we also examined the possible interaction of FcRn with tapasin. Because FcRn is expressed in the human macrophage-like cell line, THP-1, and intestinal HT-29 and T84 cell lines (17, 32), we first showed that tapasin was also expressed in both types of cell lines (Fig. 8A). Second, we performed an immunoprecipitation of THP-1 cell lysates with an FcRn serum Ab or MHC class I mAbs HC10 and W6/32. The W6/32 mAb recognizes only native  $\beta_2m$ -associated MHC H chain (33), whereas HC10 mAb only interacts with free native and denatured MHC class I H chain (34). The immunoprecipitates were blotted with a tapasin-specific Ab. The results showed that tapasin was only detected in the immunoprecipitates with mAb HC10 (Fig. 8B, *lane 4*), but not that from normal rabbit serum (*lane 1*), a human FcRn  $\alpha_2$  domain-specific serum Ab (*lane 2*), an IgG1 control (*lane 3*), or W6/32 mAb (*lane 5*). We also expressed FcRn in the 721.220 (Fig. 8C, *bottom panel*), a tapasin-deficient cell line, and 721.174 (Fig. 8C, *top panel*), a TAP-deficient cell line. Furthermore, we performed an IgG-binding assay for FcRn in both cell lines. These studies showed that the FcRn bound IgG at pH 6.0, but not at pH 7.5, in the absence of tapasin and TAP expression (Fig. 8D). To further show that an FcRn- $\beta_2m$  complex can exit the ER in tapasin- and TAP-deficient cells, we performed staining with detection by flow cytometry. FcRn could be detected on the cell surface of both the 721.220-FcRn (Fig. 8E, *top panel*) and 721.174-FcRn (Fig. 8E, *bottom panel*) transfectants by staining with the FLAG M2 mAb.

## Discussion

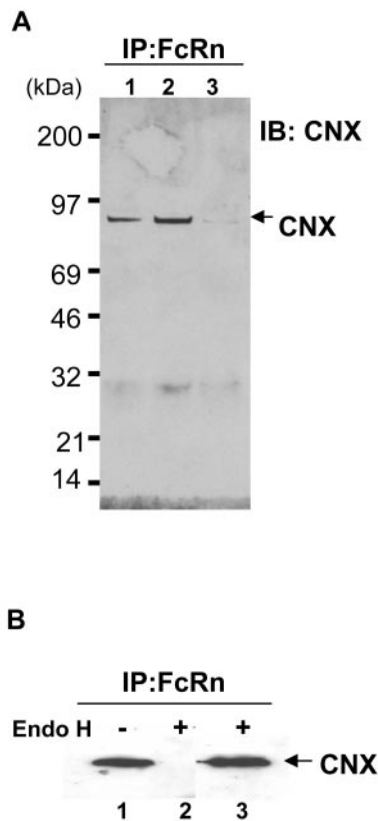
To fold or assemble correctly, newly synthesized proteins are facilitated by transient interactions with a number of chaperones that reside in the ER lumen (35). Two homologous lectins, an integral membrane protein CNX and its luminal homologue CRT, bind



