Cotranslational endoplasmic reticulum assembly of FcεRI controls the formation of functional IgE-binding receptors

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The human high affinity receptor for IgE (FcεRI) is a cell surface structure critical for the pathology of allergic reactions. Human FcεRI is expressed as a tetramer ($\alpha\beta\gamma_2$) on basophils or mast cells and as trimeric ($\alpha\gamma_2$) complex on antigen-presenting cells. Expression of the human α subunit can be down-regulated by a splice variant of FcεRIβ (β_{var}). We demonstrate that FcεRIα is the core subunit with which the other subunits assemble strictly cotranslationally. In addition to $\alpha\beta\gamma_2$ and $\alpha\gamma_2$, we demonstrate the presence of αβ and $\alpha\beta_{var}\gamma_2$ complexes that are stable in the detergent Brij 96. The role of individual FcεRI subunits for the formation of functional, immunoglobulin E–binding FcεRI complexes during endoplasmic reticulum (ER) assembly can be defined as follows: β and γ support ER insertion, signal peptide cleavage and proper N-glycosylation of α, whereas β_{var} allows accumulation of α protein backbone. We show that assembly of FcεRI in the ER is a key step for the regulation of surface expression of FcεRI. The ER quality control system thus regulates the quantity of functional FcεRI, which in turn controls onset and persistence of allergic reactions.

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Abbreviations used: CC, U373; EndoH, endoglycosidase H; ITAM, immunoreceptor tyrosine–based signaling motifs.

A significant fraction of the population (~20%) in the Western world is affected by allergies and the numbers of affected individuals is on the rise (1, 2). Convincing evidence exists that FcεRI is one of the key molecules in the pathophysiology of all allergic reactions (3–6). As a member of the antigen receptor superfamily, FcεRI shares the organizational principles of a ligand binding immunoglobulin-type protein associated with signaling subunits that regulate cellular activation via conserved immunoreceptor tyrosine–based signaling motifs (ITAMS; 7). BCR, TCR, and other Fc receptors fall in the same class (7–10). FcεRI was initially described as a tetrameric receptor composed of a high-affinity ligand-binding α chain, one β chain, and a pair of disulphide-linked γ subunits (5, 9). The FcεRI complexes on the surface of basophils and mast cells are tetrameric structures ($\alpha\beta\gamma_2$). The $\alpha\beta\gamma_2$ is the only receptor isoform formed in rodents (5). Human antigen-presenting cells additionally display a trimeric form of FcεRI that lacks the β subunit (5, 11, 12). A new splice variant of FcεRIβ (β_{var}, formerly referred to as β_T) exerts a dominant negative effect on β function (13).

The structural integrity of FcεRI is maintained by the noncovalent interactions of its various subunits. The extracellular domain of FcεRIα forms the binding site for the C_H3 domain of IgE. It binds its ligand in 1:1 ratio, with an affinity of $\sim 10^{10}$ M^{-1}. The β chain contains four potential transmembrane spanning regions with both the NH_2 and the COOH terminus protruding into the cytosol. FcεRIγ forms a dimer and is a member of the ζ gene family. IgE-dependent cross-linking of FcεRI induces cellular activation regulated via ITAMs, which are present in one copy in the β as well as in each of the γ chains (5, 9, 10). The α subunit, when expressed in the absence of β and γ, is retained in the ER. The ER retention signal of human α can be overcome by the presence of γ alone. FcεRIβ was defined as an amplifier for γ chain signaling in vitro and in vivo (14, 15) and as a regulator for surface expression. The β_{var} subunit is a splice variant that has lost its ITAM (13). Therefore β_{var}-containing complexes must behave significantly differently from those that contain the conventional β chain.

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Multisubunit receptor complexes, like FcεRI or the TCR, are assembled in the ER, from where they enter the secretory pathway (16, 17). The acquisition of the proper tertiary and quaternary structure in the ER is a carefully controlled sequence of events. Nascent polypeptides are subject to modifications, which often include signal–peptide cleavage, N-linked glycosylation and oligosaccharide trimming. Folding of proteins is guided by chaperones such as BiP and the lectins calnexin and calreticulin. Oxido-reductases control the formation of disulfide bonds between the correct pairs of cysteine residues to stabilize the folded structure (18). As a consequence of imperfections in protein folding, some polypeptides never attain their native conformation. Terminally misfolded proteins are singled out in the ER by a quality control process (19–21). However, their destruction takes place mostly in the cytosol. ER quality control substrates may cross the ER membrane before their degradation (22). In addition to the proper folding of the individual subunits, multimeric receptors like FcεRI must assemble in a concerted fashion. Only with all players in place, can ER retention signals be overcome. The sequence of events in FcεRI receptor assembly in its various configurations is interesting with respect to the functional differences of the receptor isoforms. These events all contribute to the control of receptor expression and thereby the outcome of allergic responses in vivo. The generally increased cell surface expression of FcεRI in allergic individuals supports this hypothesis (23, 24).

Studies on the human receptor are hampered by a lack of cell lines that express FcεRI irrespective of its isoforms. Primary human cells that express FcεRI are difficult to obtain, even in small numbers. Because these cells shut down synthesis of the receptor immediately after isolation, they cannot be used to study complex formation and regulation of surface expression. We therefore used in vitro translation in membrane-supplemented rabbit reticulocyte lysates to study the early events of FcεRI assembly in the ER. We translated the corresponding mRNAs of all FcεRI subunits and performed studies on temporal aspects of protein–protein interaction and their consequences for receptor assembly. Our results show that ER assembly of the individual FcεRI subunits is tightly controlled and indeed regulates the formation of properly formed receptors with IgE-binding epitopes.

RESULTS

In vitro translation of FcεRIα

We used in vitro translation as a method to study FcεRI receptor assembly in the ER (16, 25, 26), because aspects of multimeric receptor assembly cannot be studied in a time-resolved fashion in transfection experiments. First, we characterized the properties of the individual receptor subunits in this assembly system. The cDNAs for the human FcεRI subunits allow the generation of the corresponding mRNAs for in vitro translations. The mRNAs were translated in the presence of microsomes from different sources. FcεRIα is a type I membrane protein and requires cleavage of its signal sequence before N-glycosylation, which is in turn required for the formation of functional IgE-binding sites (5, 9).

