

Export of the High Affinity IgE Receptor From the Endoplasmic Reticulum Depends on a Glycosylation-Mediated Quality Control Mechanism¹

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The high affinity IgE receptor (FcεRI) is a multisubunit complex comprised of either αγ₂ or αβγ₂ chains. The cotranslational assembly of the IgE-binding α-chain with a dimer of γ-chains occurs in a highly controlled manner and is proposed to involve masking of a dilysine motif present at the cytoplasmic C terminus of the FcεRI α-chain that targets localization of this subunit to the endoplasmic reticulum (ER). Here, we show that ER quality control modulates export from the ER of newly synthesized αγ₂ and αβγ₂ receptors. We demonstrate that the presence of untrimmed N-linked core glycans (Glc₃Man₉GlcNAc₂) on the FcεRI α-chain activates the ER quality control mechanism to retain this subunit in the ER, despite the presence of γ-chains. At the same time, the untrimmed, ER-localized α-chain exhibits IgE-binding activity, suggesting that FcεRI α-chain folding occurs before constitutive glucose trimming. In additional experiments, we demonstrate that cell surface expression of an α-chain C-terminal truncation mutant is also dependent on glucose trimming, but not on γ-chain coexpression. We suggest that glucosidase trimming of terminal glucose residues is a critical control step in the export of FcεRIα from the ER. Finally, we show that the constitutive ER FcεRI α-chain, expressed in the absence of the other FcεRI subunits, associates with the ER lectin-like chaperone calnexin, but not the structurally similar ER chaperone calreticulin, presumably through interaction with monoglucosylated α-chain ER glycoforms. *The Journal of Immunology*, 2000, 165: 5686–5694.

The high affinity IgE receptor (FcεRI)³ is a key molecule involved in allergic reactions. The human receptor exists in two distinct forms: either as a tetrameric complex consisting of one α-chain subunit, one β-chain and a homodimeric γ-chain, or as a trimeric αγ₂ complex devoid of β-chain. Although inflammatory cells such as mast cells and basophils express the αβγ₂ receptor (reviewed in Ref. 1), recent findings have indicated that many other cell types such as monocytes (2), eosinophils (3), Langerhans cells (LC; Refs. 4 and 5), and dendritic cells (6) also express the receptor, albeit without β-chain. Each of the subunits of the receptor seems to have an individual function. Although the α-chain is exclusively involved in the binding of the IgE ligand, the γ-chain is necessary for signal transduction (7), whereas the β-chain appears to function as an amplifier of this signal (8, 9). Of the three chains, only the α-chain is N-glycosylated with ~40% of the total M_r derived from oligosaccharides (1). It appears likely that all seven of the potential N-linked glycosylation sites present in the human FcεRIα ectodomain sequence are used based on a careful glycosylation analysis of a truncated form of the α-chain

consisting of only the extracellular domain (10). Although glycosylation of truncated α-chain is not necessary for folding and IgE binding (11), it was shown to be necessary for secretion from transfected eukaryotic cells (10), suggesting an important role of N-linked glycans in α-chain transport. However, the relationship between constitutive FcεRIα glycosylation and FcεRI subunit assembly and eventual cell surface expression has not been elucidated.

The mechanistic features underlying FcεRI assembly and expression are of considerable interest. Early studies showed distinct differences in cell surface expression between rodent and human FcεRI; rodent FcεRI expression requires coexpression of all three subunits (12), whereas human FcεRI is reported to achieve cell surface expression with or without the β-subunit (13, 14). At the same time, evidence for a possible role of the β-subunit in enhancing or stabilizing FcεRI surface expression, compared with the αγ₂ complex, has also appeared (8), in accordance with our observations that the αβγ₂ receptor expresses to a higher density than αγ₂ in transfected cells (our unpublished results). Additional early studies revealed the importance of FcεRI subunit transmembrane (TM) and cytoplasmic domains (CD) in assembly and expression of the rat receptor (15–17) as well as the human FcεRI αγ₂ complex (17). More recently, a number of studies have revealed an additional complexity in FcεRI expression: regulation of FcεRI expression in FcεRI⁺ monocytes (18), mast cells (19, 20), basophils (21, 22), and LC (23). In some of these studies (18–22), IgE appears to up-regulate surface FcεRI, presumably by a mechanism involving stabilization of surface receptor. Other investigations have shown that, upon IgE depletion, FcεRI expression is down-regulated (24, 25), although the mechanistic details underlying FcεRI down-regulation in IgE-depleted cells have not been defined. FcεRI regulation in LC is particularly striking as it appears to be linked to maturation of LC to lymphoid dendritic cells (23). Another level of complexity exists for FcεRI expression in LC (23), eosinophils (26, 27), and megakaryocytes (28) whereby

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Received for publication April 24, 2000. Accepted for publication August 15, 2000.

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¹ This work was supported by National Institutes of Health Grant AI135759 (to M.W.R.) and by Grant SPR-1135 from Novartis, GmbH (to M.W.R.).

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³ Abbreviations used in this paper: FcεRI, high affinity Fc receptor for IgE; CD, cytoplasmic domain; CST, castanospermine; Endo H, endoglycosidase H; GFP, green fluorescent protein; LC, Langerhans cells; αt, cDNA truncation fragment; CHO, Chinese hamster ovary; TM, transmembrane.

the Fc ϵ RI α -chain has been shown to accumulate in intracellular compartments without showing significant cell surface expression despite the presence of Fc ϵ RI γ -chain. Thus, it has emerged that Fc ϵ RI regulation is an important and complex process that, in some forms, implicates control of Fc ϵ RI expression at the intracellular level including possibly Fc ϵ RI subunit assembly and transport.

Co- and post-translational modifications of nascent polypeptides occur in the lumen of the endoplasmic reticulum (ER), such as disulfide bond formation and core glycosylation, and are generally necessary to achieve polypeptide folding and assembly of multi-subunit complexes (29). Although the β -chain and γ -chains have only a few amino acid residues oriented to the luminal side of the ER, the α -chain ectodomain, comprised of two Ig-like domains, is oriented within this compartment. To prevent misfolding and aggregation in the ER because of the high protein concentration and oxidative environment within this compartment (30, 31), a complex quality control network exists involving a number of molecular chaperones to facilitate production of transport-competent proteins and protein complexes (reviewed in Ref. 32). A different level of ER quality control exists for many proteins by virtue of the presence of ER retention or Golgi-retrieval signal sequences, the latter thought to associate with coatomer protein complexes (33). In the case of the Fc ϵ RI α -chain, it has been proposed that a di-lysine motif positioned near the end of the CD functions as a retrieval signal that effectively directs localization of the free α -chain to the ER (34). Coexpression of the γ -chain leads to α -chain localization to the cell surface as the $\alpha\gamma$ 2 complex by a mechanism presumed to involve steric masking of the di-lysine motif by determinants in the γ -chain CD that lie in physical proximity to the α -chain di-lysine motif (34). The masking of the ER retrieval/retention signal by the γ -chain thus serves as "secondary quality control" (32) for α -chain surface expression. Here, we have studied the relationship of ER quality control and α -chain N-glycosylation and its effect on the export of new Fc ϵ RI molecules.