**FIGURE 5.** Effects of CNX on FcRn and  $\beta_2m$  assembly and interactions with IgG. All proteins were subjected to 4–12% NuPAGE electrophoresis under reducing conditions and transferred to a nitrocellulose membrane for Western blotting. Immunoblots were developed with ECL. The molecular mass markers in kilodaltons are indicated on the left. Each experiment was at least performed two times. **A**, The cell lysates from CEM (lane 1), CEM-NKR (lane 2), and Daudi (lane 3) were blotted with an anti-CN X Ab. The lysates from CEM or Daudi cell were used as positive control. **B**, The cell lysates from CEM-NKR-FcRn (lane 1) and CEM-NKR (lane 2) were blotted with the anti-FLAG M2 mAb. **C**, Association of FcRn H chain and  $\beta_2m$  in CEM-NKR cells. The cell lysates from CEM-NKR<sup>FcRn</sup> were immunoprecipitated by isotype-matched control IgG (lanes 1 and 3), anti-FcRn FLAG M2 mAb (lane 2), or anti- $\beta_2m$  BBM-1 mAb (lane 4). The immunoprecipitates were immunoblotted with HRP-conjugated rabbit anti-mouse IgG. **D**, The pH-dependent FcRn binding of IgG in CEM-NKR<sup>FcRn</sup> cells. IgG-binding assays were performed at both pH 6.0 and 7.5, as described in *Materials and Methods*. The cells were lysed in sodium phosphate buffer (pH 6.0 or 7.5) with 0.5% CHAPS. Approximately 0.5–1 mg of soluble proteins was incubated with human IgG-Sepharose at 4°C. The eluted proteins were subjected to electrophoresis for Western blotting. Proteins were probed with both anti-FLAG M2 mAb and anti- $\beta_2m$  BBM1 mAb and developed with HRP-conjugated rabbit anti-mouse Abs. Lysates of CEM-NKR cells were probed similarly as a negative control. The numbers refer to the amount of protein in cell lysates. The  $M_r$  markers in kilodaltons are indicated on the left. The location of the human FcRn H chain and  $\beta_2m$  is indicated by an arrow and arrowhead. **E**, Sensitivity of FcRn H chain to endo-H digestion in CEM<sup>FcRn</sup> and CEM-NKR<sup>FcRn</sup> cells. Total cell lysates (50  $\mu$ g) were incubated without enzymes (lanes 1, 3, and 5) or in the presence of endo-H (lanes 2, 4, and 6) for 18 h in native conditions. Proteins were probed with affinity-purified rabbit anti-FLAG Ab. **F**, The cell surface expression of FcRn in CEM<sup>FcRn</sup> and CEM-NKR<sup>FcRn</sup> analyzed by flow cytometry. CEM or CEM-NKR transfectants ( $5 \times 10^5$  cells/ml) were incubated with mAb anti-FLAG M2 Ab and with fluorescein-conjugated rabbit anti-mouse Ab. Cells were fixed with 0.5% (v/v) paraformaldehyde in PBS. Results are expressed as histograms of fluorescence intensity (log scale).

transiently to growing nascent glycoproteins that carry monoglucosylated *N*-linked oligosaccharides (36, 37). The newly synthesized glycoproteins carry three glucose residues on each glycan. ER glucosidases I and II rapidly remove two of them, thus generating the monoglucosylated forms that serve as substrates for CNX and CRT binding. The single glucose residue is subsequently removed by glucosidase II, resulting in the dissociation of CNX and CRT (35). If the glycoprotein is correctly folded, it will exit

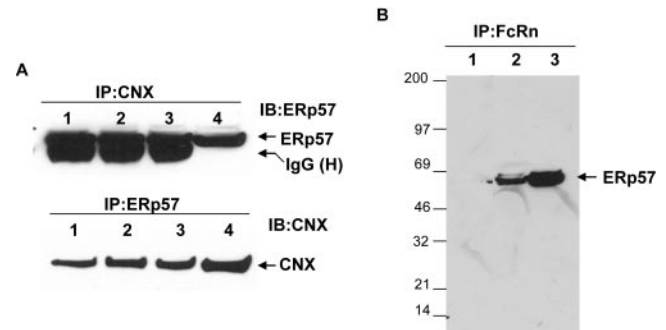
the ER. If not, it will be sensed by the ER enzyme UDP-glucose: glycoprotein glucosyltransferase (UGGT) and reglucosylated, thus regenerating a monoglucosylated glycan for reassociating with the CNX and CRT (35, 38). Once folding is complete, a protein is no longer a substrate for UGGT (38) and cannot bind CNX or CRT. Thus, by adding and removing glucose, glucosidase II and the UGGT drive their substrates through a cycle of CNX/CRT binding and release being regulated by the availability of the



**FIGURE 6.** CNX can bind the polypeptide backbone of FcRn H chain. All proteins were subjected to 4–12% NuPAGE electrophoresis under reducing condition and transferred to a nitrocellulose membrane for Western blotting. Immunoblots were developed with ECL. The molecular mass in kilodaltons are indicated on the left. Each experiment was at least performed two times. *A*, The cell lysates from FO-1<sup>FcRnN/S</sup> (lane 1), FO-1<sup>FcRn</sup> (lane 2), and FO-1<sup>mock</sup> (lane 3) were immunoprecipitated by anti-FLAG M2 mAb. The immunoprecipitates were immunoblotted with goat anti-CNX-specific Ab. *B*, Sensitivity of CNX on binding to FcRn H chain with endo-H digestion in FO-1<sup>FcRn</sup> cell. Total cell lysates (500  $\mu$ g) were immunoprecipitated by anti-FLAG M2 mAb. The immunoprecipitates were incubated without enzymes (lanes 1) or in the presence of endo-H (lanes 2 and 3) for 18 h in native conditions. Lane 2, represents the immunoprecipitate from FO-1<sup>mock</sup>. Proteins were probed with affinity-purified goat anti-CNX Ab.

