TheSignalPeptidede theIgEReceptor α-ChainPreventsSurface Expression of anImmunoreceptor Tyrosine-basedActivation Motif-freeReceptor Pool*

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The high affinity receptor for IgE, Fc epsilon receptor I (FceRI), is an activating immune receptor and key regulator of allergy. Antigen-mediated cross-linking of IgE-loaded FceRI α-chains induces cell activation via immunoreceptor tyrosine-based activation motifs in associated signaling subunits, such as FceRI γ-chains. Here we show that the human FceRI α-chain can efficiently reach the cell surface by itself as an IgE-binding receptor in the absence of associated signaling subunits when the endogenous signal peptide is swapped for that of murine major histocompatibility complex class-I H2-Kb. This single-chain isoform of FceRI exited the endoplasmic reticulum (ER), trafficked to the Golgi and, subsequently, trafficked to the cell surface. Mutational analysis showed that the signal peptide regulates surface expression in concert with other described ER retention signals of FceRI-α. Once the FceRI α-chain reached the cell surface by itself, it formed a ligand-binding receptor that stabilized upon IgE contact. Independently of the FceRI γ-chain, this single-chain FceRI was internalized after receptor cross-linking and trafficked into a LAMP-1-positive lysosomal compartment like multimeric FceRI. These data suggest that the single-chain isoform is capable of shutting IgE-antigen complexes into antigen loading compartments, which plays an important physiologic role in the initiation of immune responses toward allergens. We propose that, in addition to cytosolic and transmembrane ER retention signals, the FceRI α-chain signal peptide contains a negative regulatory signal that prevents expression of an immunoreceptor tyrosine-based activation motif-free IgE receptor pool, which would fail to induce cell activation.

Cell surface expression of FceRI,2 the high affinity receptor for IgE, regulates the magnitude of allergic responses (1–3).

FceRI is part of the family of multimeric immune recognition receptors, also referred to as activating immune receptor complexes (4–6). IgE-allergen-mediated cross-linking of the FceRI α-chain induces the release of inflammatory mediators via ITAMs of the associated signaling subunits, FceRI-β and a dimer of FceRI-γ chains (7, 8). FceRI α-chain transport from the ER to the cell surface is a tightly regulated trafficking process because susceptibility to IgE-mediated cell activation depends on the display of IgE-binding epitopes by FceRI-α (3, 9, 10).

The ER quality control system monitors correct folding as well as post-translational modifications of proteins and protein complexes (11–13). Several regulatory mechanisms that modulate the ER exit of FceRI complexes have been described. All of the ER protein quality control steps for type I membrane proteins apply to the FceRI α-chain (14). Synergistic ER retrieval signals are described for FceRI-α: two dilysine motifs, Lys212–Lys216 and Lys226–Lys230, in the cytosolic tail and the charged transmembrane amino acid Asp152 (15, 16). In human cells, these ER retrieval signals are overcome by the assembly of FceRI-α with FceRI-γ, the common Fc receptor γ-chain (3). In contrast, murine FceRI-α requires assembly with both FceRI-γ and FceRI-β to reach the cell surface. Hartman et al. (17) suggested recently that the difference in ER exit requirements between human and murine FceRI-α is encoded entirely in the extracellular domain of the protein. Furthermore, N-linked glycosylation of the IgE-binding epitopes of FceRI-α has been described as a checkpoint of the ER quality control system (18). Interestingly, the formation of IgE-binding epitopes depends only on proper core glycosylation in the ER and can occur completely independent of other receptor subunits (18).

Another key control step for the formation of FceRI complexes is the requirement for cotranslational assembly of the FceRI α-chain with its signaling subunits (19). This is different from assembly mechanisms defined for other activating immune receptors, such as the T or B cell receptors, that do not depend on coordinated translation of their subunits for receptor complex formation (4, 20). After the removal of all known transmembrane and cytosolic retention signals in FceRI-α, a substantial amount of the protein still remains intracellular (15, 18, 21). These findings suggest that an as yet undefined sequence element prevents surface expression of the FceRI α-chain in the absence of FceRI-γ. Therefore, we revisited the regulatory mechanisms for the display of FceRI-α at the cell surface.