Materials and Methods

Construction of plasmids for α - and γ -chain expression

The cDNA encoding the human Fc ϵ RI α -chain was cloned into the pIREShyg vector (Clontech, Palo Alto, CA) and the pcDNA3.1(+)-zeo vector (Invitrogen, Carlsbad, CA), both under the control of a CMV promoter. The cDNA truncation fragment (α t) lacking the coding sequence for the 16 C-terminal amino acids was cloned into the pcDNA3.1(+)-zeo. The c-myc-derived epitope tag (EQKLISEEDL) recognized by mAb 9E10 (hybridoma cell line MYC1-9E10.2; American Type Culture Collection, Manassas, VA) was positioned at the N terminus of the mature α -chain sequence (35) by placement between the constitutive leader peptide and the +1 residue of the mature sequence. The cDNA coding for the human Fc ϵ RI γ -chain was also cloned into pcDNA3.1(+)-zeo. The FLAG epitope tag (DYKDDDDK) was positioned at the N terminus of the mature γ -chain sequence and contained an Ala3 spacer between the C terminus of the FLAG tag and the start of the five-residue extracellular region (LGEPQ) of the mature γ -chain. An additional two-residue sequence (LG) was added between the constitutive leader peptide and the N terminus of the FLAG sequence to facilitate peptidase cleavage of the leader peptide. To position the FLAG tag and Ala3 spacer sequence between the native leader sequence and the +1 residue of the mature γ -chain, a series of three PCR were performed to sequentially build the desired full-length coding sequence. Each amplification used the following oligonucleotide as 3' primer: 5'-GACTCTCGAGCATATTTAGCTGGAGTTGGGAATGGG together with the 5' primers described below, using human Fc ϵ RI γ -chain plasmid as template and using Pfu polymerase (Stratagene, San Diego, CA) with 30 cycles of 94°C, 1 min; 58°C, 1 min, and 72°C 1 min, followed by one cycle of 72°C, 10 min. The following 5' PCR primers were used in reactions 1–3: 1) 5'-CTGGGAGATTATAAGGATGACGACGATAAAGCTGCAGC GCTGGGTGAGCCTCAGCTCTGCTAT; 2) 5'-GCACTGGTCTTGCTCT TACTCCTTTTGGTTGAACAAGCAGCGGCCCTGGGAGATTATAAGG AT; and 3) 5'-GCAGAAGCTTATGATTCCAGCAGTGGTCTTGCTCTTA.

PCR product 1 was excised from a 1.6% agarose gel and purified using a Qiagen (Chatsworth, CA) spin column. The purified product was then used directly in PCR 2 using the same amplification conditions as before. The spin column-purified product from this step was then used directly in the third amplification reaction using the same PCR conditions as before. The full-length PCR product was then excised from a 2% agarose gel and the purified product then cloned as a *XhoI/HindIII* fragment into the pcDNA3.1(+)-zeo vector following standard protocols (36). The nucleotide sequence of α - and γ -chain coding sequence for all expression plasmids used in this study was confirmed on an ABI model 377 version 3.0 DNA sequencer.

Cells lines and transfections

HeLa cells (Clontech) were cultured in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Irvine Scientific, Irvine, CA), 2 mM glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified 5% CO₂ incubator. For transient transfection, HeLa cells were seeded at 3×10^5 cells/35-mm well 1 day before transfection. Cells were transfected with Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's instructions. As an internal control for transfection efficiency, cells were cotransfected with an expression vector for green fluorescent protein (GFP; pGreen Lantern-1; Life Technologies). Forty-eight hours after transfection, cells were harvested by trypsinization and analyzed. Chinese hamster ovary (CHO) cells that stably express human Fc ϵ RI $\alpha\beta\gamma$ 2 were generously provided by Dr. J.-P. Kinet (Beth Israel Deaconess Medical Center, Boston, MA). Cells were grown in IMDM (Life Technologies) containing 1 mg/ml G418, 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin at 37°C, 5% CO₂. MYC 1-9E10.2 cells were kept in RPMI 1640 (Life Technologies) and 10% FBS, glutamine, and penicillin/streptomycin, at 37°C, 5% CO₂. Castanospermine (CST; Biomol, Plymouth Meeting, PA) was used at 300 μ g/ml.

Flow cytometry

Fc ϵ RI α -chain was indirectly detected using the anti-Fc ϵ RI mAb 15-1 mAb (mIgG1/ κ ; Ref. 5). Fc ϵ RI γ -chain was detected using the FLAG epitope-specific mAb M2 (mIgG1; Sigma, St. Louis, MO). Cells were incubated with 15-1 mAb, M2 mAb, or mIgG1/ κ (MOPC-21; Sigma) as an isotype control for 30 min at 4°C at a concentration of 2 μ g/10⁶ cells. Cells were washed in PBS/1% FBS and then treated with 0.5 μ g/10⁶ cells biotinylated goat F(ab')₂ anti-mouse IgG1 (γ 1 heavy chain-specific; Southern Biotechnology Associates, Birmingham, AL). After incubation for 30 min at 4°C, cells were washed and incubated with 2 μ g/10⁶ cells streptavidin-PE (PharMingen, San Diego, CA). Cells were then analyzed on a FACScalibur analyzer (Becton Dickinson, San Jose, CA) using CellQuest software for both data acquisition and analysis. Live cells were gated on forward- and side-scatter and by propidium iodide (1 μ g/ml) exclusion using the FL3 channel. GFP-fluorescence was analyzed using the FITC-channel of the 488-nm laser.

Confocal microscopy

A total of 5×10^4 cells were seeded per chamber on an eight-chamber Lab-Tec II culture slide (Nunc, Naperville, IL) and cultured overnight at 37°C, 5% CO₂. Cells were washed twice with PBS, fixed, and permeabilized with 4% paraformaldehyde for 30 min at ambient temperature in a moist chamber. Cells were then washed three times with PBS and incubated with PBS/2% goat serum (Sigma) for 30 min to block nonspecific binding. Cells were washed three times with PBS and incubated for 30 min with 40 μ g/ml 15-1 mAb (mIgG1) in PBS/2% BSA (Sigma) and anti-BiP mAb (clone 10C3, mIgG2a; StressGen Biotechnologies, Victoria, BC, Canada) at a 1:200 dilution. Cells were washed three times with PBS and then incubated with FITC-labeled goat F(ab')₂ anti-mouse IgG1 (γ 1 heavy chain-specific; Southern Biotechnology Associates) at a dilution of 1:300 and Texas Red-labeled goat anti-mouse IgG2a (γ 2a heavy chain-specific; Southern Biotechnology Associates) at a 1:50 dilution in PBS/2% BSA. Cells were then washed three times with PBS. Before sealing of the slide, Slow Fade-Light antifade (Molecular Probes, Eugene, OR) was added. The specimen was analyzed using a confocal laser microscope (MRC 600; Bio-Rad, Hercules, CA) equipped with an argon-krypton mixed gas laser (488 nm, blue line for FITC and 568 nm, yellow line for Texas Red) in association with a Zeiss IM35 M inverted microscope with objective lenses at $\times 40$ or $\times 63$. Images were collected and analyzed using Cosmos (Bio-Rad) and Adobe Photoshop software (San Jose, CA).