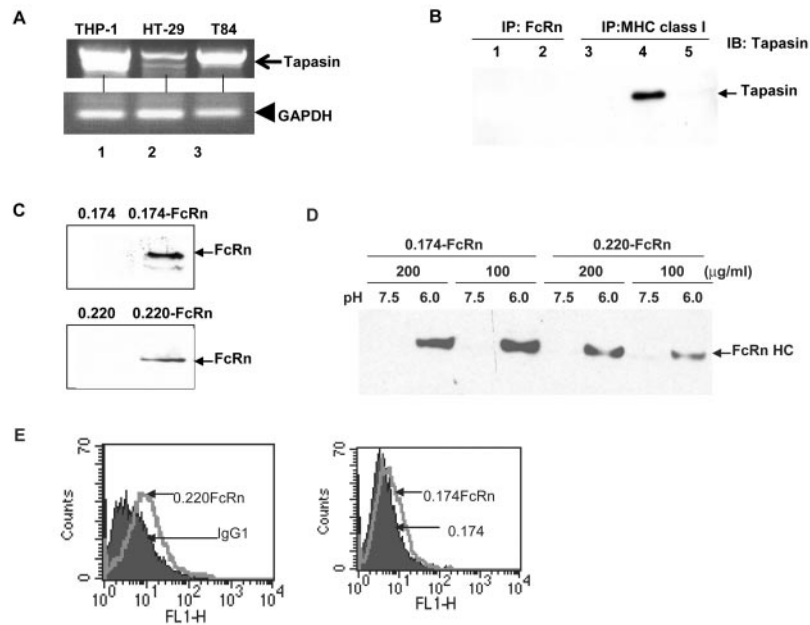
terminal glucose (35, 39). Hence, CNX, CRT, and UGGGT serve as a quality control step for the nascent glycoproteins in the ER (35, 40). Despite their extensive homology, studies with CRT- and CNX-deficient cell lines showed that their activities and substrate specificities are different (40). In the present study, we investigated functional interactions between these chaperones and the human FcRn.

CNX promotes folding of FcRn H chain and facilitates their binding to  $\beta_2m$ . This has been shown by several lines of evidence. First, CNX was found to be associated with free FcRn H chain. CNX was coimmunoprecipitated with FcRn, which was respectively confirmed by mass spectrometry of the coprecipitated CNX protein (Fig. 1) and by immunoprecipitation-Western blotting in reciprocal directions for both CNX and FcRn (Fig. 2). Second, CNX was readily detected in association with FcRn H chain in a  $\beta_2m$ -negative cell line. CNX was only detected with FcRn H chain alone, but not with an FcRn- $\beta_2m$  complex (Fig. 3A). Therefore, CNX was shown to transiently interact with the nascent FcRn H chain. Our findings thus suggest that CNX may retain FcRn H chain in the ER until assembly of the complex of H chain with



**FIGURE 7.** ERp57 binds FcRn in combination with CNX. The molecular mass in kilodaltons are indicated on the left. FO-1 transfectants were lysed for 30 min by including 2 mM final concentration of DSP in the lysis buffer PBS containing 0.5% CHAPS. Excess DSP was quenched with 50 mM glycine. The lysates were used for immunoprecipitation. All proteins were subjected to 4–12% NuPAGE electrophoresis under reducing condition and transferred to a nitrocellulose membrane for Western blotting. Immunoblots were developed with ECL. Each experiment was at least performed two times. *A*, The lysates of FO-1<sup>mock</sup> (lane 1), FO-1<sup>FcRn</sup> (lane 2), and FO-1<sup>FcRn +  $\beta_2m$</sup>  (lane 3) were immunoprecipitated by either anti-CNX Ab (top panel) or ERp57 (bottom panel). The immunoprecipitates were blotted with either anti-ERp57 (top panel) or anti-CNX (bottom panel). The cell lysates of FO-1 cells were used as a positive control for blotting (lane 4). Positions of ERp57 and CNX were labeled with arrows. IgG (H): IgG H chain. *B*, The lysates of FO-1<sup>mock</sup> (lane 1) and FO-1<sup>FcRn</sup> (lane 2) were immunoprecipitated by anti-FLAG Ab. The immunoprecipitates were blotted with anti-ERp57 mAb. The cell lysates of FO-1 cells were used as a positive control for blotting (lane 3). The molecular mass in kilodaltons are indicated on the left.