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2 The abbreviations used are: FcεRI, high affinity Fc receptor for IgE; ER, endoplasmic reticulum; LAMP-1, lysosomal-associated membrane protein-1; ITAM, immunoreceptor tyrosine-based activation motif; HA tag, hemagglutinin epitope tag; mAb, monoclonal antibody; eGFP, enhanced green fluorescent protein; endo-α, endogenous α-chain; EndoH, endoglycosidase H; PNGase F, peptide N-glycosidase F; FACS, fluorescence-activated cell sorter; RT, reverse transcription.
Type I membrane proteins, including FcεRI-α, contain a short cleavable N-terminal sequence called the signal peptide. The signal peptide sequence assures proper translocation into the ER; it binds the signal recognition particle and later is cleaved on the ER lumenal side by signal peptidases. Signal peptides have no bona fide consensus sequences, although they commonly contain a highly hydrophobic stretch (typically 10–15 amino acids) long that is preceded by a basic residue and followed by a cleavage site for the signal peptidase. A growing body of evidence suggests that signal sequences are actively involved in the quality control of type I proteins (22, 23). We thus hypothesized that the signal peptide of FcεRI-α provides a control element for ER exit and surface trafficking. Such a control module could operate in two ways: it could facilitate the cotranslational assembly of the receptor subunits, and it could prevent surface expression of a single α-chain receptor isoform without signaling subunits. Here we show that the endogenous signal peptide of FcεRI-α does indeed contain a regulatory element that controls ER exit and consecutively cell surface expression of this protein.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-human FcεRI-α monoclonal antibodies (mAb) 19-1 and 15-1 and polyclonal rabbit anti-FcεRI-α were kindly provided by Dr. J.-P. Kinet (Laboratory of Allergy and Immunology, Beth Israel Deaconess Medical Center, Boston, MA) and used as published (24, 25). The mAb 19-1 reacts only with FcεRI-α chain that expresses the IgE binding epitopes (ER and Golgi modified forms). IgE (Serotec) and anti-FcεRI-α antibody 19-1 followed by peroxidase-conjugated goat anti-mouse IgG for detection of precipitated α-chain. HA-tagged proteins were detected with peroxidase coupled 3F10 (Roche Applied Science). FcεRI-γ was detected with polyclonal anti-FcεRI-γ serum followed by peroxidase-conjugated goat anti-rabbit IgG. Peroxidase activity was detected using SuperSignal chemiluminescent substrate reagents (Pierce). Endoglycosidase H (EndoH) and PNGase F (Roche Applied Science) digestions were performed according to manufacturer’s instructions.

FACS Analysis and Cell Sorting—The cells were stained with either phycoerythrin-conjugated or allophycocyanin-conjugated anti-FcεRI-α antibody CRAI or appropriate isotype control antibodies and analyzed on a FACSscan flow cytometer (Becton Dickinson) using CellQuest software for acquisition and analysis. For staining of intracellular FcεRI-α, the cells were fixed and permeabilized using Fix & Perm reagents (CALTAG Laboratories; Invitrogen) prior to staining. Cell sorting was performed on a MoFlo cell sorter (DAKO).

RT-PCR and Real Time RT-PCR—RNA was isolated using the RNasy Mini Kit (Qiagen) performing on column DNasel digestion. cDNA synthesis was carried out with SuperScript III Reverse Transcriptase Super Mix using oligo(dT) primers (Invitrogen). To detect the human Fc receptor γ-chain, the following primers were used: forward, 5’-ACGGGCTGTAG-ACCCAGGAA-3’; and reverse, 5’-GGGTTAGGCGACT-GGTGT-3’. Human FcεRI-α chain expression was quantified by real time PCR using iQ SYBR Green Supermix (Bio-Rad) on a iCycler iQ5 (Bio-Rad). For FcεRI-α, β-actin and glyceraldehyde-3-phosphate dehydrogenase QuantiTect primers were purchased from Qiagen.

Immunofluorescence Microscopy—Immunofluorescence experiments were performed essentially as previously described (28) with minor modifications. To visualize Golgi trafficking of FcεRI-α, Melljuso cells were allowed to attach to coverslips and then fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 20 min at room temperature prior to permeabilization in 0.5% saponin, 3% bovine serum albumin, phosphate-buffered saline solution. Polyclonal rabbit serum 997 was used for

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**RESULTS**

**Efficiency of the ER Exit of the FcεRI α-Chain Depends on Its Signal Peptide**—We wanted to test the hypothesis that the signal peptide of the FcεRI α-chain contains a module to regulate ER exit of this protein in its properly folded IgE-binding form. Therefore, we compared the intracellular trafficking of FcεRI-α with its endogenous signal peptide (referred hereafter as endo-α) with a chimeric FcεRI α-chain that had its signal peptide swapped for that of H2-Kb (referred hereafter as Kb-α). A comparison of both signal peptides is shown in Fig. 1A. The H2-Kb signal peptide is highly efficient at driving expression of the murine major histocompatibility complex class I H2-Kb molecule and is thus commonly used to optimize expression of type I membrane proteins in vitro and in vivo (19, 20, 29, 30). A

