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

Edda Fiebiger,1 Domenico Tortorella,2 Marie-Helene Jouvin,3 Jean-Pierre Kinet,3 and Hidde L. Ploegh1

1Department of Pathology, Harvard Medical School, Boston, MA 02115
2Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029
3Laboratory of Allergy and Immunology, Beth Israel Deaconess Medical Center, Boston, MA 02215

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 (αβγ2) on basophils or mast cells and as trimeric (αγ2) complex on antigen-presenting cells. Expression of the human α subunit can be down-regulated by a splice variant of FcεRIβ (βvar). We demonstrate that FcεRIα is the core subunit with which the other subunits assemble strictly cotranslationally. In addition to αβγ2 and αγ2, we demonstrate the presence of αβ and αβvarγ2 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.

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 disulfide-linked γ subunits (5, 9). The FcεRI complexes on the surface of basophils and mast cells are tetrameric structures (αβγ2). The αβγ2 is the only receptor isofrom 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 (βvar, formerly referred to as βγ) 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 Cε3 domain of IgE. It binds its ligand in 1:1 ratio, with an affinity of ~1010 M–1. The β chain contains four potential transmembrane spanning regions with both the NH2 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.
Multisubunit receptor complexes, like FceRI 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 oligosaccharyltrimming. 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 FceRI must assemble in a concerted fashion. Only with all players in place, can ER retention signals be overcome. The sequence of events in FceRI 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 FceRI in allergic individuals supports this hypothesis (23, 24).

Studies on the human receptor are hampered by a lack of cell lines that express FceRI irrespective of its isoforms. Primary human cells that express FceRI 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 FceRI assembly in the ER. We translated the corresponding mRNAs of all FceRI 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 FceRI subunits is tightly controlled and indeed regulates the formation of properly formed receptors with IgE-binding epitopes.

RESULTS

In vitro translation of FceRIα

We used in vitro translation as a method to study FceRI 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 FceRI subunits allow the generation of the corresponding mRNAs for in vitro translations. The mRNAs were translated in the presence of microsomes from different sources. FceRIα 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).

FceRIα 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-Kb. 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 FceRIα (H2-Kb-FceRIα, referred to as α; Fig. 1 A). The source of microsomes proved critical for the generation of α chains with cleaved signal peptide (αsignal, Fig. 1 A) as well as for N-glycosylation (αsignal, Fig. 1 A), although no such effect was observed for FceRIγ or HLA-2A (unpublished data). Microsomes from the basophilic cell line KU812 allowed effi-
icient translation, but yielded α mostly as α\text{sig} (Fig. 1 A). Microsomes derived from canine pancreas were successful in creating Nglyc, but a sizable fraction of the translated protein was present as α\text{sig} (Fig. 1 A and unpublished data). Microsomes derived from the astrocytoma cell line U373 (CC) reproducibly generated endoglycosidase H (EndoH)-sensitive Nglyc efficiently (Fig. 1 A).

We then asked whether we could generate FcγRI 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 FcγRI (α\text{sig}, α\text{sig}, and Nglyc, Fig. 1 B), but only properly folded and N-glycosylated is recovered by IgE (Fig. 1 B).

**In vitro translation of FcγRIβ and FcγRIβ\text{var}**

The identity of the translation products from β and β\text{var} (13) was confirmed by immunoprecipitation with an anti-β serum generated against the NH\text{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 FcγRIγ**

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\text{b} (H2-K\text{b}FcγRIγ, 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 [35S]cysteine translation mix. The single methionine of γ gets cleaved after insertion into the microsomes. [35S]Cysteine present in the [35S]methionine translation mix is sufficient for labeling of K\text{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. FcγRIγ forms a dimer under nonreducing conditions. Representative experiments (n = 3).

**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
the same time (lane 4, of both translation products (lanes 1 and 2). Microsomal pellets were mixed before lysis. Direct loads of microsomes against 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 and γ. The anti-β reimmunoprecipitation unequivocally demonstrates the existence 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 also retrieved stable αγ complexes when α was translated alone. This effect is even more pronounced when β is cotranslated as well (unpublished data and see Fig. 6 A).