Immunoprecipitations and Western blot analysis

Cells were solubilized in Nonidet P-40 (Calbiochem, San Diego, CA) lysis buffer (1% Nonidet P-40, 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 10% glycerol, 20 μ g/ml aprotinin (Sigma), 10 μ g/ml leupeptin (Sigma), and 0.5 mM Pefabloc SC (4-(2-aminoethyl)-benzolsulfonylfluoride, hydrochloride; Boehringer Mannheim, Indianapolis, IN)). Lysates were kept on ice for 30 min and, subsequently, insoluble debris was removed by centrifugation at $13000 \times g$ for 30 min at 4°C. Supernatants were precleared with Sepharose 4B beads (Pharmacia, Piscataway, NJ) for 3–16 h at 4°C. For immunoprecipitation of Fc ϵ RI α -chain, 10 μ g/ml of mAb 9E10 (mIgG1/ κ) was added to lysate supernatants and allowed to incubate for 6–16 h at 4°C before addition of Protein G-Sepharose beads and continued (6–16 h) incubation. Alternatively, immune complexes containing Fc ϵ RI α -chain were isolated using murine IgE (hybridoma 26.82; Ref. 37; kindly provided by Dr. F.-T. Liu, La Jolla Institute for Allergy and Immunology, San Diego, CA) covalently coupled to CNBr-activated Sepharose 4B beads (Pharmacia). Calnexin was immunoprecipitated using an anti-calnexin C terminus polyclonal Ab (SPA-860; StressGen) at 1:200 dilution. Protein G-Sepharose-bound or IgE-Sepharose-bound proteins were washed three times in Nonidet P-40 lysis buffer and then eluted with SDS sample buffer in the absence of thiol reducing agent. Proteins were fractionated by SDS-PAGE, electrophorotransferred onto Immobilon P membranes (Millipore, Bedford, MA), and probed for the presence of Fc ϵ RI α -chain using 19-1 mAb (mIgG2a/ κ ; Ref. 5; a gift from Dr. J.-P. Kinet) at a 1:400 dilution in combination with HRP-conjugated goat anti-mouse κ light chain (GaM κ) serum (Southern Biotechnology Associates) at a 1:4000 dilution. Calnexin was detected on immunoblots using SPA860 at a dilution of 1:2000 in combination with HRP-conjugated goat anti-rabbit IgG (γ heavy chain-specific) serum at 1:2000 dilution. The HRP conjugates were visualized by using ECL Western blotting detection reagents (Amersham, Arlington Heights, IL).

Digestions with endoglycosidase H (Endo H)

Immunoprecipitated samples were denatured at 100°C for 5 min in 100 mM sodium acetate (pH 5.5), 1% SDS before 10-fold dilution in 100 mM sodium acetate buffer (pH 5.5). Samples were then incubated in the presence or absence of 10 mU recombinant Endo H (Boehringer Mannheim) for 16 h at 37°C in the presence of protease inhibitors as described above. Digested samples were concentrated using Microcon 10 spin concentrators (Amicon, Beverly, MA) according to the manufacturer's instructions, fractionated by 15% SDS-PAGE followed by immunoblot analysis.

Results

Fc ϵ RI α - and γ -chain transfection and subcellular localization

To determine the requirements for assembly of the human Fc ϵ RI $\alpha\gamma_2$ receptor, we used transient transfection of HeLa cells with expression plasmids for the Fc ϵ RI α - and γ -chains. The α -chain construct incorporated the myc epitope tag (38) and was positioned at +1 of the mature polypeptide. The γ -chain construct was expressed from the same vector as the α -chain (pcDNA3.1) and incorporated a different epitope tag (FLAG), again positioned at the N terminus of the mature protein, to allow detection of cell surface expression of the γ -chain for the first time. To monitor the efficiency of the transfection, the cell surface expression of both subunits was analyzed 48 h after transfection by flow cytometry. The percentage of cells expressing the myc-tagged α -chain and the FLAG-tagged γ -chain on the cell surface were measured by fluorescence intensity of fluorochrome-labeled secondary detection reagents. In agreement with published data (39), transfection of Fc ϵ RI α -chain alone did not produce significant surface expression as judged by comparison of anti-Fc ϵ RI α mAb staining to isotype control and a mock transfection controls (Fig. 1). As expected, cotransfection of α - and γ -chains produced high density surface expression of α -chain and with a high percentage (nearly 70%) of the transfected cells showing α -chain expression. Parallel FACS analysis revealed pronounced γ -chain surface expression (92% of cells) in cells transiently transfected with both α - and γ -chains. It is notable that γ -chain could be detected on the cell surface given that the N-terminal eight-residue FLAG epitope tag is removed

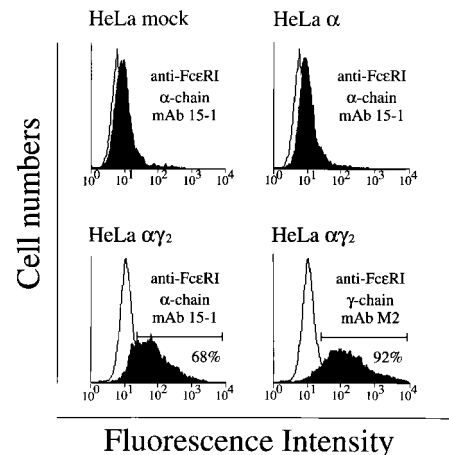


FIGURE 1. Effect of the coexpression of Fc ϵ RI γ -chain on the surface expression of Fc ϵ RI α -chain. HeLa cells were transiently transfected with either the parental (mock) expression vector (HeLa mock), or with a combination of Fc ϵ RI α -chain expression vector and mock expression vector at a 1:1 ratio (HeLa α), or with a combination of the expression vector for α -chain and for FLAG-tagged γ -chain at a 1:1 ratio (HeLa $\alpha\gamma_2$). Forty-eight hours after transfection, cells were harvested and incubated with mAb 15-1 (anti-Fc ϵ RI α -chain), mAb M2 (anti-FLAG-tagged Fc ϵ RI γ -chain), or with mIgG₁ isotype control followed by incubation with a biotin-labeled goat-anti-mouse IgG1 F(ab')₂. Cells were then stained with PE-labeled streptavidin and subjected to analysis by flow cytometry comparing isotype control staining (open histogram) to test mAb staining (shaded histogram).

from the cell membrane by only an additional eight-residue sequence (comprised of an Ala3 spacer plus the constitutive N-terminal γ -chain sequence LGEPQ). Hence, the FLAG epitope is relatively accessible to mAb association within the assembled Fc ϵ RI $\alpha\gamma_2$ receptor complex on the cell surface. The greater fluorescence intensity seen for γ -chain surface expression compared with α -chain might be explained by the simple fact that one α -chain molecule is assembled with a dimer of γ -chains thus producing a 1:2 molar ratio of α : γ at the cell surface. Alternatively, M2 mAb binding in the FACS staining protocol used may occur more efficiently than the mAb staining of α -chain.