MelJuso cells transfected with Kb-α. Immunoprecipitation was performed with IgG; IgG was used to control for specificity of IgE binding. F, RT-PCR analysis confirmed lack of γ-chain expression in 293T, HeLa, and MelJuso cells. cDNA from human tonsil tissue was used as positive control (CTRL).
summary of all constructs used in this study is given in Table 1. Because FcεRI-α becomes highly glycosylated on its way from the ER to the cell surface, modifications of N-glycans on the FcεRI α-chain can be used to monitor proper ER to Golgi trafficking of the protein. 293T cells were transiently transfected with FcεRI endo-α or Kβ-α constructs, and the molecular properties of the translation products were compared (Fig. 1, A and B). All of the constructs were tagged with HA at the C terminus to allow for detection of the FcεRI-α-chain irrespective of its folding stage. We used the molecular weight characteristics of endo-α in the presence of FcεRI-γ as the published standard for comparison (Fig. 1B, first lane) (18, 25, 31). Immunoblotting experiments showed that the protein pattern between 40 and 60 kDa obtained with single FcεRI-α-chains (Fig. 1B, second and third lanes) is comparable with the pattern in α- and γ-chain cotransfected cells (Fig. 1B, first lane). In the absence of FcεRI-γ, endo-α seems to be expressed predominantly near ~46 kDa; based on the literature this is most likely the ER glycosylated form of the protein (ER form; Fig. 1B, second lane) (19, 24). We detected some unglycosylated protein backbone at ~34 kDa, probably because of incomplete insertion into the ER. Interestingly, some higher molecular weight forms, most likely Golgi-modified protein (Golgi form; Fig. 1B, second lane), were also detected (19, 24, 31). This was not expected from the literature and indicates that a small amount of endo-α by itself can exit the ER (Fig. 1B, second lane). Exchange of the endogenous signal peptide with the H2-Kβ signal peptide (Kβ-α) dramatically enhanced the amounts of potentially Golgi-modified protein despite the absence of FcεRI-γ (Fig. 1B, third lane). Transfection with an FcεRI-α construct lacking a signal peptide resulted in the expression of a 30–34 kDa protein corresponding to the unglycosylated protein backbone (Fig. 1B, fourth lane). These results suggested that the signal peptide is involved in the control of ER exit and the amount of FcεRI α-chain that traffics to the Golgi in the absence of FcεRI γ-chains.

We next confirmed that the observed molecular weight changes of the single-chain FcεRI-α resulted from post-translational glycosylation because of receptor trafficking from ER to Golgi and not from polyubiquitination during targeting for proteasomal degradation. Thus, we immunoprecipitated FcεRI α-chain with IgE to select for properly folded protein and examined the extent of N-glycosylation by immunoblotting. Endo-α in the presence of FcεRI-γ was used as a control (Fig. 1C, left panel). The ER form of FcεRI-α was sensitive to EndoH digestion as evidenced by a drop in the molecular weight of the 46-kDa ER form to the protein backbone (34 kDa; Fig. 1C, compare first and second lanes). In the presence of FcεRI-γ, a large amount of FcεRI-α remained EndoH-resistant because of glycosylation patterns acquired in the Golgi (Fig. 1C, second lane).

Deglycosylation of FcεRI-α precipitated from endo-α or Kβ-α transfectants with EndoH and PNGase F yielded a comparable protein pattern to that of endo-α in the presence of FcεRI-γ (Fig. 1C, compare left immunoblot with middle and right immunoblots). In line with our observations in whole cell lysates (Fig. 1B), the amount of EndoH-sensitive protein varied depending on the nature of the signal peptide. The Golgi form precipitated from endo-α and Kβ-α transfectants remained sensitive to PNGase F (Fig. 1C, third, sixth, and ninth lanes). Therefore, we concluded that the single-chain isoform of FcεRI was properly glycosylated in the Golgi. Sensitivity to PNGase F digestion also excluded that single FcεRI α-chain was simply aggregated or ubiquitinated protein and targeted for degradation.