Immunoprecipitation of αβγ and αβαγ complexes

The α, β, γ, or αβαγ mRNAs were translated into microsomes, which were then solubilized 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 αβαγ complexes (Fig. 6 A). The α chain in the latter complexes seemed to be underrepresented when compared with αβγ.

Figure 4. FcεRI complexes form cotranslationally. (A) Cotranslational formation of αγ complexes. α and γ RNA were translated consecutively (lanes 1 and 3, αγ) or at the same time (lane 2 and 4, α + γ). (B) 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). (C) α and γ were translated separately and microsomes were mixed before lysis. Direct loads of microsomes showed the presence of both proteins. No αγ complexes were precipitated. (D) Immunoprecipitations of αγ complexes with serum directed against γ. The anti-control serum successfully precipitated complexes 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 αNglyc 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 and γ. The anti-β reimmunoprecipitation unequivocally demonstrates the existence 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 also retrieved stable αγ complexes when α was translated alone. This effect is even more pronounced when β is cotranslated as well (unpublished data and see Fig. 6 A). The anti-γ but not the control serum successfully precipitated αβγ complexes (Fig. 6 A). The anti-γ reagent also retrieved stable αβαγ complexes (Fig. 6 A). The α chain in the latter complexes seemed to be underrepresented when compared with αβγ.
var induces the accumulation of sig

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

Var down-regulates surface IgE-binding epitopes

We subcloned var into a bicistronic vector with EGFP (pIRES2-β-EGFP and pIRES2-βvar-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 CHO was assessed by FACS with biotinylated IgE (Fig. 7 B, filled black). CHO 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 mean fluorescence intensity (MFI) of IgE-reactivity drops from 370 to levels of the negative control (Fig. 7 B, black line, MFI = 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).

βvar induces the accumulation of α+sig

We next examined the fate of α when translated in the presence of βvar. For this purpose αβvarγ 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 αNglyc and α−sig were coprecipitated. For unknown reasons, we were unable to directly demonstrate βvar in these precipitates. This finding might again reflect a decrease in the stability of αβvarγ complexes, with βvar dissociating before γ, or equally likely, a more general problem of detection of βvar. As in cellular expression systems (13), βvar is rapidly lost from in vitro translation mixtures (unpublished data).

βvar down-regulates surface IgE-binding epitopes

We subcloned β and βvar into a bicistronic vector with EGFP (pIRES2-β-EGFP and pIRES2-βvar-EGFP). Next,
Although we observed surface expression of IgE-binding epitopes in cells transfected with pIRE2-β-EGFP, this surface marker was significantly down-regulated in cells transfected with pIRE2-β<sub>var</sub>-EGFP (Fig. 7 A, representative experiment). Transfections in CHOαγ cells yield the same results (unpublished data). Our experiments confirm that β<sub>var</sub> 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<sub>var</sub> induces accumulation of α<sub>+</sub> in vivo**

We next explored the mechanism by which β<sub>var</sub> might interfere with the generation of IgE-binding epitopes. For this purpose we generated a COOH-terminally HA-tagged version of Kβ-α (α<sub>HA</sub>) 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<sub>HA</sub> confirmed that α<sub>HA</sub> is properly N-glycosylated in the absence of β or γ subunits (Fig. 7 A; lane 1; 31). The presence of unglycosylated α protein backbone was specific for the presence of β<sub>var</sub> (Fig. 7 B, lane 3).

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

For more extended studies of the intracellular fate of α, NH<sub>2</sub>-terminal EGFP fusion proteins of β or β<sub>var</sub> (GFP-β or GFP-β<sub>var</sub>) 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 and GFP-β<sub>var</sub> demonstrates that both proteins are stabilized when inhibitors of the proteasome are present (Fig. 8 A). Pretreatment of cells with proteasome inhibitor ZL<sub>3</sub>VS (5 μm, 1 h; reference 32) and its presence throughout the pulse chase stabilize β as well as β<sub>var</sub> throughout the chase (Fig. 8 A). We infer that the β subunits are subject to proteasomal proteolysis with β<sub>var</sub> more susceptible to proteasomal degradation. GFP-β and GFP-β<sub>var</sub> 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<sub>var</sub> and analyzed by epifluorescence. Cells were treated with ZL<sub>3</sub>VS 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 6. Cotranslational formation of tetrameric FcεRI complexes.**