$\beta_2m$ . This is in agreement with the role of CNX in retaining unassembled MHC class I and CD1 H chains in the ER (18, 41–43). Third, CNX was implicated in the assembly of FcRn H chain and  $\beta_2m$ . Addition of purified  $\beta_2m$  to detergent lysates of FO-1<sup>FcRn</sup>, or compared with FO-1<sup>FcRnN/S</sup> cells, resulted in stable association of FcRn H chain with  $\beta_2m$  at low concentrations of  $\beta_2m$ . The removal of the *N*-linked glycan decreased the affinity of FcRn H chain with  $\beta_2m$ . However, it should be noted that the glycan mutant did not eliminate the binding of FcRn H chain for  $\beta_2m$ , especially under high concentrations of  $\beta_2m$  (Fig. 4B). Fourth, CNX was implicated in the initial folding of the FcRn H chain. This is suggested by the fact that ERp57, a thiol-dependent reductase and cysteine protease, was detected in association with an FcRn-CNX complex. Specifically, both CNX and FcRn H chain were coprecipitated with ERp57 under nondenaturing conditions. Because ERp57 has the potential to influence disulfide-bond formation, it may be that the role of CNX is to direct newly synthesized FcRn H chains into specific disulfide bond formation as mediated by ERp57 and presumably other chaperones. In this aspect, CNX might thus act as a scaffold to recruit ERp57 to the FcRn H chain. Therefore, CNX and ERp57 may help the FcRn H chain fold properly, and thereby increase the pool of fully conformational FcRn H chain available to bind  $\beta_2m$ . This is consistent with the proposal that ERp57 interacts with glycoproteins, such as MHC class I and CD1, in combination with CNX (18, 43–46). However, CNX operates as chaperone in the quality control of newly synthesized glycoproteins in two opposing ways. CNX ensures that correctly folded glycoproteins leave the ER, but also can mediate glycoprotein degradation in cases in which correct protein folding is impossible (47). Given that CNX is functioning at different levels, it is of interest to determine how these various effects are ultimately manifest in terms of the expression and degradation of FcRn. This



**FIGURE 8.** Effects of tapasin and TAP on the folding of FcRn and  $\beta_2m$  and IgG binding. *A*, RT-PCR amplification of FcRn cDNA from THP-1 and human intestinal epithelial cell lines (HT-29 and T84). First-strand cDNA was prepared as described in *Materials and Methods*. Amplified PCR products were electrophoresed in 1.2% agarose gels and stained with ethidium bromide. Similar PCR products amplified with a GAPDH-specific primer pair were also fractionated in 1.2% agarose gels as internal controls. The arrow indicates the location of the amplification products for human tapasin and GAPDH. *B*, The lysates of THP-1 were immunoprecipitated by normal rabbit serum (lane 1), FcRn-specific serum Ab (lane 2), IgG1 (lane 3), HC10 mAb (lane 4), and W6/32 mAb (lane 5). The immunoprecipitates were subjected to 12% SDS-PAGE electrophoresis under reducing condition and transferred to a nitrocellulose membrane. MHC class I molecules interacting with tapasin were used as a positive control. The membranes were immunoblotted with anti-tapasin Ab. *C*, The cell lysates from 0.174 and 0.174<sup>FcRn</sup> (top) and 0.220 and 0.220<sup>FcRn</sup> (bottom) were subjected to 12% SDS-PAGE electrophoresis under reducing condition and transferred to a nitrocellulose membrane for Western blotting with rabbit anti-FLAG Ab. Immunoblots were developed with ECL. *D*, pH-dependent FcRn binding of IgG in 0.174<sup>FcRn</sup> and 0.220<sup>FcRn</sup>. IgG-binding assays were performed at both pH 6.0 and 7.5, as described in *Materials and Methods*. The cells were lysed in sodium phosphate buffer (pH 6.0 or 7.5) with 0.5% CHAPS. Approximately 1–2 mg of soluble proteins was incubated with human IgG-Sepharose at 4°C. The eluted proteins were subjected to electrophoresis for Western blotting. Proteins were probed with both anti-FLAG M2 mAb and developed with HRP-conjugated rabbit anti-mouse Abs. The  $M_r$  markers in kilodaltons are indicated on the left. The location of the human FcRn H chain is indicated by an arrow. *E*, The cell surface expression of FcRn in 0.174<sup>FcRn</sup> and 0.220<sup>FcRn</sup> analyzed by flow cytometry. The 0.220-FcRn (top) or 0.174-FcRn (bottom) ( $5 \times 10^5$  cells/ml) were incubated with mAb anti-FLAG M2 Ab and with FITC-labeled rabbit anti-mouse Ab. Cells were fixed with 0.5% (v/v) paraformaldehyde in PBS. Results are expressed as histograms of fluorescence intensity (log scale).

may be especially important because FcRn plays a role in maintaining IgG homeostasis. The regulation of FcRn expression is therefore predicted to be critical for this function.