We next visualized the ER exit of the single FcεRI α-chain. We transiently transfected MelJuso cells with Kβ-α and detected the α-chain with the polyclonal serum 997 (Fig. 1D, left panel). Immunofluorescence double staining with the Golgi marker GM130 (Fig. 1D, middle panel) showed a significant amount of FcεRI α-chain in a Golgi compartment in the absence of FcεRI-γ (Fig. 1D, overlay, right panel). Immunoprecipitation with IgE confirmed that the α-chain was properly modified in the Golgi in these cells (Fig. 1E). We confirmed by RT-PCR and immunoblot that neither 293T, HeLa, nor MelJuso express FcεRI-γ (Fig. 1F and data not shown). In summary, this set of data demonstrated that the FcεRI α-chain by itself can exit the ER and traffics to the Golgi where N-linked glycans are modified in the absence of FcεRI-γ transcripts.

**TABLE 1**

| Construct name | Description |
|----------------|-------------|
| Endo-α         | Human FcεRI α-chain with endogenous signal peptide |
| Kβ-α           | Human FcεRI α-chain with signal peptide of murine major histocompatibility complex class I H2-Kβ |
| Endo-α<sup>endominus</sup> | Truncation mutant of endo-α lacking the cytosolic tail of the α-chain |
| Kβ-α<sup>D/N</sup> | Truncation mutant of Kβ-α lacking the cytosolic tail of the α-chain |
| Kβ-α<sup>RI</sup> | Transmembrane mutant of Kβ-α; the negatively charged aspartic acid in the transmembrane region was exchanged to the neutral asparagine |
| Endo-α<sup>NC</sup> | Signal peptide mutant; the negatively charged glutamic acid at position 6 was exchanged to the neutral alanine |
| Endo-α<sup>DE</sup> | Signal peptide mutant; the negatively charged glutamic acid at position 6 was exchanged to the positively charged lysine |

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different analysis regions: eGFP-negative cells (Fig. 2B, upper plot), low eGFP-expressing cells (eGFP<sub>low</sub>), and high eGFP-expressing cells (eGFP<sub>high</sub>). eGFP<sub>high</sub> cells expressed significantly more FcεRI-α at the cell surface than eGFP<sub>low</sub> cells (i.e. 68% versus 15%, respectively).

To analyze cells with equal transfection efficiencies, we next sorted endo-α and K<sup>b</sup>-α transfected cells based on equal levels of eGFP expression and analyzed the protein characteristics of the FcεRI-α chain by immunoblotting with the mAb 19-1 (Fig. 2C). This reagent reacts only with the properly folded IgE-bind-
transfected with empty vector (6.0 ± 1.5% versus 1.0 ± 0.2%, respectively; means ± S.E., n = 7). Consistent with the more effective ER-to-Golgi transport (Fig. 2C), Kβ-α transfected cells showed a 3.45-fold increase in expression when compared with endo-α transfectants (20.7 ± 2.9% versus 6.0 ± 1.5%, respectively; means ± S.E., n = 7; Fig. 2E).

Because surface expression of FcεRI-α was substantially higher using constructs with the H2-Kβ signal peptide (Fig. 2E), it is fair to conclude that these findings argue for a strong retention of properly folded FcεRI-α chain in the ER by its endogenous signal peptide and imply that this signal peptide is a control module to prevent surface display of a single-chain isoform of FcεRI.

**The Signal Peptide Controls Surface Expression of FcεRI-α in the Absence of Cytoplasmic ER Retention Signals—**ER retention/retirval by dileucine motifs in the cytosolic tail of FcεRI-α has been proposed as a key regulatory mechanism to prevent ER exit in the absence of the FcεRIγ chain (15, 16). Thus, we generated cytosolic tail truncations of endo-α and Kβ-α to dissect the contributions of the signal peptide from the ER retention signals of the cytosolic tail of the α-chain.

Endo-α and Kβ-α constructs that lack their cytosolic tails were generated by introducing stop codons following the transmembrane domain of FcεRI-α at position serine199. These constructs are referred to as endo-αtail-minus and Kβ-αtail-minus.