(A) αβγ, γ, or αβ<sub>var</sub>γ mRNA were simultaneously translated into CC microsomes. Microsomal pellets were solubilized in 1% Brij96 lysis buffer and immunoprecipitations with anti-γ or control serum were performed. Stable αβγ as well as αβ<sub>var</sub>γ complexes were retrieved by anti-γ. (B) β<sub>var</sub> induces the accumulation of α<sub>+</sub>γ. αβ<sub>var</sub>γ mRNA were cotranslated and direct loads of in vitro translations were compared with anti-α immunoprecipitates. All translated proteins with a prominent band of ~33 kDa are present in direct loads of CC microsomes translated with αβ<sub>var</sub>γ. Anti-α immunoprecipitation shows an overrepresentation of α<sub>+</sub>γ 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 pIRE2-β-EGFP or pIRE2-β<sub>var</sub>-EGFP (Fig. 7 A, graph refers to β [red] and β<sub>var</sub> [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
Figure 7. FcεRIβ VAR induces the accumulation of αsig in vivo. (A) βvar down-regulates surface IgE-binding epitopes. β and βvar were subcloned into a bicistronic vector expressing EGFP to control for equal expression levels (pRES2-β-EGFP and pRES2-βvar-EGFP). 293 cells were transiently transfected and treated with proteasome inhibitor for 2 h. After SDS lysis, immunoblots with anti-β serum were performed 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). Mean fluorescence is shown on the abscissa. (C) CHOβγ transfectants were transiently transfected with pRES2-β-EGFP or pRES2-βvar-EGFP (β red and βvar black). Reactivity of mAb 15–1 (reactivity shown in blue) inhibits subsequent IgE binding (red). Mean fluorescence is shown on the abscissa. (D) CHOβγ transfectants were transiently transfected with pRES2-β-EGFP or pRES2-βvar-EGFP (β red and βvar black). Reactivity of mAb 15–1 (reactivity shown in blue) inhibits subsequent IgE binding (red). Mean fluorescence is shown on the abscissa. (E) CHOβγ transfectants were transiently transfected with pRES2-β-EGFP or pRES2-βvar-EGFP (β red and βvar black). Reactivity of mAb 15–1 (reactivity shown in blue) inhibits subsequent IgE binding (red). Mean fluorescence is shown on the abscissa.

DISCUSSION

Allergen- and IgE-dependent cross-linking of FcεRIα is responsible for the immediate as well as the chronic inflammatory responses observed in atopic patients (5, 6). Surface expression of FcεRIα critically determines the sensitivity to an allergic stimulus and is therefore pivotal for the ensuing clinical responses observed in atopic patients (5, 6). Metabolic labeling of cells transiently transfected with αvar, β and γ followed by anti-HA immunoprecipitations and EndoH digestion. Most αvar is rapidly transformed into its fully N-glycosylated modification (αNglyc). Susceptibility to EndoH treatment defines αsig.