In agreement with our FACS data (Fig. 1) and with another study employing transiently transfected COS cells (34), we observed pronounced surface expression of Fc ϵ RI α -chain in HeLa cells cotransfected with both Fc ϵ RI α - and γ -chains, but not α -chain-only transfectants, by immunofluorescence microscopy (data not shown). To identify the fate of the α -chain, we examined the subcellular localization of transiently transfected Fc ϵ RI α -chain in the absence of γ -chain. Transfected and subsequently permeabilized HeLa cells were simultaneously labeled with an Ab specific for the ER-resident chaperone BiP (Ig heavy chain binding protein) and a mAb, 15-1, recognizing native human Fc ϵ RI α -chain. Using confocal microscopy, we observed distinct colocalization (yellow) of α -chain (green) with the ER-resident protein Grp 94 (BiP), which was visualized with a red emitting fluorochrome (Fig. 2). Binding of mAb 15-1 is an indication of correct folding of nascent α -chain because this mAb is thought to bind to a conformationally sensitive epitope (40) that also overlaps with the conformationally sensitive IgE-binding site (5, 40). These data suggest that, in the absence of γ -chains, expression of the α -chain leads to measurable accumulation in the ER compartment and in a state that appears to be folded properly, as judged by anti-Fc ϵ RI α mAb binding.

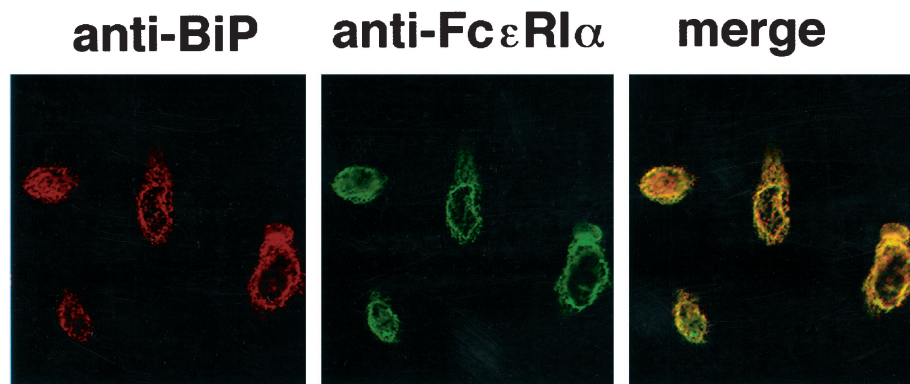


FIGURE 2. Analysis of subcellular localization of FcεRI α-chain in the absence of γ-chains by confocal laser-scanning microscopy. Fixed and permeabilized HeLa cells transfected with FcεRI α-subunit only were incubated with an Ab (mIgG2a isotype) specific for the ER-resident protein BiP (Grp 78) and anti-FcεRI α-chain mAb 15-1 (mIgG1 isotype). The primary Abs were then detected with Texas Red-labeled anti-mouse IgG2a (red) and FITC-conjugated anti-mouse IgG1 (green) secondary Abs. Confocal red and green images (anti-BiP and anti-FcεRI α-chain) were collected and merged to afford yellow color corresponding to Ab colocalization.

FcεRI α-chain immunoprecipitation with 9E10 or IgE

FcεRI α-chain expressed in the absence of γ-chain was then isolated from the ER using an Ab (9E10) specific for the c-myc derived epitope tag. Placement of this tag at the N terminus of the mature α-chain was designed to allow detection of both folded and partially folded forms of the α-chain, in analogy to the use of a polyclonal anti-peptide Ab specific to a sequence proximal to the α-chain N terminus (10). Immunoblot analysis revealed a single sharp band of 54 kDa specific for α-chain, compared with a mock transfection control (Fig. 3, lanes 2 and 1, respectively). Importantly, immunoprecipitation of the ER-resident α-chain could also be accomplished with IgE-Sepharose (Fig. 3, lane 4). In agreement with the data shown in Fig. 2, binding of nascent α-chain to IgE provides evidence that a significant portion of ER-localized α-chain exists in a properly folded state, and without a requirement of FcεRI γ-chain coexpression and αγ2 assembly. As a final characterization effort, we confirmed that the isolated ER-resident α-chain had achieved constitutive N-linked core glycosylation (41) by determination of sensitivity to treatment with either jack bean mannosidase (data not shown) or Endo H (see below), which in both cases afforded an α-chain product that migrated with a lower apparent K_d (~10 kDa) by SDS-PAGE.

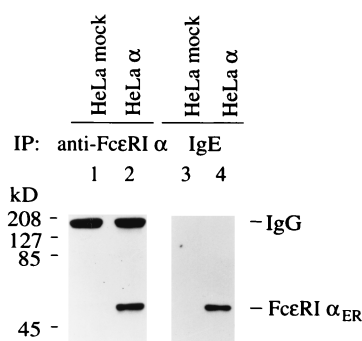


FIGURE 3. IgE binding to ER-localized FcεRI α-chain. HeLa cells were either mock transfected or transfected with FcεRI α-chain containing an N-terminal c-myc epitope tag. Forty-eight hours after transfection, FcεRI α-chain was immunoprecipitated using anti-c-myc 9E10 mAb (designated anti-FcεRIα) or IgE and then fractionated under nonreducing conditions by 12.5% SDS-PAGE. FcεRIα was detected on subsequent immunoblots using anti-FcεRI α-chain mAb 19-1. The position of the m.w. marker as well as the positions of ER-resident FcεRI α-chain (FcεRI α_{ER}) and IgG are indicated.

The role of glucose trimming on FcεRI α-chain expression properties

We then investigated the effect of glucose trimming on the intracellular expression properties of the FcεRI α-chain. Initial experiments evaluated α-chain transfection in HeLa cells cultured with CST, a well characterized inhibitor of the ER-resident enzymes glucosidase I and II, that blocks trimming of glucose residues from constitutive G₃ N-linked core glycans (Ref. 42; see Fig. 4A). HeLa cells were transiently transfected with α-chain and cultured in the presence of CST before immunoprecipitation with IgE and analysis by immunoblotting. In CST-treated cells, an α-chain band (FcεRIα_{ER}^{G₃}) was observed migrating with a distinctly higher apparent K_d than that (FcεRIα_{ER}) detected from untreated cells (Fig. 4B, lanes 2 and 1, respectively), consistent with the expectation of a higher m.w. for the untrimmed G₃ glycoform. Significantly, the untrimmed G₃ α-chain glycoform could be readily isolated by binding to an IgE-Sepharose matrix suggesting that the nascent α-chain had achieved a native fold capable of ligand binding capacity, before initiation of constitutive glucose trimming and ER quality control processes. Furthermore, under the immunoprecipitation conditions employed (excess IgE), the untrimmed G₃ α-chain and the constitutive G₁ (or G₀) form accumulated in the ER to approximately the same extent, as judged by the similar immunoblot band intensities, suggesting that the G₃ and G₁ (or G₀) glycoforms may exhibit similar intracellular stability characteristics.