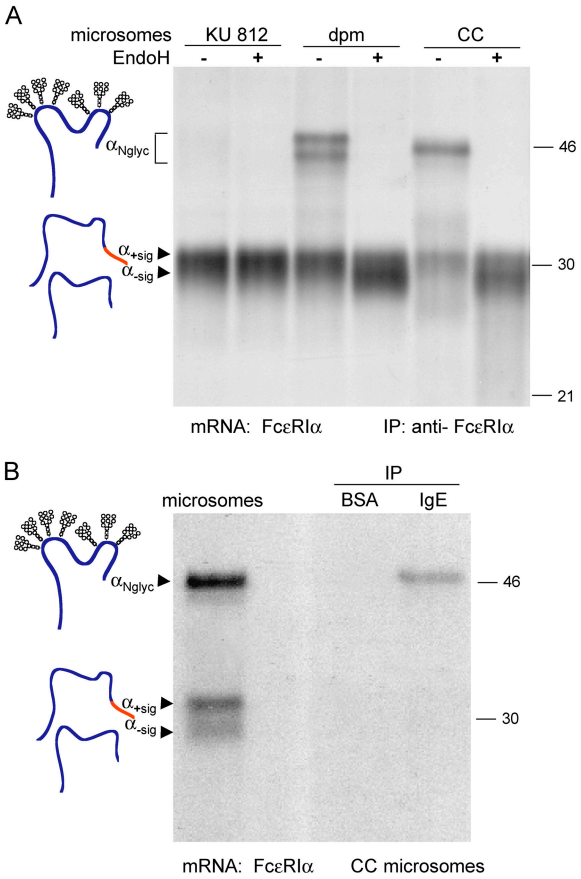

Figure 1. In vitro translation of IgE-binding FcεRIα. (A) Signal peptide cleavage and N-glycosylation of FcεRIα depends on the source of microsomes used during in vitro translation. Transcripts of K^b-FcεRIα were translated for 1 h at 30°C in the presence of microsomes derived from KU812, dog pancreas (dpm), or U373 (CC). After lysis of the microsomal pellets in 1% Brij96 lysis buffer, immunoprecipitates were obtained with polyclonal anti-α serum and either mock digested or digested with Endo H. Subsequently immunoprecipitates were analyzed by 12.5% SDS-PAGE under reducing conditions. Conversion of α_{+sig} to α_{Nglyc} occurs most efficiently in CC microsomes. (B) α_{Nglyc} generated with CC microsomes expresses properly folded IgE-binding epitopes. IgE selectively precipitates the fully N-glycosylated form of FcεRIα. Representative experiments ($n = 3$).

FcεRIα cDNA equipped with its endogenous signal peptide translated poorly. Although we could detect the expected polypeptides reactive with anti–α serum, cotranslation of the α construct with β and γ mRNAs would have rendered further assembly studies technically difficult (unpublished data). We therefore exchanged the signal sequence of the α subunit for that of H2-K^b. The latter has proved efficient for translation as well as for adjustment of insertion efficiencies of different subunits during TCR assembly (16). This swap of signal peptides allowed efficient translation of FcεRIα ($H2\text{-}K^bFc\varepsilon RI\alpha$, referred to as α; Fig. 1 A). The source of microsomes proved critical for the generation of α chains with cleaved signal peptide (α_{-sig}, Fig. 1 A) as well as for N-glycosylation (α_{Nglyc}, Fig. 1 A), although no such effect was observed for FcεRIγ or HLA-2A (unpublished data). Microsomes from the basophilic cell line KU812 allowed effi-

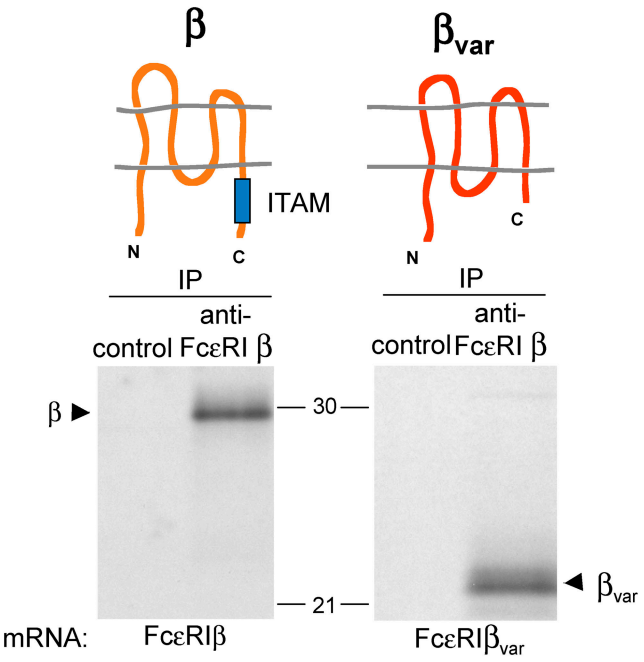

Figure 2. In vitro translation of FcεRIβ and FcεRIβ$_{var}$. (A) Transcripts of FcεRIβ or FcεRIβ$_{var}$ were translated for 1 h at 30°C in the presence of CC microsomes. After lysis of the microsomal pellets in 1% Brij96 lysis buffer, immunoprecipitates were obtained with polyclonal anti-β serum and analyzed by 12.5% SDS-PAGE under reducing conditions. Representative experiments ($n = 3$).

cient translation, but yielded α mostly as α_{+sig} (Fig. 1 A). Microsomes derived from canine pancreas were successful in creating α_{Nglyc}, but a sizable fraction of the translated protein was present as α_{+sig} (Fig. 1 A and unpublished data). Microsomes derived from the astrocytoma cell line U373 (CC) reproducibly generated endoglycosidase H (EndoH)-sensitive α_{Nglyc} efficiently (Fig. 1 A).

We then asked whether we could generate FcεRIα with proper IgE-binding epitopes, using IgE as the bait to recover translation products from microsomal pellets (Fig. 1 B). The direct analysis of the microsomal extracts shows the presence of all forms of FcεRIα (α_{+sig}, α_{-sig}, and α_{Nglyc}, Fig. 1 B), but only properly folded and N-glycosylated α is recovered by IgE (Fig. 1 B).

In vitro translation of FcεRIβ and FcεRIβ$_{var}$

The identity of the translation products from β and β_{var} (13) was confirmed by immunoprecipitation with an anti-β serum generated against the NH_2 terminus of β, therefore reactive with both splice variants (Fig. 2 A). Indeed, both proteins migrate at their expected molecular weight of ~28,000 and 22,000, respectively (Fig. 2 A; reference 13).

In vitro translation of FcεRIγ

The γ chain is a type I membrane protein (3, 8, 9). To achieve comparable translation efficiencies, we exchanged the signal sequence of γ that of H2-K^b (H2-K^{b}FcεRIγ, referred to as γ; Fig. 3). Fig. 3 A shows the proper insertion and signal peptide cleavage of γ in the in vitro translation assay with a