visualize all forms of α. CHOαγ transfectanted with GFP-β are positive for both mAb 15–1 and the anti-α serum (Fig. 8B). In contrast, CHOαγ transfectanted with GFP-βvar do not stain with mAb 15–1 but still remain positive with the anti-α serum (Fig. 8B). 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-βvar interferes with proper folding and the formation of IgE-binding epitopes on the α subunit. Cells transfected with GFP-βvar still express readily detectable unfolded α chain, suggestive of the mechanism by which βvar and GFP-βvar down-regulate IgE-binding epitopes. In experiments without inhibition of proteasomal activity, GFP-βvar 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 βvar. FcεRIαHA was transiently transfected into 293 cells in the presence of βγ or βvar cDNA (Fig. 8C). Immunooprecipitations 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 αβvarγ. In correlation with the results presented earlier in this study, the only significant difference was the persistence of αsig in the presence of βvar. The slight and progressive decrease in the molecular weight of αNglyc observed in all conditions is attributable to mannose trimming (32, 34). We failed to detect αmod in this experimental setting. Next we compared FcεRIα protein levels in αβvarγ transfectants in the presence and absence of proteasome inhibitors (Fig. 8D). Although proteasomal inhibition stabilizes βvar (reference 13; Fig. 8A; 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 βvar do not change the fate of FcεRIα α is not targeted to proteasomal degradation. We consider it unlikely that the short half-life of βvar is of functional importance for this mechanism.
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 chain signaling as well as for receptor surface expression (14, 15). In contrast, βvar 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 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-βvar 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 βvar. FcεRIαcDNA was transiently transfected into 293 cells in the presence of βγ or βvarγ cDNA. Transfection, immunoprecipitations and analysis were performed as described in (A). αβγ is stabilized by the presence of βvar selectively (C). (D) Comparison of FcεRI protein levels in αβvarγ transfectants in the presence and absence of proteasome inhibitors. No alterations in the amount or expression pattern of FcεRI in induced by the inhibitor.

Figure 8. FcεRIβvar induces the accumulation of improperly folded FcεRI in vivo. (A) NH₂-terminal EGFP-fusion proteins of β or βvar (GFP-β or GFP-βvar) 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 βvar. Pulse-chase analysis: cells were analyzed untreated or pretreated with proteasome inhibitor ZLVS (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-βvar. (B) CHOαγ were transiently transfected with GFP-β and GFP-βvar and analyzed by epifluorescence. Cells were treated with ZLVS 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-βvar 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 βvar. FcεRIαcDNA was transiently transfected into 293 cells in the presence of βγ or βvarγ cDNA. Transfection, immunoprecipitations and analysis were performed as described in (A). αβγ is stabilized by the presence of βvar selectively (C). (D) Comparison of FcεRI protein levels in αβvarγ transfectants in the presence and absence of proteasome inhibitors. No alterations in the amount or expression pattern of FcεRI in induced by the inhibitor.
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 αβvarγ 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 βvar 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 stoichiometry 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 binding epitopes at the cell surface. It may thereby affect the receptor stochiometry make cotranslational assembly of the also confirmed at the protein level (11, 23). The demands of

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 βvar 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 stoichiometry 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 binding epitopes at the cell surface. It may thereby affect the receptor stochiometry make cotranslational assembly of the also confirmed at the protein level (11, 23). The demands of
into pcDNA3.1. After linearization, T7 polymerase was used for in vitro transcription (Promega). RNA was capped as previously described and stored as alcohol precipitates at −80°C. Before translation, RNA was decapped. 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 at 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 [35S]methionine/cysteine (1,200 Ci/mM; NEN)/ml at 37°C for the time indicated. Pulse-chase experiments, cell lysis, and immunoprecipitations were performed as previously described (33). 1% Brij 96 lys 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 for the allergy-associated FcεRIβ gene. Immunoblotting

Flow cytometry analysis. Quantitative flow cytometry analysis of cells expressing constructs in pRS2-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/VS; 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).

This study was supported by the Sandler Program for Asthma Research. During the course of this study Edda Fiebiger was supported by the APART Program of the Austrian Academy of Sciences and the Charles A. King Trust, Freet National Bank, a Bank of America Company, Co-Trustee (Boston, MA). The authors have no conflicting financial interests.

Submitted: 12 July 2004
Accepted: 8 December 2004

REFERENCES
1. Sutton, B.J., and H.J. Gould. 1993. The human IgE network. Nature. 366:421–428.
2. Cookson, W.O., and M.F. Moffatt. 1997. Asthma: an epidemic in the absence of infection? Science. 275:41–42.
3. Metzger, H. 1991. The high affinity receptor for IgE on mast cells. Clin. Exp. Allergy. 21:269–279.
4. Muddle, G.C., I.G. Reischl, N. Corvaja, A. Hren, and E.M. Poellabauer. 1996. Antigen presentation in allergic sensitization. Immunol. Cell Biol. 74:167–173.
5. Kinet, J.P. 1999. The high-affinity IgE receptor (Fc εpon RI): from physiology to pathology. Annu. Rev. Immunol. 17:931–972.
6. Kinet, J.P. 1999. Atopic allergy and other hypersensitivities. Curr.