The full-length and tail-minus constructs were transiently transfected into 293T cells. Using bicistronic expression of eGFP as a marker, a gate was set for the analysis of cells with comparable transfection efficiencies (Fig. 3A, *upper panel*). Both the endo-αtail-minus and Kβ-αtail-minus reached the cell surface more efficiently than the full-length constructs (Fig. 3, *A* and *B*). Importantly, Kβ-αtail-minus was exported far more efficiently than endo-αtail-minus (88 ± 12% versus 37 ± 16%, respectively; mean ± S.D., n = 4; Fig. 3B). Additionally, we sorted transfected cells based on their eGFP expression and performed immunoblot analysis with the mAb 19-1 (Fig. 2C). We detected, again, more Golgi form of FcεRI-α in cells transfected with Kβ-α than endo-α (Fig. 3C, *first and second lanes*). Golgi-modified FcεRI-α chains were also more abundant in cells trans-

**FIGURE 3.** The signal peptide regulates cell surface expression of the FcεRI-α-chain independently of ER retention signals in the cytosolic tail. A, 293T cells were transfected with constructs lacking the cytosolic tail of the α-chain (i.e. endo-αtail-minus and Kβ-αtail-minus) or with the full-length constructs, endo-α, and Kβ-α, in pIRES2-eGFP. Transfected cells expressing equal levels of eGFP were gated (upper dot plots) and analyzed for α-chain at the cell surface (lower dot plots). Empty vector transfected cells were used as control (CTRL). B, quantification (means ± S.E.) of cell surface expression of 4 independent experiments as shown in A. C, cells expressing equal levels of eGFP were FACS-sorted and cell lysates were analyzed for protein characteristics of FcεRI-α by immunoblotting with mAb 19-1. Detection of eGFP was used as loading control. D, an Asp → Asn mutation in the transmembrane domain increases surface expression of FcεRI-α-chain. HeLa cells were transiently transfected with Kβ-α or the Asp → Asn transmembrane mutant (i.e. Kβ-αD/N). FACS histogram overlay depicts surface α-chain expression. Kβ-α, filled, gray histogram; Kβ-αD/N, striped, black histogram; empty vector, light gray line. E, relative FcεRI-α mRNA levels of Kβ-α and Kβ-αD/N were determined by real time PCR. The values were determined in triplicate and normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Representative data of three independent experiments are shown in *D* and *E*.

The full-length and tail-minus constructs were transiently transfected into 293T cells. Using bicistronic expression of eGFP as a marker, a gate was set for the accuracy of the cell sorting, eGFP levels were determined (Fig. 2C). As an additional control we sorted cells with equal levels of eGFP and showed that those cells also expressed equal levels of FcεRI-α mRNA by quantitative RT-PCR (Fig. 2D). We next studied surface expression efficiency of the single FcεRI α-chain in the bicistronic expression system. 293T cells were transiently transfected with endo-α, Kβ-α, or the empty pIRES-eGFP vector as a control. Cells expressing equal levels of eGFP were gated and analyzed for cell surface expression of FcεRI-α (Fig. 2E). Cells transfected with the endo-α construct showed low but significant surface expression levels compared with cells transfecting FcεRI-α chain (25). We confirmed that Kβ-α transfectants contained significantly higher levels of Golgi-modified protein than endo-α transfectants (Fig. 2C). To control for the accuracy of the cell sorting, eGFP levels were determined (Fig. 2C). As an additional control we sorted cells with equal levels of eGFP and showed that those cells also expressed equal levels of FcεRI-α mRNA by quantitative RT-PCR (Fig. 2D). We next studied surface expression efficiency of the single FcεRI α-chain in the bicistronic expression system. 293T cells were transiently transfected with endo-α, Kβ-α, or the empty pIRES-eGFP vector as a control. Cells expressing equal levels of eGFP were gated and analyzed for cell surface expression of FcεRI-α (Fig. 2E). Cells transfected with the endo-α construct showed low but significant surface expression levels compared with cells transfecting FcεRI-α chain (25). We confirmed that Kβ-α transfectants contained significantly higher levels of Golgi-modified protein than endo-α transfectants (Fig. 2C). To control for the accuracy of the cell sorting, eGFP levels were determined (Fig. 2C). As an additional control we sorted cells with equal levels of eGFP and showed that those cells also expressed equal levels of FcεRI-α mRNA by quantitative RT-PCR (Fig. 2D). We next studied surface expression efficiency of the single FcεRI α-chain in the bicistronic expression system. 293T cells were transiently transfected with endo-α, Kβ-α, or the empty pIRES-eGFP vector as a control. Cells expressing equal levels of eGFP were gated and analyzed for cell surface expression of FcεRI-α (Fig. 2E). Cells transfected with the endo-α construct showed low but significant surface expression levels compared with cells
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fected with K^b-α^tail-minus than endo-α^tail-minus (Fig. 3C, third and fourth lanes).