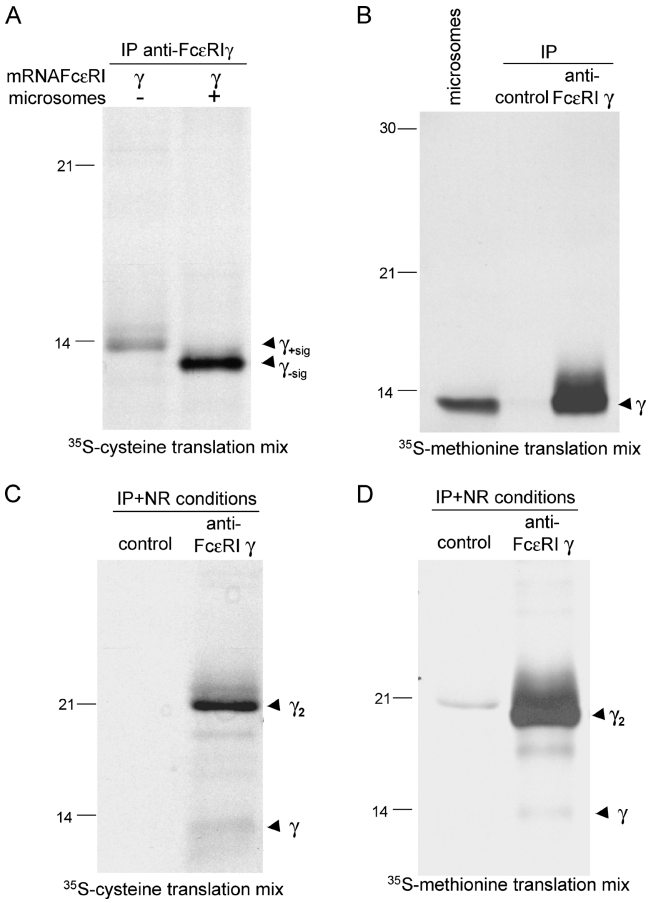

Figure 3. ER insertion and proper signal peptide cleavage of FcεRIγ. (A) Proper insertion and signal peptide cleavage of K^b-γ in an in vitro translations with a [^{35}S]cysteine translation mix. (B) In vitro translations of K^b-γ with a [^{35}S]methionine translation mix. The single methionine of γ gets cleaved after insertion into the microsomes. [^{35}S]Cysteine present in the [^{35}S]methionine translation mix is sufficient for labeling of K^b-γ. In vitro translations were performed in the absence and presence of CC microsomes. Translation products were analyzed after immunoprecipitation from 1% Brij96 lysates of in vitro translations performed in the absence of microsomes (−) or lysates of microsomal pellets (+) and analyzed by 12.5% SDS-PAGE under reducing (A and B) or nonreducing (C and D) conditions. FcεRIγ forms a dimer under nonreducing conditions. Representative experiments ($n = 3$).

[^{35}S]cysteine translation mix. The single methionine present in γ is removed upon insertion into the microsomes. However, labeling with the [^{35}S]methionine translation mix (containing both [^{35}S]Met and [^{35}S]Cys) allows sufficient labeling as shown in anti-γ immunoprecipitations (Fig. 3 B). For reasons that remain unclear, in vitro translations using [^{35}S]cysteine did not allow visualization of β_{var} (unpublished data). Thus, all subsequent translation assays were performed with the [^{35}S]methionine translation mix. Under nonreducing conditions, γ runs as a dimer with both translation mixes (Fig. 3, C and D).

Receptor assembly studies: αγ complexes form cotranslationally

The next set of experiments addressed the existence of αγ complexes and their assembly. Anti-α immunoprecipitations from Brij 96 lysates of microsomal pellets demonstrate the

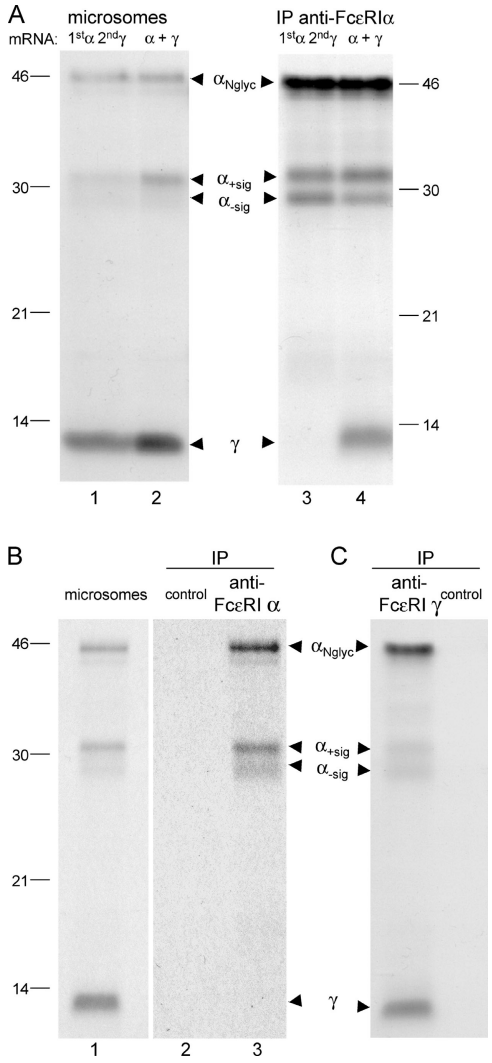

Figure 4. FcεRI complexes form cotranslationally. (A) Cotranslational formation of αγ complexes. α and γ RNA were translated consecutively (lanes 1 and 3, 1st α 2nd γ) or at the same time (lane 2 and 4, α + γ) into CC microsomes. Direct loads of the microsomal pellets confirmed the presence of both translation products (lanes 1 and 2). αγ complexes were only retrieved by anti-α immunoprecipitation, if both proteins were translated at the same time (lane 4, α + γ). (B) α and γ were translated separately and microsomal pellets were mixed before lysis. Direct loads of microsomes showed the presence of both proteins. No αγ complexes were precipitated. (C) Immunoprecipitations of αγ complexes with serum directed against γ.

presence of stable αγ complexes (Fig. 4 A, lane 4). Such complexes arise only if both proteins are translated at the same time. Microsomal pellets from assay mixtures in which α and γ RNA were translated consecutively are devoid of αγ complexes, despite the presence of both proteins in the direct load of the microsomes (Fig. 4 A, lanes 1 and 3). Complexes of αγ where absent when proteins were translated separately and then mixed before or after lysis (Fig. 4 B; and unpublished data). Direct loads of microsomes from translation mixtures in which α and γ were present concurrently generally show more properly folded α_{Nglyc} when compared with samples in which α was translated alone. This effect is even more pronounced when β is cotranslated as well (unpublished data and see Fig. 6 A). Immunoprecipitations of αγ complexes with serum directed against γ confirmed the association of α_{+sig}, α_{-sig}, and α_{Nglyc} with γ (Fig. 4 C).

Cotranslation of β and γ chains resulted in efficient insertion of both proteins into microsomes (Fig. 5 A, lane 1). Neither serum coprecipitated the other protein. These experiments control for proper solubilization under the necessary mild lysis conditions and further show that βγ complexes do not occur in the absence of α (Fig. 5 A, lanes 2 and 3). We also failed to detect βγ complexes when β and γ mRNAs were translated separately and microsomes were mixed before lysis (Fig. 5 D and E, lanes 2) or when microsomal pellets of cotranslation experiments were lysed in 1% digitonin (unpublished data).