Opin. Immunol. 11:603–605.
7. Roth, M. 1989. Antigen receptor tail clue. Nature. 338:383–384.
8. Ravench, J.V., and J.-P. Kinet. 1991. Fc Receptors. Annu. Rev. Immunol. 9:457–492.
9. Kinet, J.-P., M.-H. Jouvin, R. Paolini, R. Numerof, and A. Scharenberg. 1996. IgE receptor (FcεRI) and signal transduction. Eur. Respir. J. 9:116–118.
10. Turner, H., and J.P. Kinet. 1999. Signalling through the high-affinity IgE receptor Fc εpon RI. Nature. 402:B24–B30.
11. Maurer, D., E. Fiebiger, C. Ebner, B. Reininger, G.F. Fischer, S. Wichlas, M.-H. Jouvin, M. Schmitt-Egenolf, D. Kraft, J.-P. Kinet, and G. Stingl. 1996. Peripheral blood dendritic cells express FcεRI as a complex composed of FcεRIα- and FcεRIβ-chains and can use this receptor for IgE-mediated allergen presentation. J. Immunol. 157:607–613.
12. Maurer, D., C. Ebner, B. Reininger, E. Fiebiger, D. Kraft, J.-P. Kinet, and G. Stingl. 1995. The high affinity IgE receptor (FcεRI) mediates IgE-dependent allergen presentation. J. Immunol. 154:6285–6290.
13. Donnadieu, E., M.H. Jouvin, S. Rana, M.F. Moftatt, E.H. Mockford, W.O. Cookson, and J.P. Kinet. 2003. Competing functions encoded in the allergy-associated FcεRIβ gene. Immunity. 18:665–674.
14. Donnadieu, E., M.H. Jouvin, and J.P. Kinet. 2000. A second amplifier function for the allergy-associated FcεRIβ-subunit subunit. Immunity. 12:515–523.
15. Dombrowicz, D., S. Lin, V. Flamand, A.T. Brini, B.H. Keller, and J.P. Kinet. 1998. Allergy-associated FcεRIβ is a molecular amplifier of IgE- and IgE-mediated in vivo responses. Immunity. 8:517–529.
16. Huppa, J.B., and H.L. Ploegh. 1997. In vitro translation and assembly of a complete T cell receptor-CD3 complex. J. Exp. Med. 186:393–403.
17. Call, M.E., J. Pyrdol, M. Wiedmann, and K.W. Wucherpfennig. 2002. The organizing principle in the formation of the T cell receptor-CD3 complex. Cell. 111:967–979.
18. Jahn-Schmid, B., U. Wiedermann, B. Bohle, A. Repa, D. Kraft, and C. Ebner. 1999. Oligodeoxynucleotides containing CpG motifs modulate the allergen: TH2 response of BALB/c mice to Bet v 1, the major birch pollen allergen. J. Allergy Clin. Immunol. 104:1015–1023.
19. Dobson, C.M. 2004. Principles of protein folding, misfolding and aggregation. Semin. Cell Dev. Biol. 15:3–16.
20. Goldberg, A.L. 2003. Protein degradation and protection against misfolded or damaged proteins. Nature. 426:895–899.
21. Sitta, R., and I. Braakman. 2003. Quality control in the endoplasmic reticulum protein factory. Nature. 426:891–894.
22. Benaroudj, N., E. Tarcsa, P. Casio, and A.L. Goldberg. 2001. The unfolding of substrates and ubiquitin-independent protein degradation by proteasomes. Biochimie. 83:311–318.
23. Maurer, D., E. Fiebiger, B. Reininger, B. Wolff-Winisiki, M.-H. Jouvin, O. Kilgus, J.-P. Kinet, and G. Stingl. 1994. Expression of functional high-affinity immunoglobulin E receptors (FcεRI) on monocytes of atopic individuals. J. Exp. Med. 179:745–750.
24. Bieber, T. 1994. Fc εpon RI on human Langerhans cells: a receptor in search of new functions. Immunol. Today. 15:52–53.
25. Bjñlakers, M.J., P. Benaroch, and H.L. Ploegh. 1994. Assembly of HLA DR1 molecules translated in vitro: binding of peptide in the endoplasmic reticulum presequence association with invariant chain. EMBO J. 13:2699–2707.
26. Furman, M.H., H.L. Ploegh, and D. Tortorella. 2002. Membrane-specific, host-derived factors are required for US2- and US11-mediated degradation of major histocompatibility complex class I molecules. J. Biol. Chem. 277:3258–3267.
27. Hirsch, C., S. Mitsaghi, D. Blom, M.E. Pacold, and H.L. Ploegh. 2004. Yeast N-glycanase distinguishes between native and non-native glycoproteins. EMBO Rep. 5:201–206.
28. Wang, B., A. Rieger, O. Kilgus, K. Ochiai, D. Maurer, D. Fodinger, J.P. Kinet, and G. Stingl. 1992. Epidermal Langerhans cells from normal human skin bind monomeric IgE via Fc εpon RI and G. Stingl. 1994. Immunomorphologic characterization of Fc εpon RI-bearing cells within the human dermis. J. Invest. Dermatol. 102:315–320.
30. Khubal, R., B. Osterhoff, B. Wang, J.P. Kinet, D. Maurer, and G. Stingl. 1997. The high-affinity receptor for IgE is the predominant IgE-binding structure in lesional skin of atopic dermatitis patients. *J. Invest. Dermatol.* 108:336–342.

