

Different Stabilities of the Structurally Related Receptors for IgE and IgG on the Cell Surface Are Determined by Length of the Stalk Region in Their α -Chains¹

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A variant of the high affinity IgE receptor Fc ϵ RI, which is composed of α - and γ -chains without the β -chain, is expressed on human APC, such as dendritic cells, and has been suggested to facilitate Ag uptake through IgE and hence to facilitate Ag presentation to T cells. The level of Fc ϵ RI on these cells is correlated with the serum IgE concentration, suggesting IgE mediates the up-regulation of the $\alpha\gamma$ 2-type Fc ϵ RI. The IgE-mediated Fc ϵ RI up-regulation on mast cells and basophils has been shown to enhance the ability of these cells to release chemical mediators and cytokines that are responsible for allergic inflammatory reactions. Here, to elucidate the mechanism controlling Fc ϵ RI expression, we compared two structurally related Ig receptors, human Fc ϵ RI and Fc γ RIIIA, which carry different α -chains but the same γ -chains. The half-life of Fc ϵ RI on the cell surface was short unless it bound IgE, whereas Fc γ RIIIA was stably expressed without IgG binding. Shuffling of the non Ig-binding portions of the Fc ϵ RI α and Fc γ RIIIA α chains revealed that the stalk region was critical in determining the difference in their stability and ligand-induced up-regulation. Unexpectedly, analyses with added or deleted amino acids in the stalk region strongly suggested that the length rather than the amino acid sequence of the stalk region was of major importance in determining the different stabilities of Fc ϵ RI and Fc γ RIIIA on the cell surface. This finding provides new insights into the mechanism regulating surface Fc ϵ RI expression. *The Journal of Immunology*, 2006, 176: 7008–7014.

The high affinity receptor for IgE, Fc ϵ RI, composed of α -, β -, and γ -chains, is expressed on the surface of mast cells and basophils as a critical component in allergic responses (1, 2). The ligation of IgE-bound Fc ϵ RI by multivalent Ags results in the activation of multiple signaling pathways leading to diverse effector responses, including the release of chemical mediators, cytokines, and chemokines, that are responsible for allergic inflammatory reactions (3, 4). IgE binding to Fc ϵ RI induces a marked up-regulation of Fc ϵ RI expression on mast cells and basophils in both humans and mice (5, 6). The IgE-mediated Fc ϵ RI up-regulation has been shown to enhance the ability of these cells to release chemical mediators and cytokines such as histamine, leukotrienes, IL-4, and IL-6 (6–8). In accord with this, the i.v. administration of nonanaphylactogenic humanized anti-IgE mAb (omalizumab) to atopic patients resulted in down-regulation of Fc ϵ RI on basophils in parallel with the reduction of mediator release from activated basophils (9, 10). Thus, the IgE-mediated Fc ϵ RI up-regulation appears to be one of the critical factors determining the severity of allergic diseases.

In humans, a variant Fc ϵ RI composed of α - and γ -chains without the β -chain is expressed on APC, such as monocytes and dendritic cells including Langerhans cells in the skin (11, 12). It has

been suggested that the cross-linking of Fc ϵ RI on these cells induces the production of inflammatory cytokines and that the Fc ϵ RI-IgE-Ag complex facilitates Ag uptake and Ag presentation to T cells, hence contributing to T cell-mediated allergic inflammation (13, 14). The level of Fc ϵ RI on dendritic cells and monocytes is highly correlated with the serum IgE concentration, as in the case of basophils (15, 16). Therefore, the β -chain of Fc ϵ RI appears to be dispensable for IgE-mediated Fc ϵ RI up-regulation.

We and others have recently demonstrated that stabilization and accumulation of Fc ϵ RI on the cell surface through IgE binding is the major mechanism of the IgE-mediated Fc ϵ RI up-regulation on mast cells (17, 18). Cell surface Fc ϵ RI is unstable and quickly removed, but Fc ϵ RI bound to IgE is more stable and stays on the cell surface longer. In the presence of excess IgE, every new Fc ϵ RI transported to the cell surface is loaded with IgE and stabilized, leading to increased surface Fc ϵ RI expression. Fc γ RIIIA, a low affinity IgG receptor, has a structure similar to that of Fc ϵ RI and two forms, $\alpha\beta\gamma$ 2 and $\alpha\gamma$ 2 (19). Fc γ RIIIA and Fc ϵ RI carry different α -chains but share β - and γ -chains (20). We previously compared the levels of mouse Fc γ RIIIA and Fc ϵ RI on bone marrow-derived mast cells over time and found that Fc γ RIIIA was stably expressed on the cell surface even in the absence of ligand binding, in contrast to Fc ϵ RI (17). This indicated that a difference in the α -chains of these two receptors accounted for their different stabilities on the cell surface in the absence of ligands. Overall, the α -chains display a similar structure; both have two Ig-like domains (D1 and D2) that are responsible for Ig binding, a stalk region that includes membrane-proximal and transmembrane portions, and a cytoplasmic tail. Therefore, close examination of structural differences between the α -chains of the two receptors was needed to determine what caused this difference in stability.

Here, we defined the region of human Fc ϵ RI α and Fc γ RIIIA α chains that regulates their stability on the cell surface by generating a panel of chimeric or mutant Fc ϵ RI α /Fc γ RIIIA α chains and expressing them in mouse fibroblast cells together with human

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Fc γ R. In contrast to our expectations, the stalk region but not the cytoplasmic tail was involved in determining the basal expression level and stability of the α -chain on the cell surface. Further analysis strongly suggested that the length rather than the amino acid sequence of the stalk region was important for determining the different half-lives of Fc ϵ RI α and Fc γ RIIIA α on the cell surface.