These results fit with the assumption that the α chain is the core of all FcεRI complexes. This hypothesis also implies a direct interaction of α with β. We therefore attempted to demonstrate the existence of such complexes by in vitro translation. As shown in Fig. 5 B, the αβ complex is stable in Brij96 and is generated only cotranslationally (Fig. 5, D and E, lanes 3). Due to its molecular weight, β is difficult to distinguish from α_{+sig} and α_{-sig}. The anti-β reimmunoprecipitation unequivocally demonstrates the existance of αβ complexes (Fig. 5 B, lane 2). We could also demonstrate the presence of these complexes on a cellular level by immunoprecipitations from 293 cells transiently transfected with α and β complexes (Fig. 5 C). The use of tagged versions of both proteins allowed the detection of the individual subunits by immunoblotting after immunoprecipitation. The αβ complexes can be retrieved specifically with an anti-HA reagent via α_{HA} (Fig. 5 C, lanes 2–4), but not with a control Ab (Fig. 5 C, lane 1). A considerable amount of this α protein becomes EndoH resistant, indicative of modifications of N-glycans in the Golgi apparatus (α_{mod}; Fig. 5 C, lane 3) and thus proper ER exit of αβ complexes. Digestion of immunopreciptated αβ complexes with PNGase was performed to provide further evidence for proper folding of α. PNGase preferentially attacks improperly folded glycoproteins (27), and the resistance of α to digestion by this enzyme thus supports our findings that α is expressed as properly folded protein in αβ complexes.

Cotranslation of HLA-A2 with β and γ were performed as specificity controls (Fig. 5 D, lanes 4 and 6). As expected, HLA-A2 failed to form a complex with β and γ (Fig. 5 E, lanes 4 and 6).

Immunoprecipitation of αβγ and $\alpha\beta_{var}\gamma$ complexes

The α,β, γ, or α,$\beta_{var,}$ γ mRNAs were translated into microsomes, which were then solibilized in 1% Brij96 and subjected to immunoprecipitation. The anti-γ but not the control serum successfully precipitated αβγ complexes (Fig. 6 A). The anti-γ reagent also retrieved stable $\alpha\beta_{var}\gamma$ complexes (Fig. 6 A). The α chain in the latter complexes seemed to be underrepresented when compared with αβγ.

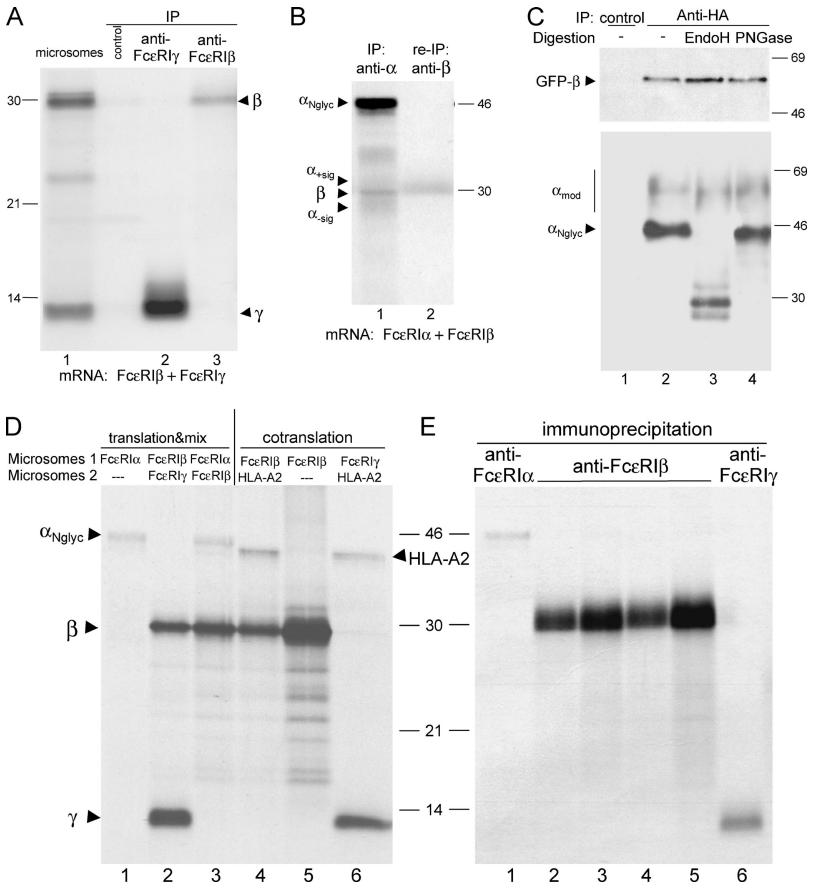

Figure 5. Association studies of different FcεRI subunits. (A) Cotranslation of β and γ chains results in efficient expression of both proteins in microsomes. Anti-β and -γ serum both failed to coprecipitate βγ complexes. (B) Demonstration of αβ complexes. Anti-α serum precipitates stable αβ complexes. The presence of β is in the complex is confirmed by anti-β reimmunoprecipitations. (C) Confirmation of αβ complexes in 293 cells transiently transfected with α_{HA} and GFP-β cDNA. (D and E) The β and γ subunits associate with α but not HLA-A2. FcεRI complexes form only cotranslationally. Direct loads of microsomes (D) in which proteins were translated separately and mix before lysis (lanes 1–3) and microsomes from cotranslations (lanes 4–6) were compared with immunoprecipitations. Complexes were precipitated from 1% Brij96 lysates of in vitro translations from CC pellets and analyzed by 12.5% SDS-PAGE under reducing conditions. Representative experiments ($n = 3$).

β_{var} induces the accumulation of α_{+sig}

We next examined the fate of α when translated in the presence of β_{var}. For this purpose $\alpha\beta_{var}\gamma$ mRNAs were cotranslated and direct loads of microsomal pellets were compared with anti-α immunoprecipitates to assess more carefully all forms of α present in the translation mix (Fig. 6 B). We detected the presence of all translated proteins, with a prominent band of ~33 kD. Anti-α immunoprecipitation confirmed the nature of this polypeptide as α_{+sig} (Fig. 6 B). The γ chain as well as α_{Nglyc} and α_{-sig} were coprecipitated. For unknown reasons, we were unable to directly demonstrate β_{var} in these precipitates. This finding might again reflect a decrease in the stability of $\alpha\beta_{var}\gamma$ complexes, with β_{var} dissociating before γ, or equally likely, a more general problem of detection of β_{var}. As in cellular expression systems (13), β_{var} is rapidly lost from in vitro translation mixtures (unpublished data).

β_{var} down-regulates surface IgE-binding epitopes

We subcloned β and β_{var} into a bicistronic vector with EGFP (pIRES2-β-EGFP and pIRES2-β_{var}-EGFP). Next, 293 cells were transiently transfected and treated with proteasome inhibitor for 2h. After SDS lysis, immunoblots with anti-β serum were performed to confirm the proper expression of both proteins (Fig. 7 A).

We verified that mAb 15–1 recognizes the IgE-binding epitope of FcεRIα (13, 23, 28–30). IgE binding capacity of CHOαγ was assessed by FACS with biotinylated IgE (Fig. 7 B, filled black). CHOαγ show comparable reactivity when stained with 15–1 (Fig. 7 B, blue). Preincubation of cells with 15–1 inhibits subsequent IgE binding (Fig. 7 B, red). The Δ mean fluorescence intensity (ΔMFI) of IgE-reactivity drops from 370 to levels of the negative control (Fig. 7 B, black line, ΔMFI=10). This result is in accordance with the literature (13, 23, 28–30) and confirms that 15–1 recognizes the IgE-binding site of FcεRIα. The fact that both reagents recognize the same epitope also accounts for the misinterpretation of cellular distribution patterns of FcεRIα in humans. Endogenous IgE bound to FcεRIα precludes recognition with mAb 15–1 or biotinylated IgE unless the natural ligand is removed by acid stripping (23, 28–30).