To extend the foregoing analysis we evaluated the effect of CST on CHO cells stably transfected with the FcεRI α-, β- and γ-subunits (CHO αβγ2; Ref. 6). A profound difference in the immunoblot pattern of IgE immunoprecipitated α-chain was found between CST-treated and -untreated CHO αβγ2 cells (Fig. 4C). In the absence of CST treatment, a broad 60- to 70-kDa signal was found that is characteristic of the heterogeneous, Golgi-processed α-chain glycoforms (FcεRI α_{Golgi}, lane 1) that are typically expressed on the cell surface. In addition, a discrete band of ~54 kDa, characteristic of the ER-resident α-chain glycoform, was also detected (FcεRI α_{ER}, lane 1). Treatment of the immunoprecipitated product corresponding to lane 1 with Endo H had no discernable effect on the Golgi-processed FcεRIα form (FcεRI α_{Golgi}), as expected, whereas the 54-kDa band was converted to a lower m.w. form of less than 30 kDa (FcεRIα_{ER}^{degly}, lane 2), corroborating the ER localization of this α-chain isoform. In contrast immunoprecipitation of α-chain from cells treated with CST

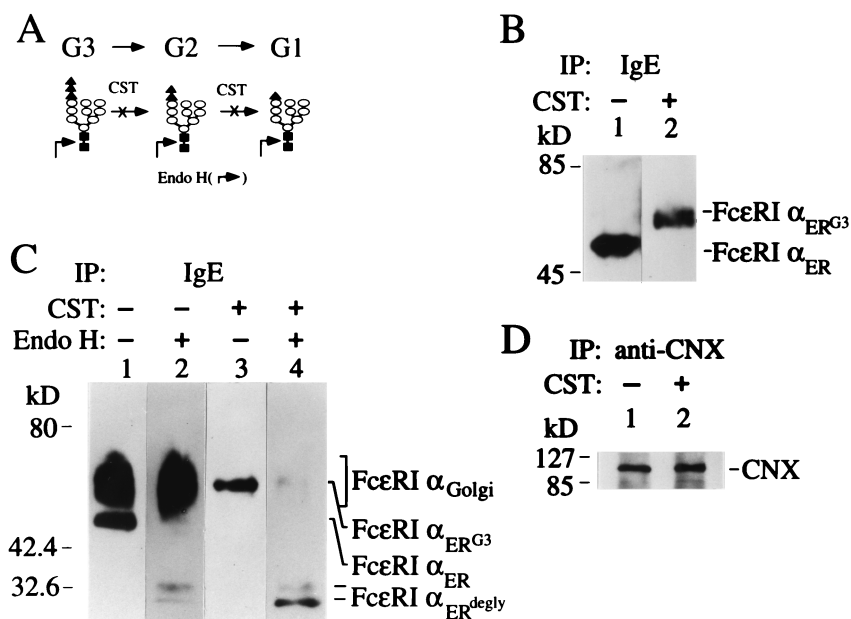


FIGURE 4. Inhibition of glucose trimming of *N*-linked core glycans with CST and its effect on the electrophoretic pattern of FcεRI α-chain. **A**, Schematic structure of G₃, G₂, and G₁ *N*-glycans. Trimming of the terminal glucose residues on *N*-linked core glycans in the ER is inhibited by CST. All ER-localized *N*-glycans (G₃-G₀) are sensitive to treatment with Endo H. **B**, HeLa cells transiently expressing FcεRI α-chain alone were cultured for 48 h in the presence or absence of 300 μg/ml CST. FcεRI α-chain was isolated from cell lysates with IgE and then analyzed on a nonreducing 12.5% SDS-polyacrylamide gel. FcεRI α-chain was detected on the immunoblot using the anti-FcεRI α-chain mAb 19-1. **C**, CHO αβγ₂ cells were cultured in the presence or absence of 300 μg/ml CST for 24 h. Cells were lysed and FcεRI α-chain was immunoprecipitated with IgE. Samples were heat-denatured and then incubated at 37°C for 16 h in the presence or absence of Endo H before analysis on a nonreducing 15% SDS-polyacrylamide gel. FcεRI α-chain was detected on the immunoblot using the anti-FcεRI α-chain mAb 19-1. The positions of the m.w. markers are indicated as well as the position of Golgi-processed, cell surface FcεRI α-chain (FcεRI α_{Golgi}), ER-resident FcεRI α-chain (FcεRI α_{ER}), ER-resident FcεRI α-chain with untrimmed glucose residues (FcεRI α_{ER}G₃), and deglycosylated ER-resident FcεRI α-chain (FcεRI α_{ER}degly). **D**, Calnexin was immunoprecipitated from lysates of CHO αβγ₂ cells that were cultured in the presence or absence of 300 μg/ml CST for 24 h. Immunoprecipitates were analyzed on a nonreducing 12.5% SDS-polyacrylamide gel using anti-calnexin polyclonal serum. The positions of the m.w. markers as well as the position of the calnexin band are indicated.

afforded only a relatively sharp band of ~64 kDa (FcεRI α_{ER}G₃, lane 3). As before, evidence that this α-chain glycoform corresponds to an ER and not Golgi product was demonstrated by sensitivity to Endo H treatment, which afforded an α-chain product with a markedly reduced *M_r* (compare lanes 3 and 4). To control for potential CST-derived artifacts, we determined that essentially equivalent amounts of a constitutive ER protein (calnexin) could be immunoprecipitated from cells cultured with or without CST (Fig. 4D), indicating that CST treatment did not intrinsically alter protein biosynthesis.

Effect of glycosidase inhibition on FcεRI αβγ₂ cell surface expression

Aliquots of the CST-treated and -untreated CHO αβγ₂ cells were also analyzed by flow cytometry to appraise the effect of glycosidase inhibition on α-chain surface expression. The number of receptors expressed on the cell surface was reduced by nearly 50% upon CST treatment (Fig. 5, +CST). After a 24-h incubation with CST, a new population of FcεRI α⁺-positive cells appeared expressing approximately an order of magnitude lower mean fluorescence intensity than that found for the untreated receptor. A similar observation was made upon coculture of CHO αβγ₂ cells with tunicamycin (data not shown). In parallel experiments, we also observed a qualitative increase in ER localization of α-chain in CST-treated CHO αβγ₂ cells, as assessed using confocal microscopy (data not shown). Taken together, these data suggest that inhibition of α-chain glucose trimming affects either the assembly or transport competency of the αβγ₂ complex leading to attenuated cell surface expression.