31. Albrecht, B., M. Woisetschlager, and M.W. Robertson. 2000. Export of the high-affinity IgE receptor from the endoplasmic reticulum depends on a glycosylation-mediated quality control mechanism. *J. Immunol.* 165:5686–5694.

32. Fiebiger, E., C. Story, H.L. Ploegh, and D. Tortorella. 2002. Visualization of the ER-to-cytosol dislocation reaction of a type I membrane protein. *EMBO J.* 21:1041–1053.

33. Fiebiger, E., C. Hirsch, J.M. Vyas, E. Gordon, H.L. Ploegh, and D. Tortorella. 2004. Dissection of the dislocation pathway for type I membrane proteins with a new small molecule inhibitor, eeyarestatin. *Mol. Biol. Cell.* 15:1635–1646.

34. Tokunaga, F., C. Brostrom, T. Koide, and P. Arvan. 2000. Endoplasmic reticulum (ER)-associated degradation of misfolded N-linked glycoproteins is suppressed upon inhibition of ER mannosidase I. *J. Biol. Chem.* 275:40757–40764.

35. Ra, C., M.H. Jouvin, U. Blank, and J.P. Kinet. 1989. A macrophage Fc gamma receptor and the mast cell receptor for IgE share an identical subunit. *Nature.* 341:752–754.

36. Kurosaki, T., I. Gander, U. Wirthmueller, and J.V. Ravetch. 1992. The beta subunit of the Fc epsilon RI is associated with the Fc gamma RIIC on mast cells. *J. Exp. Med.* 175:447–451.

37. Dombrowicz, D., V. Flamand, I. Miyajima, J.V. Ravetch, S.J. Galli, and J.P. Kinet. 1997. Absence of Fc epsilonRI alpha chain results in upregulation of Fc gammaRIIC-dependent mast cell degranulation and anaphylaxis. Evidence of competition between Fc epsilonRI and Fc gammaRIIC for limiting amounts of FcR beta and gamma chains. *J. Clin. Invest.* 99:915–925.

38. Ploegh, H.L. 1995. Current Protocols in Protein Science. Wiley, New York. 10.12.11–10.12.18.

39. Bertho, N., J. Cerny, Y.M. Kim, E. Fiebiger, H. Ploegh, and M. Boes. 2003. Requirements for T cell-polarized tubulation of class II compartments in dendritic cells. *J. Immunol.* 171:5689–5696.

40. Wiertz, E.J., T.R. Jones, L. Sun, M. Bogyo, H.J. Geuze, and H.L. Ploegh. 1996. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell.* 84:769–779.