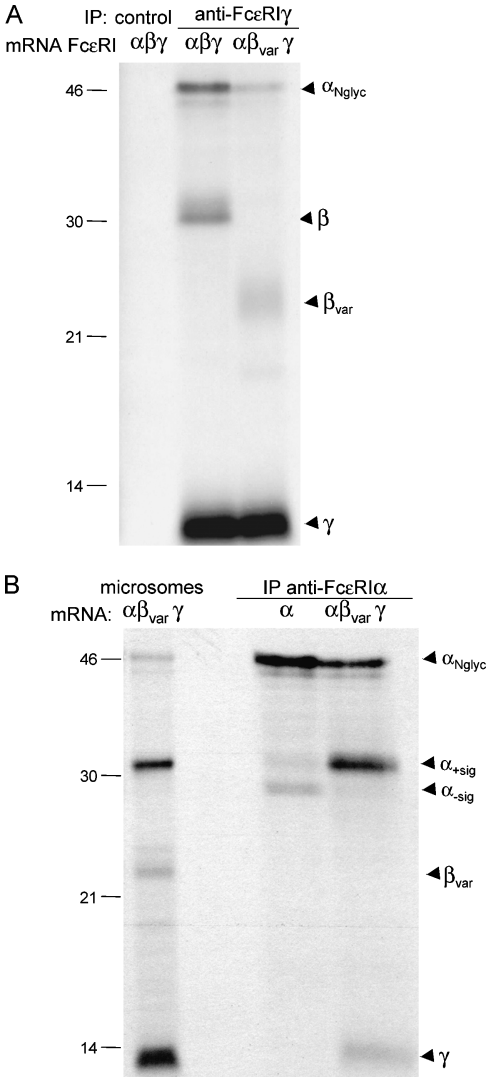

Figure 6. Cotranslational formation of tetrameric FcεRI complexes. (A) α,β, γ, or α,$\beta_{var,}$ γ mRNAs were simultaneously translated into CC microsomes. Microsomal pellets were solibilized in 1% Brij96 lysis buffer and immunoprecipitations with anti-γ or control serum were performed. Stable αβγ as well as $\alpha\beta_{var}\gamma$ complexes were retrieved by anti-γ. (B) β_{var} induces the accumulation of α_{+sig}. $\alpha\beta_{var}\gamma$ mRNAs were cotranslated and direct loads of in vitro translations were compared with anti-α immunoprecipitates. All translated proteins with a prominent band of ~33 kD are present in direct loads of CC mircosomes translated with $\alpha\beta_{var}\gamma$. Anti-α immunoprecipitation shows an overrepresentation of α_{+sig} when compared with translations of α alone. Proteins were precipitated from 1% Brij96 lysates of in vitro translations from CC pellets and analyzed by 12.5% SDS-PAGE under reducing conditions. Representative experiments ($n = 3$).

We show that our bicistronic constructs regulate the surface expression of IgE-binding epitopes as previously described (13). For this purpose, CHOαβγ were transiently transfected with pIRES2-β-EGFP or pIRES2-β_{var}-EGFP (Fig. 7 A, graph refers to β [red] and β_{var} [black]). Reactivity with mAb 15–1, which is specific for the IgE-binding epitope (5, 23), was monitored in a population gated for EGFP expression as a marker for successful transfection with β or β_{var}. Although we observed surface expression of IgE-binding epitopes in cells transfected with pIRES2-β-EGFP, this surface marker was significantly down-regulated in cells transfected with pIRES2-β_{var}-EGFP (Fig. 7 A, representative experiment). Transfections in CHOαγ cells yield the same results (unpublished data). Our experiments confirm that β_{var} impairs formation of surface expressed IgE-binding epitopes in vivo and functions in a dominant way when co-expressed with β in CHOαγ cells (13).

β_{var} induces accumulation of α_{+sig} in vivo

We next explored the mechanism by which β_{var} might interfere with the generation of IgE-binding epitopes. For this purpose we generated a COOH-terminally HA-tagged version of K^b-α (α_{HA}) because the commonly used anti-α reagents failed to detect the 30-kD α protein backbone and yielded poor results when used for immunoprecipitation in pulse-chase experiments. Anti-HA immunoprecipitation followed by anti-HA immunoblotting on 293 cells transiently transfected with α_{HA} confirmed that α_{HA} is properly N-glycosylated in the absence of β or γ subunits (Fig. 7 B, lane 1; 31). The presence of unglycosylated α protein backbone was specific for the presence of β_{var} (Fig. 7 B, lane 3).

Metabolic labeling experiments were then performed to shown that the α protein that accumulates in the presence of β_{var} is indeed α_{+sig}. Anti-HA immunoprecipitations followed by EndoH digestion were performed in cells transiently transfected with $\alpha_{HA}\beta_{var}\gamma$ (Fig. 7 C). These experiments shown that most α_{HA} is transformed into its fully N-glycosylated modification irrespectively of the presence of the β_{var} subunits. Comparing its characteristic with EndoH-treated protein, the remaining α_{HA} protein can be identified as α_{+sig} (Fig. 7 C). We could thus confirm by both immunoblotting and by pulse labeling that β_{var} allows accumulation of α_{+sig}.

For more extended studies of the intracellular fate of α, NH_2-terminal EGFP fusion proteins of β or β_{var} (GFP-β or GFP-β_{var}) were generated. The fusion adds the expected 28 kD to the molecular mass but otherwise does not interfere with the molecular characteristics of either protein (reference 13; Fig. 8 A; and unpublished data). Pulse-chase analysis of GFP-β or GFP-β_{var} demonstrates that both proteins are stabilized when inhibitors of the proteasome are present (Fig. 8 A). Pretreatment of cells with proteasome inhibitor ZL_3VS (5 μm, 1 h; reference 32) and its presence throughout the pulse chase stabilize β as well as β_{var} throughout the chase (Fig. 8 A). We infer that the β subunits are subject to proteasomal proteolysis with β_{var} more susceptible to proteasomal degradation. GFP-β and GFP-β_{var} should be informative reagents for the analysis of the fate of the α subunit at the single cell level.