We also investigated whether the modification of the transmembrane ER retention signal Asp^{192} (15) would have an effect on surface trafficking of K^b-α and generated a K^b-α^{D/N} mutant. The removal of the transmembrane ER retention signal resulted in more efficient surface expression of K^b-α^{D/N} versus K^b-α (Fig. 3D) at equal mRNA expression levels (Fig. 3E).

In summary, we showed that a single α-chain isofrom of FcεRI reached the cell surface alone. The K^b-signal peptide chimera trafficked more efficiently to the cell surface even in the absence of cytosolic ER retention motifs, indicating that the K^b-signal peptide does not simply override ER retention. This set of data suggests that the signal peptide provides an additional regulatory element that controls FcεRI α-chain trafficking in a concerted way with recently described ER retention signals.

**Point Mutations in the Endogenous Signal Peptide Argue against Interference with Signal Particle Recognition as a Regulatory Mechanism for Cell Surface Transport**—Signal peptides do not have a well defined consensus sequence; however, one common feature is a positively charged residue preceding the hydrophobic stretch. This N-terminal part of the signal peptide sequence is critical for recognition of nascent proteins by the signal recognition particle to initiate translocation across the ER membrane (32–34). The endogenous signal peptide of the FcεRI α-chain does not contain this positively charged amino acid. Instead, a negatively charged glutamic acid is found in position 6 (Figs. 1A and 4A). The H2-K^b signal peptide is devoid of any charged amino acids (Figs. 1A and 4A). To investigate whether the introduction of a positively charged amino acid or removal of the negative charged amino acid in the endogenous signal peptide of the α-chain could modulate intracellular trafficking, we generated point mutants where the glutamic acid (E) at position 6 was exchanged to lysine (K) or alanine (A). The generated constructs were termed endo-α/E/K and endo-α/E/A. Neither endo-α/E/K nor endo-α/E/A was able to reach the cell surface more efficiently than endo-α (Fig. 4B). We also compared the ER to Golgi transport and found no significant difference between endo-α and the mutants (Fig. 4C and data not shown). Thus, mutating the endogenous FcεRI signal peptide to match the signal peptide consensus sequence or to resemble that of H2-K^b did not affect trafficking. These results argue for signal peptide cleavage rather than ER insertion as the mechanism that regulates ER exit of the FcεRI α-chain in the absence of the FcεRI γ-chains.

**Single FcεRI α-Chain Is Stabilized upon Monovalent Interaction with IgE**—Multimeric isofroms of FcεRI are stabilized at the cell surface by monovalent interactions with IgE (35). We next wanted to investigate whether the single-chain isofrom of FcεRI also shares this key feature of the multimeric FcεRI complexes. We cultured endo-α and K^b-α transfecants with or without IgE for 16 h. Interaction with IgE stabilized the single FcεRI α-chain at the cell surface as measured by flow cytometry (Fig. 5). The degree of stabilization was comparable between both constructs (2.3-fold versus 1.7-fold; Fig. 5B). These data showed that single FcεRI-α interacts with and is stabilized by its natural ligand IgE at the cell surface like multimeric receptor isofroms.

**Single FcεRI α-Chain Internalizes after Cross-linking**—Cross-linking of multimeric FcεRI isofroms (i.e., αγ2 and αβγ2) induces signaling via the ITAM motifs in the γ- and β-chains as...
This indicates that the Fc α-chain for internalization and intracellular trafficking after Fc receptor internalization (data not shown). Next, internalization of FcR-I at the cell surface. We monitored α-chain internalization in HeLa cells. First, FcRI was loaded with the α-chain-specific antibody CRA1 at the cell surface. This incubation step does not result in receptor internalization (data not shown). Next, internalization of FcRI-α was induced by cross-linking with a fluorescently labeled secondary antibody. Irrespective of the presence of the γ-chain, we found FcRI-α inside the cell within 30 min (Fig. 6A). HeLa cells stably transfected with a LAMP-1-eGFP reporter were used to study whether the single-chain receptor isoform traffics into endo/lysosomal compartments. We found that a single FcRI α-chain shuttles to a lysosomal compartment as characterized by LAMP-1 expression comparable with multimeric FcRI containing the common γ-chain (Fig. 6B). This indicates that the FcRI signaling subunits are not essential for internalization and intracellular trafficking after FcRI cross-linking.

DISCUSSION

FcRI is an activating immune receptor complex that must accomplish the following tasks: assembling properly in the ER, reaching the cell surface, binding IgE, and inducing ITAM-based cell activation as well as internalization of receptor complexes upon antigen-mediated receptor cross-linking (schematic in Fig. 7A).