The roles of chaperones in assembling FcRn H chain and  $\beta_2m$  may overlap. It is very intriguing that in our IgG-binding assay, FcRn H chain could assemble with  $\beta_2m$ , be expressed at the cell surface, as well as bind human IgG at acidic pH in the CNX-deficient cell line, CEM-NKR (Fig. 5). These observations are in agreement with previous findings from the assembly of MHC class I- $\beta_2m$  and TCR complex. In CEM-NKR, CNX is not an absolute requirement for proper folding and assembly of MHC class I chain- $\beta_2m$  or TCR complexes (21, 22, 48). There were no significant differences in the transport rate and surface expression of MHC class I between CEM and CEM-NKR by using W6/32 (33) and HC10 (34) mAbs. Studies using glucosidase II-deficient cells confirmed this observation (49). The lack of similar mAbs for recognizing only native  $\beta_2m$ -associated FcRn H chain or free native and denatured FcRn H chain limits the further dynamic assessment of the assembly of FcRn- $\beta_2m$  complex. However, the acquisition of endo-H resistance (Fig. 5) and detection of FcRn H chain on the cell surface (Fig. 5) suggest that the FcRn- $\beta_2m$  complex exits the ER. FcRn assembly and binding to IgG appear normal in a CNX-deficient mutant cell line, suggesting that alternative chaperones or a backup mechanism might exist in CEM-NKR cells to compensate for FcRn quality control under conditions in which CNX is

absent. This may not be surprising that there is considerable overlap of the substrate glycoproteins and ER chaperones such that they are able to associate with the same proteins simultaneously or sequentially. In the absence of CNX expression, CRT hence may be the first candidate to bind FcRn H chains and facilitate H chain folding and assembly with  $\beta_2m$ . However, our results have shown that CRT only interacted with the FcRn H chain- $\beta_2m$  complex (Fig. 3B) and is bound to FcRn in CEM-NKR cells (data not shown). In addition, it was also shown that CRT expression was markedly increased at least 3-fold in CEM-NKR relative to CEM cells (48). It remains unclear whether CRT can bind to FcRn H chain alone in CEM-NKR cells. Therefore, CNX and CRT may function redundantly in the ER of CEM-NKR cells. However, it is unknown whether they perform the same or distinct molecular functions in the folding/assembly of newly synthesized FcRn H chain. We reason that CNX first interacts with the nascent chains of N-linked FcRn because CNX is located near the ER translocon to confer convenient access of CNX to glycans. In addition, CNX, rather than CRT, could be easily detected in a coprecipitation with the FcRn H chain (Fig. 1), suggesting that CNX is quantitatively a major chaperone that interacts with the FcRn H chain. Because chaperones usually compete for binding sites on the incoming nascent chain, whatever binds first apparently dominates during early stages of co- and posttranslational folding (50). It might be possible that association of CNX with a newly synthesized FcRn H

chain may restrict the access of alternative components such as CRT. In addition to CRT, however, we cannot rule out that there may be redundancy in other chaperone-assisted assembly of FcRn in CEM-NKR cells, such as the Ig-binding protein, Bip. Human class I molecules can stably interact with Bip proteins (51). Some chaperones may also have a low affinity of interaction that does not survive detergent solubilization before immunoprecipitation. Overall, these possibilities may account for the lack of any observable effect of CNX absence on FcRn expression in the CEM-NKR cell line. These results indicate that redundant proteins may substitute for the function of CNX in the assembly of FcRn and  $\beta_2m$  or that CNX is not absolutely required for the assembly and transport of human FcRn molecules.