To this end, CHOαγ cells were transiently transfected with GFP-β and GFP-β_{var} and analyzed by epifluorescence. Cells were treated with ZL_3VS to inhibit proteasomal degradation for 2 h, fixed, and stained with mAb 15–1 to visualize the IgE-binding form of α as previously described (23, 33). Staining with the anti-α polyclonal serum was performed to

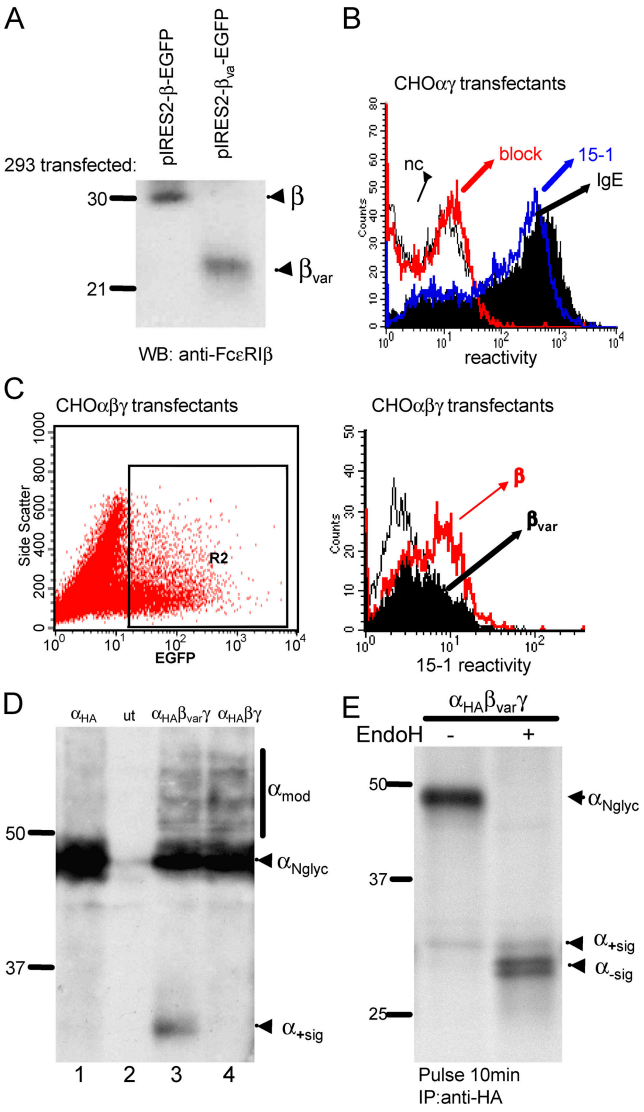

Figure 7. FcεRIβ$_{var}$ induces the accumulation of α$_{+sig}$ in vivo. (A) β$_{var}$ down-regulates surface IgE-binding epitopes. β and β$_{var}$ were subcloned into a bicistronic vector expressing EGFP to control for equal expression levels (pIRES2-β-EGFP and pIRES2-β$_{var}$-EGFP). 293 cells were transiently transfected and treated with proteasome inhibitor for 2 h. After SDS lysis, immunoblots with anti-β serum were preformed to demonstrate the expression of both proteins. (B) mAb 15–1 recognizes the IgE-binding epitope of FcεRIα. IgE binding capacity of CHOαγ was assessed with biotinylated IgE (black). Preincubation with mAb 15–1 (reactivity shown in blue) inhibits subsequent IgE binding (red). ΔMean fluorescence is shown on the abscissa. (C) CHOαβγ were transiently transfected with pIRES2-β-EGFP or pIRES2-β$_{var}$-EGFP (β (red) and β$_{var}$(black)). Reactivity of mAb 15–1, which is specific for the IgE-binding epitope, was monitored on the surface of a cell population gated for EGFP-expression as a marker for expression of β and β$_{var}$. pIRES2-β$_{var}$-EGFP specifically down-regulates surface 15–1 reactivity. (D) β$_{var}$ induces accumulation of unglycosylated α protein backbone in vivo. A HA-tagged version of K^b-α (α$_{HA}$) was transiently transfected into 293 cells in the presence of the indicated FcεRI subunits. Transfection was followed by anti-HA immunoprecipitation and anti-HA immunoblotting. α$_{HA}$ folds properly and becomes N-glycosylated (α$_{Nglyc}$) in the absence of other complex subunits (lane 1). Cotransfection of βγ (lane 4) or β$_{var}$γ (lane 3) induces Golgi-associated glycosylation modification of α$_{HA}$ (α$_{mod}$). Unglycosylated protein backbone accumulates specifically in the presence of β$_{var}$. (E) β$_{var}$ induces the accumulation of α$_{+sig}$. Metabolic labeling of cells transiently transfected with α$_{HA}$, β and γ followed by anti-HA immunoprecipitations and EndoH digestion. Most α$_{HA}$ is rapidly transformed into its fully N-glycosylated modification (α$_{Nglyc}$). Susceptibility to EndoH treatment defines α$_{+sig}$.

visualize all forms of α. CHOαγ transfected with GFP-β are positive for both mAb 15–1 and the anti-α serum (Fig. 8 B). In contrast, CHOαγ transfected with GFP-β$_{var}$ do not stain with mAb 15–1 but still remain positive with the anti-α serum (Fig. 8 B). Experiments performed with CHOαβγ cells show identical results (unpublished data). It is important to note that inclusion of the proteasome inhibitor did not rescue the expression of IgE-binding epitopes. In agreement with our in vitro translation results, this experiment demonstrates that expression of GFP-β$_{var}$ interferes with proper folding and the formation of IgE-binding epitopes on the α subunit. Cells transfected with GFP-β$_{var}$ still express readily detectable unfolded α chain, suggestive of the mechanism by which β$_{var}$ and GFP-β$_{var}$ down-regulate IgE-binding epitopes. In experiments without inhibition of proteasomal activity, GFP-β$_{var}$ is more difficult to detect but can still be visualized. Such cells also contain unfolded α chain but are devoid of IgE-binding epitopes, as visualized by staining with anti-α serum or 15–1, respectively (unpublished data).

Additionally we performed a set of pulse-chase experiments to confirm on the cellular level, that FcεRIα is indeed not targeted to proteasomal degradation by β$_{var}$. FcεRIα$_{HA}$ was transiently transfected into 293 cells in the presence of βγ or β$_{var}$γ cDNA (Fig. 8 C). Immunoprecipitations were performed with anti-HA in 1% NP-40 lysis buffer to assure access to the total cellular pool of FcεRIα. No enhanced degradation of any form of FcεRIα was observed in cells transfected with αβ$_{var}$γ. In correlation with the results presented earlier in this study, the only significant difference was the persistence of α$_{+sig}$ in the presence of β$_{var}$. The slight and progressive decrease in the molecular weight of α$_{Nglyc}$ observed in all conditions is attributable to mannose trimming (32, 34). We failed to detect α$_{mod}$ in this experimental setting. Next we compared FcεRIα protein levels in αβ$_{var}$γ transfectants in the presence and absence of proteasome inhibitors (Fig. 8 D). Although proteasomal inhibition stabilizes β$_{var}$ (reference 13; Fig. 8 A; and unpublished data), we do not detect alterations in the amount or expression pattern of FcεRIα. Because inclusion of proteasome inhibitor and consequent stabilization of β$_{var}$ do not change the fate of FcεRIα α is not targeted to proteasomal degradation. We consider it unlikely that the short half-life of β$_{var}$ is of functional importance for this mechanism.