The fidelity of intracellular trafficking of FcRI complexes has so far been considered a function of cytosolic and transmembrane ER retention signals in the FcRI α-chain that are masked by association with the other receptor subunits. Here we demonstrated that the signal peptide of FcRI-α contains an additional regulatory element that contributes to the retention

FIGURE 5. IgE-induced stabilization of FcεRI at the cell surface does not require FcεRI-γ-chains. A, 293T cells were transfected with Kb-α in pRES2-eGFP, incubated with 200 ng/ml IgE for 18 h, and analyzed by flow cytometry. Transfected cells were identified by eGFP expression and gated to determine cell surface expression of the α-chain. B, IgE stabilization of the single FcεRI α-chain is signal peptide-independent. 293T cells were transfected with endo-α or Kβ-α in pRES2-eGFP, incubated with 200 ng/ml IgE for 18 h before analysis by flow cytometry. The data are presented as the means ± S.E. of three independent experiments. CTRL, control.

FIGURE 6. Single FcεRI α-chain internalizes after receptor cross-linking. A, Kβ-α was transiently transfected into HeLa cells (left images) or into HeLa cells stably expressing the FcεRI γ-chain (right images). For FcεRI-α cross-linking, the cells were incubated with the mouse anti-human FcεRI-α antibody, CRA1, which was next cross-linked with an anti-mouse Alexa-Fluor 568-F(ab')2 fragment (shown in red). The cells were incubated for 30 min at 37°C (lower panels). For time point (t) = 0, the cells were fixed before cross-linking with anti-mouse Alexa Fluor 568-F(ab')2 (upper panels). Cell surface membranes were visualized using Alexa Fluor 647-labeled wheat germ agglutinin (shown in blue). Confocal micrographs are presented as overlays. Representative experiment is shown (n = 3). B, FcεRI α-chain traffics into LAMP-1-positive lysosomal compartments independently of the presence of FcεRI-γ. HeLa cells expressing LAMP-1-eGFP (shown in green) were transfected with Kβ-α (shown in red). Receptor cross-linking and internalization was performed as described above. The left panel shows cells transfected with Kβ-α alone. The right panel shows cells that co-express FcεRI α- and γ-chains. The bottom panel depicts higher magnifications of the lysosomal regions of the images shown in the upper panel. The bottom panel shows single channel images for LAMP-eGFP in green (left), FcεRI-α in red (middle), and the merged image (right). The cells were analyzed 90 min after cross-linking.
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FIGURE 7. Schematic of intracellular trafficking of FceRI and receptor-mediated cell activation. A, the subunits of multimeric FceRI complexes assemble in the ER, traffic to the Golgi and reach the cell surface. Antigen-induced cross-linking of IgE-loaded FceRI complexes induces ITAM-mediated cell activation and internalization of the receptor. Cross-linked FceRI shuttles antigen-IgE immune complexes to lysosomal compartments. B, single FceRI \alpha-chain can exit the ER, traffic to the Golgi and reach the cell surface by itself. Trafficking of this single receptor isoform is tightly regulated by the signal peptide. Antigen-induced cross-linking of IgE-loaded single FceRI \alpha-chain induces receptor internalization in the absence of ITAM-mediated cell activation. Like multimeric FceRI complexes, cross-linked single FceRI \alpha-chain isoforms reach lysosomal compartments.

of the \alpha-chain in the ER. We found that human FceRI-\alpha inefficiently reaches the cell surface by itself and that a swap of the endogenous signal peptide for that of H2-Kb allows for significantly more efficient surface expression.

We show that the single FceRI \alpha-chain binds IgE at the cell surface and stabilizes upon IgE binding. This observation supports an earlier report by Kubota et al. (35) describing that surface stabilization of FceRI is a function of the stalk region of the \alpha-chain. This conclusion was derived from the analysis of chimeric \alpha-chains in the presence of FceRI \gamma-chains. Our experiments show unambiguously that the IgE-mediated surface stabilization affects wild type FceRI-\alpha as a single-chain receptor. We further demonstrate that single FceRI \alpha-chain is efficiently targeted into lysosomal compartments after receptor cross-linking. Thus, the single-chain isoform of the receptor could act as an IgE-receptor, which reacts to serum IgE levels and shuttles antigen into antigen presentation compartments. Removal of the cytosolic ER retention signals as well as mutating the transmembrane ER retention signal suggests that the signal peptide regulates ER retention in a concerted fashion with formerly described ER retention motifs (15, 18). The fact that the regulatory influence of the signal peptide could still be observed when other ER retention signals were removed argues against a simple overexpression artifact.