CNX can interact with the backbone of FcRn H chains in addition to binding the glycans. The removal of the *N*-linked glycosylation addition site of FcRn (Fig. 6A) failed to disrupt the interaction, suggesting that CNX can directly interact with the polypeptide backbone of the FcRn H chain. In addition, the FcRn H chain glycan mutant still assembled with  $\beta_2m$  in vitro, although a high concentration of  $\beta_2m$  was necessary (Fig. 4). Recently, a dual binding model has been proposed in which unfolded glycoproteins interact with both the lectin site and a polypeptide binding site in CNX and CRT (52, 53), although a lectin-only model remains controversial. It is possible that CNX uses its lectin site to bind oligosaccharides first, thereby bringing the nascent FcRn H chain into proximity with the membrane-constrained CNX. This glycan binding may cause a conformational change that allows CNX to recognize polypeptide segment(s) of the unfolded FcRn H chain. Subsequently, CNX may associate directly with the unfolded FcRn H chain, making the CNX-oligosaccharide interaction less important for maintaining the complex. For example, removal of the glycan from CNX-associated FcRn H chain by digestion with endo-H did not disrupt the interaction (Fig. 6B). It is likely that the CNX-oligosaccharide interaction acting in conjunction with polypeptide-based associations could increase overall binding avidity. A glycan-independent association with CNX has been observed by others for nonglycosylated CD3 $\epsilon$  and MHC class I and II, and vesicular stomatitis virus G glycoproteins (41, 54, 55). Indeed, sites of interactions between CNX and mouse MHC class I H chain or P-glycoprotein have been mapped in proximity to the transmembrane region, which is free of glycans (23, 56). This also raises the interesting issue of how the FcRn H chain associates with CNX. Further mapping will be required to determine more precisely this issue.

Our studies also suggest that tapasin and TAP are not involved in the assembly of FcRn and  $\beta_2m$ . Based on previous immunoprecipitation analyses of detergent lysates of mammalian cells, MHC class I H chain- $\beta_2m$  are in complex with CRT, TAP, and tapasin following release from CNX (14, 30, 31, 57). Together, these proteins have been referred to as the MHC class I peptide-loading complex (14). Tapasin probably stabilizes and bridges the peptide-free H chain- $\beta_2m$  and TAP. Upon binding of peptide, the MHC class I complex is released from TAP and tapasin and exits the ER. Tapasin interactions with TAP, therefore, may guarantee efficient capture of translocated peptides for MHC class I complexes. However, FcRn lacks the Ag-binding groove (16). Therefore, peptide loading is not important and required for FcRn assembly and exit to the ER. This implies the tapasin and TAP may not be pivotal for FcRn biogenesis. To test this hypothesis, we immunoprecipitated FcRn and MHC class I in THP-1 cells. The detection of tapasin association with MHC class I H chain, but not FcRn H chain (Fig. 8B), suggests that tapasin was unable to interact with the FcRn H chain. Furthermore, we expressed FcRn in both TAP-1-deficient .174 and tapasin-deficient .220 cell lines

(Fig. 8C). These results showed that lack of either tapasin or TAP-1 did not affect the assembly of FcRn and  $\beta_2m$  by showing pH-dependent binding of IgG (Fig. 8D) and expression of FcRn at the cell surface (Fig. 8C). All of these data suggest that during evolution, FcRn might have lost the need for an interaction with tapasin and TAP as its antigenic binding groove narrowed.

The requirement for chaperones to the folding and assembly of human FcRn and other FcRn species may be different. Among the FcRn homologues, to date cloned, human and bovine FcRn H chain have a single *N*-linked glycosylation site (6, 58), whereas rodents have four such sites (7). Interestingly, it was found that the selection of a particular chaperone pathway by a nascent glycoprotein was partially dependent on the numbers of *N*-linked glycans in a glycoprotein (36, 50, 59). For example, both mouse and human class I H chains associate with CNX before binding  $\beta_2m$ . However, mouse MHC class I proteins remain bound to CNX after binding  $\beta_2m$  (14), whereas the human MHC class I H chain appears to dissociate from CNX either before or while binding  $\beta_2m$  (41, 51, 60). This may also be in part explained by a potential difference in the affinity of  $\beta_2m$  for its class I H chain between the human and murine species (61). Murine MHC class I H chain has weak affinity for murine  $\beta_2m$ . In mice, a CNX-glycan interaction might be required to stabilize this association until peptide is bound, whereas human MHC class I may not require CNX because  $\beta_2m$  binds with sufficient affinity. Furthermore, the positions of *N*-linked glycans in a glycoprotein may also affect chaperone selection. The closer the oligosaccharide moieties are to the N terminus, the higher are the chances that the protein engages with CNX (51, 60). Therefore, a detailed examination of interactions between FcRn and chaperones in other species is underway to better understand the nature of their interactions with FcRn (X. Zhu, unpublished data).

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## Disclosures

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