DISCUSSION

Allergen- and IgE-dependent cross-linking of FcεRI is responsible for the immediate as well as the chronic inflammatory responses observed in atopic patients (5, 6). Surface expression of FcεRI critically determines the sensitivity to an allergic stimulus and is therefore pivotal for the ensuing clin-

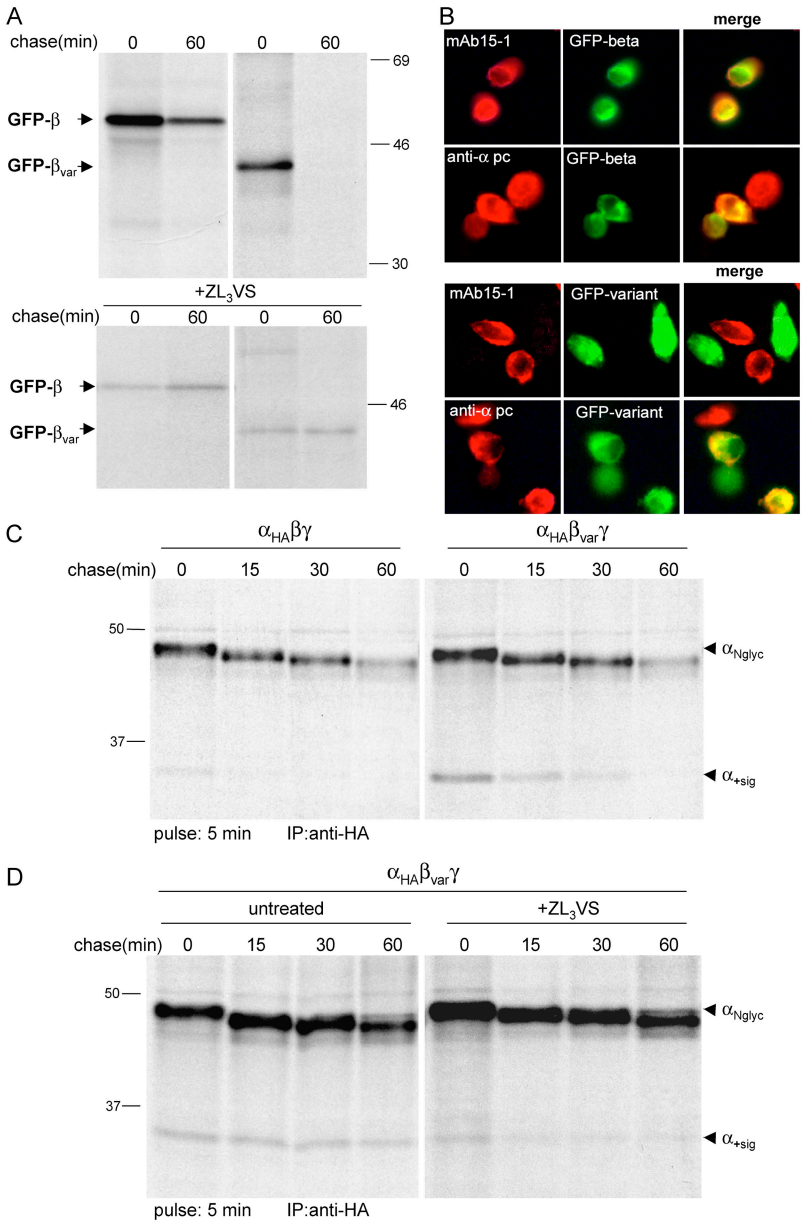

Figure 8. FcεRIβ$_{var}$ induces the accumulation of improperly folded FcεRIα in vivo. (A) NH$_2$-terminal EGFP-fusion proteins of β or β$_{var}$ (GFP-β or GFP-β$_{var}$) were transiently transfected into 293 cells with lipofectamine. The fusion adds the expected 28kD to the molecular weight but otherwise does not interfere with the molecular characteristics of β or β$_{var}$. Pulse-chase analysis: cells were analyzed untreated or pretreated with proteasome inhibitor ZL$_3$VS (5 μm, 2 h). Anti-GFP immunoprecipitates were obtained from 1% NP-40 lysates and analyzed by 12.5% SDS-PAGE under reducing conditions. Inhibition of the proteasome stabilizes GFP-β$_{var}$. (B) CHOαγ were transiently transfected with GFP-β and GFP-β$_{var}$ and analyzed by epifluorescence. Cells were treated with ZL$_3$VS for 2 h, fixed and stained with mAb 15–1 to visualize IgE-binding epitopes. Staining with the anti-α polyclonal serum was performed to visualize all forms of α. Transfection with the GFP-β does not influence mAb 15–1 reactivity. Cells transfected with GFP-β$_{var}$ do not show mAb 15–1 reactivity but still remain positive with the anti-α serum indicative for the presence of unfolded α chain. Representative experiment (n = 4). (C, D) Pulse-chase experiments to confirm that FcεRIα not targeted to proteasomal degradation by β$_{var}$. FcεRIα$_{HA}$ was transiently transfected into 293 cells in the presence of βγ or β$_{var}$γ cDNA. Transfection, immunoprecipitations and analysis were performed as described in (A). α$_{+sig}$ is stabilized by the presence of β$_{var}$ selectively (C). (D) Comparison of FcεRIα protein levels in αβ$_{var}$γ transfectants in the presence and absence of proteasome inhibitors. No alterations in the amount or expression pattern of FcεRIα in induced by the inhibitor.

ical response. The only defined extracellular regulator of FcεRI surface expression defined so far is IgE, its natural ligand (5). With regard to intracellular regulation of receptor expression, FcεRIβ was described as an amplifier for γ chain signaling as well as for receptor surface expression (14, 15). In contrast, β$_{var}$ has been shown to down-regulate surface expression of α (13). The mechanistic basis of these regulatory events is poorly understood. Glycosylation-mediated quality control regulates ER export of FcεRI (31). Here we show the efficiency of ER assembly is controlled by the

presence of the different subunits. We were able to define the IgE-binding α chain as the core of the receptor that pairs with the other subunits in a strictly cotranslationally regulated assembly event. These experiments establish ER assembly as a rate-limiting step in the expression of functional surface receptors, with obvious consequences for the onset of allergic diseases. In addition to the well-described FcεRI isoforms, we were also able to demonstrate the existence of $\alpha\beta_{var}\gamma$ and αβ complexes, not previously documented.

Protein synthesis and folding in the ER are not always efficient: improperly folded structures are cleared from the ER and directed toward degradation (19–21). In addition to the proper folding of the individual subunits, multimeric receptors must assemble in a concerted fashion. Receptors such as FcεRI or the TCR assemble in the ER and maintain their integrity by noncovalent interaction of the various subunits. Only with all players in place can ER retention signals be overcome (5, 9). For the TCR this process is well established and occurs in three consecutive assembly steps (17). Although FcεRI and TCR share the same principal structure of ligand-binding and signal-transducing units and can even use the same γ chain for signaling (5), we show that their assembly is regulated differently. FcεRI complexes form strictly cotranslationally. The presence of β and γ clearly favors a conversion of α into its IgE-binding form when compared with translation in the presence of γ alone. The β_{var}, on the other hand, slows down this conversion and induces the accumulation of unglycosylated α with the signal peptide still in place.