To our knowledge, this is the first report showing that a signal peptide can regulate the surface expression of an Fc receptor. However, several reports in the literature have shown that signal peptides can function as more than just ER targeting sequences (14). A polymorphism in the luteinizing hormone receptor protein improves its signal peptide function and increases protein expression. The consequences of the higher expression levels of this luteinizing hormone receptor protein are unfavorable, and consequently, this polymorphism serves as a predictor for adverse outcome in breast cancer patients (37). Along this line, genetic polymorphism in the signal peptide of FceRI-\alpha could influence IgE receptor expression levels and allergy. Whether such a polymorphism in the FceRI-\alpha signal peptide indeed exists in humans needs to be addressed in future studies.

The ER assembly of FceRI complexes is regulated more tightly than that of other activating immune receptor complexes (19). Although other receptor complexes assemble in consecutive steps (4, 5, 20, 30), assembly of FceRI complexes occurs strictly cotranslationally. It is also important to keep in mind that FceRI-\gamma is a signaling subunit shared by multiple activating immune receptors. Therefore, several receptor complexes compete for this protein in the ER. Competition for limiting amounts of FceRI-\gamma has been demonstrated in vivo. The absence of FceRI \alpha-chain enhances FcYRIII-dependent mast cell degranulation and anaphylaxis (38). FceRI seems to be at a competitive disadvantage because it needs to fulfill its cotranslational assembly requirement. Therefore, the assembly machinery has to assure both temporal and spatial coordination of subunit translation. In this context, ER retention of FceRI-\alpha by the signal peptide could facilitate this cotranslational event. One possibility for signal peptides to modulate ER retention is by regulating the activity of type I signal peptidase (39). Because signal peptide cleavage is a cotranslational event, a slow cleavage rate might impede folding of FceRI-\alpha in a way that provides more time for assembly. Given that we were not successful in destroying the regulatory property of the endogenous signal peptide through a targeted mutation, however, we cannot formally rule out that the signal peptide controls \alpha-chain trafficking via regulating its recognition and translocation into the ER.

We suggest that this additional regulatory sequence element is important to prevent the formation of an FceRI \gamma-chain-free IgE receptor pool at the cell surface (schematic in Fig. 7B). In human antigen presenting cells, such as dendritic cells and macrophages, intracellular accumulation of FceRI-\alpha is frequently found (21, 39–41). This is probably because FceRI-\alpha alone folds properly and forms IgE-binding sites (9) and is therefore not efficiently recognized by the ER-associated degradation machinery. A slow rate of signal peptide processing might however help the ER-associated degradation system to recognize some of this FceRI-\alpha and target it for degradation. Although FceRI-\alpha is mainly restricted to the ER in the absence of the \gamma-chain, we show that some properly folded protein reaches the cell surface and forms a signaling-deficient IgE receptor isoform. This receptor pool lacks ITAM-based signaling modules yet retains its ability to bind IgE and to endocytose upon receptor activation. This single-chain IgE-receptor also reaches lysosomal compartments like tetrameric and trimeric FceRI (36).³ FceRI shuttles IgE-antigen complexes into lysoso-

³ B. Platzer, manuscript in preparation.
nal compartments for antigen presentation, most likely to facilitate allergen presentation via major histocompatibility complex class II (36, 42). We show here that this intracellular trafficking is not dependent on signaling subunits, which carry ITAM motifs in their cytosolic tails. We therefore conclude that signals via ITAMs in FcεRI-β or FcεRI-γ are dispensable for intracellular trafficking of IgE-antigen complexes to lysosomal compartments.

In the context of allergy, this FcεRI-γ and ITAM-free receptor appears attractive. Such an IgE receptor could remove IgE or IgE-allergen complexes in an immunologically silent form, because cross-linking would not initiate cell activation via ITAMs (Fig. 7B). IgE-FcεRI-mediated cell activation during allergy is, however, not the physiological function of IgE-mediated response. This pathway is highly active during helminth parasite infections and critical for the development of protective immunity. In this context, expression of an IgE-receptor that fails to signal is undesirable, because it could compromise the development of protective immunity. In summary, based on our findings, we propose that the endogenous signal peptide of FcεRI-α represents a critical control element preventing unwanted expression of a signaling-deficient IgE receptor isoform.

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