Effect of glycosidase inhibition on FcεRI αγ₂ cell surface expression

We then appraised the effect of CST-treatment on α-chain surface expression in αγ₂-transfected HeLa cells. HeLa cells were transiently cotransfected with α- and FLAG-γ-chain plasmids as well as a plasmid encoding GFP using logarithmically growing HeLa

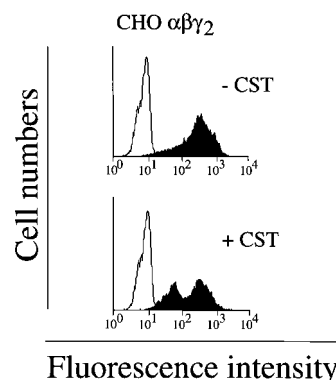


FIGURE 5. Inhibition of glucose trimming of *N*-linked core glycans with CST and its effect on the cell surface expression of FcεRI α-chain. CHO αβγ₂ cells were cultured for 24 h in the presence or absence of 300 μg/ml CST. Cells were harvested and incubated at 4°C with anti-FcεRI α-chain mAb 15-1 (shaded histogram) or mIgG1 isotype control (open histogram), followed by the incubation with biotin-labeled goat-anti mouse IgG1 F(ab')₂. Cells were labeled with streptavidin-PE and analyzed for FcεRI α-chain surface expression by flow cytometry.

cells that had been precultured with CST for 4 h. After transfection and additional culture (48 h) in the presence of CST, the cells were tested for both α - and γ -chain surface expression by flow cytometry. As shown in Fig. 6A (HeLa $\alpha_{wt}\gamma_2$), α -chain surface expression was significantly reduced in CST-treated cells compared with untreated cells (18% and 28% α -chain positive cells, respectively). At the same time, the level of γ -chain expression showed a discrete increase (from 49% to 58% of transfected cells) in the CST-treated cells. To establish whether these results were derived from different transfection efficiencies resulting from CST-treatment, compared with untreated control cells, we measured the level of transfection efficiency based on intracellular expression of GFP. As shown in Fig. 6B, the percentage of GFP-positive cells was essentially the same with or without CST-treatment, suggesting that α - and γ -chain transfection efficiency was not intrinsically affected by CST treatment.

Cell surface expression of a Fc ϵ RI α -chain truncation mutant lacking the C-terminal half of the CD

As described earlier, the wild-type Fc ϵ RI α -chain cannot achieve cell surface expression without coexpression and assembly with the Fc ϵ RI γ_2 -subunit. We hypothesized that deletion of the putative di-lysine ER localization motif might allow α -chain expres-

sion in the absence of the γ -subunit. A truncated α -chain was constructed lacking the C-terminal 16 residues of the CD that included the -7 and -3 lysine residues. Expression of this truncated α -chain, designated α_t , in transfected HeLa cells was detected on the cell surface in the absence of γ -chain cotransfection (Fig. 6A; HeLa α_t , -CST). This finding indicates that the deleted CD sequence represents most or all of the principle determinant involved in ER localization and appears to show that the TM domain contributes relatively little to the ER retention mechanism. As such, the truncated α -chain represents a simplified model for the study of α -chain biosynthesis and cell surface transport. Therefore, we tested the effect of glucosidase inhibition on α_t expression in cells cultured with or without CST. We observed a prominent reduction (75%) in α_t surface expression (Fig. 6A; HeLa α_t , +CST) in cells cocultured with CST. Importantly, the CST-treated cells showed a similar transfection efficiency compared with the untreated control cells, based on the similar GFP expression characteristics (Fig. 6B; α_t). Furthermore, we also measured a significant population of double-positive cells staining for surface α_t and intracellular green fluorescence from cotransfected GFP in non-CST-treated cells compared with CST-treated cells (Fig. 6C, compare *middle* and *right panels*, respectively). Taken together, a consistent cell surface expression pattern was found for α_t and the $\alpha\gamma_2$ and $\alpha\beta\gamma_2$ receptor complexes, suggesting a strong link between constitutive glucose trimming and Fc ϵ RI α cell surface expression.

Association of the ER chaperone calnexin with Fc ϵ RI α -chain

The presence and importance of deglycosylated α -chain glycoforms such as G_1 and G_0 in the transport pathway leading to cellular expression is strongly implied from the foregoing studies. It is well established that the G_1 ER glycoform is the substrate recognized by ER lectin-like chaperones calnexin and calreticulin (43–45). Therefore, we tested whether the α -chain transfected in HeLa cells could specifically associate with calnexin as measured by the capacity to coimmunoprecipitate the two proteins. As

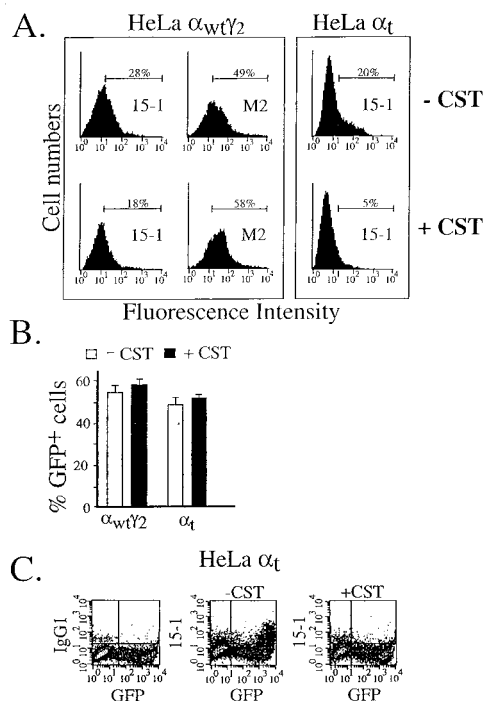


FIGURE 6. Inhibition of glucose trimming of *N*-glycans and its effect on the cell surface coexpression of wild-type Fc ϵ RI α -chain (α_{wt}) and γ -chains or a Fc ϵ RI α -chain truncation mutant lacking 16 residues from the carboxy terminus of the cytoplasmic domain (α_t). A, HeLa cells, cultured in the presence or absence of CST, were transiently cotransfected with an expression vector for GFP together with expression vectors for α_{wt} and γ -chain ($\alpha_{wt}\gamma_2$) or α_t only. Forty-eight hours after transfection, cells were harvested and stained with mAb 15-1, mAb M2, or with a mIgG1 isotype control for analysis by flow cytometry. GFP-fluorescence was analyzed in the FITC-channel of the 488-nm laser. B, The percent of GFP⁺ cells was analyzed as an internal control to monitor the transfection efficiency of HeLa cells ($\alpha_{wt}\gamma_2$ and α_t) cultured with (filled bars) or without (open bars) of CST ($n = 3$). C, A representative example of two-color dot-blot analysis of the intensity of GFP and PE fluorescence after 15-1 staining or isotype (IgG1) control staining of HeLa cells transiently transfected with α_t and GFP and cultured for 48 h in the presence or absence of CST.