Not all FcεRI receptor subunit RNAs are generated in the same quantities in primary cells. When compared with α, β is always underrepresented (5). These observations were also confirmed at the protein level (11, 23). The demands of receptor stochiometry make cotranslational assembly of the receptor a key step that controls expression of functional IgE-binding epitopes at the cell surface. It may thereby affect the susceptibility to allergic stimuli in vivo. Receptor α subunits can still fold properly, but do so at a less efficient rate when compared with folding in the presence of β and γ. Such α chains lack the necessary partners for complex formation during translation and consequently for ER exit. Our finding thus provides an explanation for the intracellular accumulation of α in cell types that express high levels of γ, which in principle should allow surface expression (5). It is possible that in such cells, translation of α and γ might not occur in synchrony and thus ER exit may be compromised. In addition to its critical role for FcεRI signaling and ER exit, FcεRIγ is a subunit of Fcγ receptors (35). Functional association of FcεRIβ with CD16, FcγRIII, has also been demonstrated (36, 37). Both Fc receptor types might thus compete for γ and β subunits, which adds an additional level of complexity and importance to assembly control. Because various Fcγ receptor subtypes and FcεRI are expressed simultaneously in a variety of cell types (e.g., monocytes, dendritic cells, Langerhans cells, mast cells), this event is of physiological importance. Antigen presenting cells and monocytes of all individuals express Fcγ receptors. The proper assembly of Fcγ receptors could indirectly control the expression of FcεRI by depleting the pool of receptor units that are required for FcεRI ER exit. The functional consequences of β_{var} expression for cotranslational assembly need to be interpreted carefully, in particular with regard to the fact that so far transcripts of β_{var} were not depicted in absence of the classic β chain.

Improperly assembled FcεRI subunits are subjected to the ER quality control system and must be directed toward degradation. FcεRIβ_{var} is a natural substrate for proteasomal degradation and is itself rapidly destroyed, irrespective of the presence of FcεRI's other subunits (13). Whereas the α subunit is also degraded by the proteasome, we found no evidence for a redirection of α toward proteasomal degradation by β_{var} (unpublished data). When expressed alone, α_{Nglyc} is a rather stable protein. It remains to be established whether primary cells such as Langerhans cells, eosinophils, or monocytes of allergic patients have a mechanism to direct preformed pools of α toward the cell surface. In view of our results, this possibility appears at present unlikely.

Our study establishes cotranslational assembly of FcεRI as the first quality control mechanism for the generation of functional IgE-binding FcεRI. The dysregulation of this quality control process might contribute to the expression of high FcεRI levels in allergic patients. We show that the ER quality control system regulates quantities of functional FcεRI. It thereby controls onset and persistence of allergic reactions and might thus be a target for therapeutic interventions.

MATERIALS AND METHODS

DNA constructs. The cDNAs encoding FcεRIα, FcεRIβ, FcεRIβ_{var}, and FcεRIγ were provided by the laboratory of J.-P. Kinet (Laboratory of Allergy and Immunology, Beth Israel Deaconess Medical Center, Boston, MA) (13). Signal peptide encoding sequences of α and γ were exchanged for the signal peptide of mouse class I heavy chain H2-K^b (16). All cDNAs were cloned into pcDNA3.1 under the control of the T7 promotor. β and β_{var} were also subcloned into pIRES2-EGFP (CLONTECH Laboratories, Inc.). NH_2-terminal EGFP fusion proteins of β and β_{var} were generated in pEGFP-C1 (CLONTECH Laboratories, Inc.). A COOH-terminal K^b-α_{HA} fusion protein was generated and expressed in pcDNA3.1.

Antibodies and antisera. cIgE (Serotec) and mAb 15–1 recognize the IgE-binding epitope of α and were used for the detection of properly folded and N-glycosylated form of the protein (11, 12). Polyclonal rabbit anti-α recognizes all forms of the protein irrespective of its folding status. Antisera against α, β, and γ are published and were used as described in the literature (13). αGFP was generated by immunizing rabbits with the bacterially expressed GFP and used as previously described (32). HA-tagged proteins were precipitated with mAb 12CA5 and detected with HRP-conjugated rat mAb 3F10 (Roche).

Cell lines and transient transfections. 293, CHOαβγ and CHOαγ cells were maintained in DMEM as previously described (13, 23). Various constructs of β and β_{var} were expressed in 293, CHOαγ, or CHOαβγ cells by transient transfection, using a liposome-mediated transfection protocol (5–10 μg of DNA, 20 μl of lipofectamine, 10 cm dish; Lipofectamine; GIBCO BRL) as previously described (33). Cells were analyzed between 24 and 48 h after transfection.

In vitro transcription and translation. Both methods were essentially performed as previously described (16, 25, 26). In vitro transcriptions were performed using T7 polymerase (Promega). All cDNAs were subcloned

into pcDNA3.1. After linearization, T7 polymerase was used for in vitro transcription (Promega). RNA was caped as previously described and stored as alcohol precipitates at −80°C. Before translation, RNA was decaped. Optimal amount of the individual RNAs was determined empirically for each individual receptor subunit and each stock of RNA. RNAs were stored as alcohol precipitates at −80°C. The optimal reaction time of the in vitro translation was determined empirically as 1 h. Reticulocyte lysate was purchased from Promega. Microsomes were prepared from various cell lines as previously described and pelleted after in vitro translations for further analysis as previously described (26). Complex precipitations of FcεRI were performed in 1% Brij 96 lysates as previously described (11).

Metabolic labeling of cell, pulse-chase experiments, immunoprecipitation, enzymatic digestion, and immunoblotting 293 cells were detached, followed by starvation in methionine-/cysteine-free DME for 60 min at 37°C. Cells were metabolically labeled with 500 μCi of [^{35}S]methionine/cysteine (1,200 Ci/mM; NEN)/ml at 37°C for the times indicated. Pulse-chase experiments, cell lysis, and immunoprecipitations were performed as previously described (33). 1% Brij96 lysis buffer was used to maintain the integrity of FcεRI complexes as previously described (11). The immunoprecipitates were analyzed by SDS-PAGE followed by fluorography (38). Endo H (New England Biolabs, Inc.) digestions was performed as described by the manufacture. αHA immunoblots were performed with SDS lysates under nonreducing conditions (23).

Flow cytometry analysis. Quantitative flow cytometry analysis of cells expressing constructs in pIRES2-EGFP in living cells was performed by FACS® (FACS®Calibur; BD, Mountain View, CA) supported by CellQuest software (BD). IgE-binding epitopes of FcεRIα were stained with mAb15-1 or biotinylated IgE as previously described (13, 23, 28–30).

Immunostaining and epifluorescence microscopy. Immunofluorescence experiments were performed essentially as previously described (39) with minor modifications as follows. Cells were allowed to attach to slides overnight before inhibitor incubation (ZL_3VS; reference 40; 4 h 10 μM final from a DMSO stock). DMSO was used as solvent control. After fixation with 3.7% paraformaldehyde for 20 min at room temperature immunohistochemistry was performed in a 0.5% saponin/3% BSA/PBS solution. mAbs 15–1 was used to define α chains that exhibit properly folded IgE-binding epitopes (13, 23). Polyclonal anti-α serum was used to show all forms of α irrespective of their folding or glycosylation status (13). Anti-mouse Alexa Fluor 568 (Molecular Probes) and anti-rabbit Alexa Fluor 568 (Molecular Probes) were used as the fluorescent probe. Further analysis was performed with a Bio-Rad epifluorescence microscope as previously described (39).

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