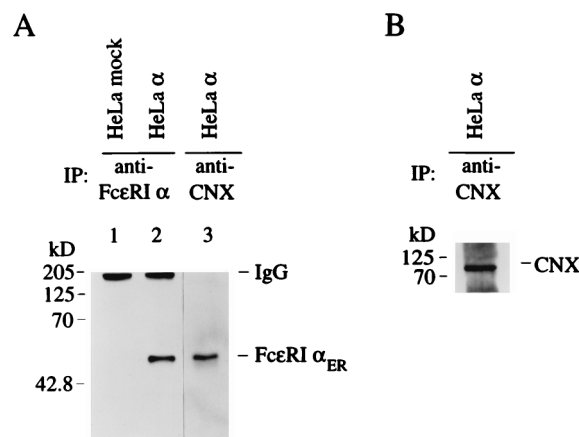


FIGURE 7. Fc ϵ RI α -chain is coimmunoprecipitated with the ER-chaperone calnexin. A, HeLa cells were transiently transfected with an expression vector for the c-myc-tagged Fc ϵ RI α -chain alone. Cells were lysed and immunoprecipitations were performed with either 9E10 mAb or with commercial polyclonal rabbit anti-calnexin sera. Immunoprecipitates were separated on a nonreducing 12.5% SDS-polyacrylamide gel. Fc ϵ RI α -chain was detected on the immunoblot using the anti-Fc ϵ RI α -chain mAb 19-1 after 9E10 (lane 2) and anti-CN (lane 3) immunoprecipitation but not in mock transfected control (lane 1). The position of the molecular mass markers and the ER-resident Fc ϵ RI α -chain (Fc ϵ RI α_{ER}) are indicated. B, Calnexin was detected on the immunoblot using anti-calnexin serum. The position of molecular mass markers and the calnexin band (CNX) are indicated.

shown in Fig. 7A, the characteristic myc-tagged α -chain could be immunoprecipitated from lysates of transiently transfected cells with mAb 9E10 (*lane 2*). At the same time, immunoprecipitation with polyclonal anti-calnexin sera afforded coprecipitation of the α -chain, visualized in an immunoblot with an anti-Fc ϵ RI α mAb (19-1; *lane 3*). As expected, the same anti-calnexin sera was effective in immunoprecipitation of the endogenous 90-kDa calnexin protein as revealed by immunoblot analysis (Fig. 7B) using the same anti-calnexin sera. In parallel immunoprecipitation experiments, we could not detect coprecipitation of α -chain with polyclonal anti-calreticulin sera (data not shown). To our knowledge, the foregoing analysis is the first demonstration of ER chaperone association with a nascent Fc ϵ RI subunit or for any subunit within the Fc receptor family.

Discussion

In this study, we examined aspects of intracellular expression and transport of the individual Fc ϵ RI α -chain as well as Fc ϵ RI $\alpha\gamma_2$ and $\alpha\beta\gamma_2$ receptor complexes. Our experimental approach employed transient transfection of Fc ϵ RI subunits followed by flow cytometry and/or immunoprecipitation. In agreement with previous studies (39), Fc ϵ RI α -chain cell surface expression was dependent on coexpression of γ -chain with no detectable surface α -chain found in α -chain-only transfected cells using an anti-Fc ϵ RI α mAb for detection (Fig. 1). We probed the same transfected cells with a mAb (9E10) specific for the myc epitope tag that had been placed at the N terminus of the mature α -chain and again could not detect surface expression of the α -chain (data not shown). It was anticipated that the N-terminal tag would be accessible for mAb binding in either the folded or unfolded state, and thus the absence of 9E10 binding suggests that little if any α -chain can achieve surface expression. It has been hypothesized that the α -chain is retained in the ER because of the presence of a nonclassical dilysine ER-retrieval signal located near the intracellular COOH terminus, and that the α -chain is subsequently rapidly targeted for degradation (34). Our data using confocal scanning microscopy demonstrate a significant signal for ER-localized human Fc ϵ RI α -chain expressed without γ -chains (Fig. 2). Furthermore, the intracellular α -chain could be isolated from cell lysates through binding to an IgE-Sepharose affinity matrix (Fig. 3), suggesting that the α -chain can fold properly in the ER before assembly with the γ -subunit. Moreover, the quantity of intracellular α -chain isolated with IgE was comparable to that isolated with the 9E10 mAb (Fig. 3, *lanes 4 and 2*, respectively) suggesting that a high proportion of the ER-localized α -chain must exist in a native-like form.

The ER-resident Fc ϵ RI α -chain migrates as a distinct 54-kDa band by SDS-PAGE suggestive of a homogeneous product, in contrast to the broad signal of the post ER compartment-processed α -chain, containing heterogeneous complex oligosaccharides derived from Golgi glycosylation. The lack of complex glycosylation suggests that the 54-kDa α -chain glycoform has likely been actively retained in the ER instead of transport to and retrieval from the Golgi compartment (33). It is interesting to compare the foregoing α -chain characteristics to intracellular α -chain isolated from cells shown to be devoid of cell surface Fc ϵ RI. A considerably more complex SDS-PAGE/immunoblotting profile of α -chain immunoprecipitated from either LC (23) or eosinophils (26) was found suggestive of additional carbohydrate processing that, in turn, implies that the α -chain in these cells is retrieved to the ER from post-ER compartments.

We then analyzed cells cotransfected with α - and γ -chains for cell surface expression of $\alpha\gamma_2$ receptors by flow cytometry using mAbs specific for the extracellular domains of the individual receptor subunits. Direct detection of γ -chains on the cell surface is

hampered by the presence of only five extracellular residues and indeed no mAbs have been described for this epitope, although polyclonal sera specific for the extracellular domain of the rat γ -chain have been reported (46). To overcome this limitation, we placed an epitope tag (FLAG) at the N terminus of the mature γ -chain and showed that the tagged γ -chain was functional in assembly and transport of the α -chain. Transport competency also implied that constitutive cleavage of the γ -chain leader peptide had likely occurred in the expected manner. Interestingly, a γ -chain construct with a similarly positioned RGS6 epitope tag was found to be at least as functional as the FLAG-tagged γ -chain in directing α -chain surface expression but could not be detected using a panel of different anti-His6 mAbs (not shown).

We then focused our analysis on the role of N-linked glycosylation on expression of $\alpha\gamma_2$ and $\alpha\beta\gamma_2$ receptors in eukaryotic cells by targeted inhibition of ER glycosylation. Inhibition of glucosidases I and II leads to accumulation of G_3 glycoforms (47). In CST-treated, α -chain-only transfectants the G_3 α -chain glycoform exhibited IgE binding activity indicating that the nascent G_3 α -chain has achieved a functional fold capable of ligand binding. As an important comparison, we evaluated IgE immunoprecipitation of glucosidase-treated CHO $\alpha\beta\gamma_2$ cells and found a homogeneous SDS-PAGE band of higher apparent m.w. than the ER α -chain glycoform (Fig. 4C). Evidence that this product was localized to the ER was demonstrated by sensitivity to Endo H treatment (*lane 4*). The apparent lack of highly glycosylated, Golgi-processed α -chain (*lane 3*) suggests that comparatively little cell surface α -chain was present after 24 h exposure to CST, at least in a form that was detergent soluble and capable of binding IgE. Although the turnover rate of Fc ϵ RI $\alpha\beta\gamma_2$ in these cells has not been quantitated, it seems likely that over a 24-h period constitutive Fc ϵ RI internalization and subsequent degradation of preexisting cell surface receptor will occur, in analogy to rat Fc ϵ RI turnover in RBL cells, which occurs to a significant extent in a 24 h period, in the absence of bound IgE (minimum half life, 8 h; Refs. 48 and 49).

Initial evidence for an important role of N-linked oligosaccharides in folding and transport of the Fc ϵ RI α -chain came from a study that showed the dependence of N-linked glycosylation on the secretion profile of a truncated α -chain consisting of only the extracellular domain (9). We have now analyzed the role of N-linked glycosylation for export of Fc ϵ RI to the cell surface. Using the same strategy as described above, we used CST to block constitutive glucose trimming and appraised the effect on Fc ϵ RI cell surface expression. CST-treated CHO $\alpha\beta\gamma_2$ cells displayed an order of magnitude reduction of α -chain cell surface density on more than 50% of the cells (Fig. 5). In addition, the overall decrease in constitutive surface receptor is accompanied by the appearance of a new population of cells with reduced mean fluorescence intensity, a finding consistently observed in replicate experiments. The appearance of a discrete Fc ϵ RI α^{dim} population is intriguing but presently not well understood. What is clear from the FACS analysis is that glucosidase inhibition afforded a profound reduction in overall Fc ϵ RI α -chain cell surface expression. This finding is in agreement with the reported decrease in cell surface expression of rodent Fc ϵ RI $\alpha\beta\gamma_2$ in CST-treated rat basophilic leukemia cells (50) and CST-treated cells expressing the insulin receptor (51). In the latter study, a 24-h incubation period resulted in a 50% reduction of surface receptors. Similarly, a CST-induced decrease (40%) in expression of the integral membrane low-density lipoprotein receptor has been reported and ascribed to a mechanism involving receptor redistribution (52).

CST-treatment of HeLa cells cotransfected with α - and γ -chains also produced a pronounced reduction of Fc ϵ RI α -chain cell surface expression (Fig. 6). In these experiments, we also monitored the level of FLAG- γ -chain surface expression and observed comparable γ -chain expression with or without CST treatment, suggesting that glucosidase inhibition does not intrinsically alter de novo protein synthesis. From this, we infer that the CST-dependent differences in α -chain expression are derived solely from differences in the level of glucose trimming. At the same time, we observed a reproducible increase in γ -chain expression in the CST-treated cells. This finding suggested the possibility that the γ -chain might be transport competent in the absence of constitutively processed α -chain. Indeed, in preliminary studies, we have observed that γ -chain-only transfected HeLa cells show a strong pattern of cell surface expression measured by flow cytometry (data not shown), a finding that we are currently analyzing in further detail.

We also detected a significant level of cell surface expression of a truncated α -chain (α t in Fig. 6) without γ -chain cotransfection. The capacity of α t to achieve surface expression suggests that the ER localization motif must be the principle determinant involved in ER retention and that the TM domain exerts comparatively little effect. Furthermore, we observed a sharp reduction (75%) in cell surface expression of α t in cells treated with CST, suggesting that the same glucose-trimming mechanism operative in ER quality control of the constitutive α -chain is also operative in α t surface expression. Taken together, our data suggest that the ER-export signal for Fc ϵ RI is stringently regulated by both 1) the number of terminal glucose residues present on core oligosaccharide units of the α -chain, and 2) the presence of an ER localization signal in the α -chain CD. Achievement of a transport competent α -chain may be more under the control of the α -chain ER dilysine localization signal than TM domain-mediated effects although, like the structurally related Fc γ RIII α γ 2 receptor (53), the stable assembly of the Fc ϵ RI α - and γ -chains is likely mediated through the TM domains of the two subunits (34). Assuming that the α -chain glycoform can assemble normally with homodimeric γ -chains in the untrimmed G₃ state, then masking of the α -chain dilysine ER localization motif by the γ -chain is a necessary but insufficient signal for export of Fc ϵ RI complexes.

Glucose trimming of the N-linked glycans likely serves as a critical checkpoint for continuation along the secretory pathway. Monoglucosylated G₁ glycoforms are potential substrates for lectin-like ER-chaperones such as calnexin and calreticulin, well established components of the ER quality control network (45) that act to assist and promote nascent polypeptide folding before further deglycosylation (to the G₀ form) and export from the ER (54). We postulated that the constitutive ER Fc ϵ RI α -chain G₁ glycoform might associate with one or more of the lectin-like chaperones and demonstrated that anti-calnexin antisera could coimmunoprecipitate α -chain and calnexin from lysates of HeLa cells transiently transfected with only the α -chain (Fig. 7). At the same time we could not detect coprecipitation of α -chain using antisera against the 60-kDa ER lumenal protein calreticulin and Grp78 (data not shown). The extent of calnexin and calreticulin association with α -chain expressed as either an α γ 2 or α β γ 2 receptor is currently under investigation.

It is informative to compare the requirements for Fc ϵ RI export to that of other multisubunit receptor complexes such as TCR or MHC class I proteins. The comparison of Fc ϵ RI with TCR is especially interesting because both receptors can use either the ζ -chain dimer or the Fc γ γ -chain dimer in assembly and as a signal transduction subunit (55). In this case, glucose trimming was demonstrated to be essential for the stability of nascent TCR α -chain in the ER and its association with TCR β -chain (56). However, for

the assembly of the MHC class I complex, glucose trimming of glycans is not necessary for assembly and export to the cell surface, although important for the association of free heavy chains with calnexin (57). Studies using either a calnexin-deficient cell line (58) or glucosidase II-deficient cells (57) demonstrated efficient expression of MHC class I proteins. Thus, alternative pathways within the ER quality control system exist to assist MHC class I assembly. Inhibition of Fc ϵ RI α glucose trimming by CST led to reduced cell surface expression of α β γ 2, α γ 2 and an α t truncation mutant. The possibility that α -chain association with ER lectin chaperones such as calnexin may serve as a critical checkpoint in the generation of new transport-competent receptor molecules is currently under further investigation.

Acknowledgments

We thank A. Forcada-Lowrie for technical assistance and Dr. F. Zenke for helpful discussions. CHO cells that stably express the human Fc ϵ RI α β γ 2 receptor were generously provided by Dr. J.-P. Kinet. The preparation of oligonucleotides and DNA sequencing was performed in the MEM DNA core laboratory funded through the Stein Foundation. This is The Scripps Research Institute manuscript number 12517-MEM.

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