Materials and Methods

Antibodies

Biotinylated mouse mAbs specific for human Fc ϵ RI α (CRA-1), Fc γ RIII (3G8), IgE (G7-26), and their isotype-matched control mAbs, MPC-11 (IgG2b), MOPC-21 (IgG1), and G155-178 (IgG2a), respectively, and allophycocyanin-conjugated streptavidin were purchased from BD Pharmingen. Rabbit anti-Fc γ R chain, HRP-conjugated goat anti-rabbit IgG, mouse anti- α -tubulin mAb, and HRP-conjugated goat anti-mouse IgG were purchased from Upstate Biotechnology, Cell Signaling Technology, Sigma-Aldrich, and Santa Cruz Biotechnology, respectively. Purified human IgE was from Yamasa Shoyu (Chiba, Japan).

Cell lines and culture

The mouse fibroblast cell line NIH3T3 was grown in DMEM (Sigma-Aldrich) supplemented with 10% FCS (Invitrogen Life Technologies), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C under 5% CO₂. The retroviral packaging cell line Plat-E (21) was cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin-streptomycin, 5 μ g/ml puromycin (Sigma-Aldrich), and 5 μ g/ml blasticidin (Invitrogen Life Technologies).

Construction of vectors for expressing human Fc γ R, Fc ϵ RI α , Fc γ RIIIA α , and their mutants

cDNAs coding for human Fc γ R, Fc ϵ RI α , and Fc γ RIIIA α were generated by RT-PCR from peripheral blood and subcloned into pBluescript II (Stratagene). The Fc γ R cDNA insert was then cloned into the expression vector BCMGSHyg (22). Recombinant cDNAs encoding chimeric Fc ϵ RI α /Fc γ RIIIA α chains or mutant forms of each α -chain were generated by splice overlap extension using two-step PCRs (23). These cDNA inserts were then subcloned into a retroviral vector pMX-IRES-GFP (21).

Transfection of cell lines with expression vectors

NIH3T3 cells in 100-mm-diameter dishes were transfected with BCMGSHyg-Fc γ R (10 μ g) using Fugene6 (Roche Diagnostics), and selected in complete DMEM containing 0.5 mg/ml hygromycin B (Calbiochem) to obtain clones that stably expressed human Fc γ R. Plat-E cells were cultured in DMEM supplemented with 20% FCS and 100 U/ml penicillin-streptomycin at 2×10^6 cells/60-mm dish for 24 h, followed by transfection with pMX-IRES-GFP carrying the indicated cDNA using Effectene (Qiagen), and their culture supernatants were collected 48 h later. NIH3T3-Fc γ R cells were incubated with 1 ml of the culture supernatant for 24 h in the presence of 10 μ g/ml polybrene (Sigma-Aldrich).

Flow cytometry

NIH3T3 transfectants were cultured with or without 3 μ g/ml human IgE for 12 h to examine the IgE-mediated up-regulation of Fc ϵ RI. To examine the stability of Fc ϵ RI and Fc γ RIIIA α on the cell surface, the cells were cultured in the presence or absence of 5 μ g/ml brefeldin A (BFA³; Sigma-Aldrich) for 12 h. For flow cytometric analysis, single-cell suspension was prepared by treating cells with 0.25% trypsin-EDTA (Sigma-Aldrich) and preincubated with normal rat serum (Rockland) at 4°C for 15 min to prevent the nonspecific binding of other Abs. To detect human Fc ϵ RI α , Fc γ RIIIA α , and IgE on the cell surface, cells were stained with biotinylated CRA-1, 3G8, G7-26, respectively, followed by allophycocyanin-streptavidin. Stained cells were analyzed with a FACSCalibur (BD Biosciences).

Immunoprecipitation and immunoblotting

NIH3T3 transfectants were cultured with human IgE for 12 h before being lysed with lysis buffer (1% digitonin (Sigma-Aldrich), 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 10 mM NaF, 1 mM Na₃VO₄, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin). The Fc ϵ RI/IgE

complexes were immunoprecipitated with anti-human IgE mAb and protein G-Sepharose (Amersham Pharmacia Biotech) and resolved by SDS-PAGE. The proteins were electrotransferred to PVDF membranes and probed with rabbit anti-Fc γ R Ab followed by HRP-conjugated goat anti-rabbit IgG Ab. In parallel, aliquots of whole cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti- α -tubulin Ab, as a loading control.

Results

$\alpha\gamma$ 2-type Fc ϵ RI but not Fc γ RIIIA is unstable on the cell surface in the absence of ligand binding

The level of Fc ϵ RI on dendritic cells and monocytes is closely related with the serum IgE concentration in allergic patients (15, 16). To study the mechanisms underlying Fc ϵ RI stability on the cell surface, we used mouse fibroblast NIH3T3 cells transfected with human α - and γ -chains, which formed a reconstituted Fc ϵ RI. In accordance with the clinical observations, culturing the transfectant NIH3T3 cells with IgE for 12 h induced a 4-fold up-regulation of the cell surface Fc ϵ RI (Fig. 1A). Thus, the β -chain of Fc ϵ RI was dispensable for the IgE-mediated up-regulation of human Fc ϵ RI, and no molecules specific to mast cells and basophils seemed necessary for the IgE-mediated Fc ϵ RI up-regulation.

We previously showed that culturing mouse bone marrow-derived mast cells with BFA, an inhibitor of intracellular protein transport that prevents transport of newly synthesized molecules to the cell surface, induces a drastic reduction in $\alpha\beta\gamma$ 2-type Fc ϵ RI but not $\alpha\beta\gamma$ 2-type Fc γ RIIIA on the cell surface (17). Culturing the NIH3T3 transfectants in the presence of BFA without IgE for 12 h resulted in as much as 90% reduction in the $\alpha\gamma$ 2-type Fc ϵ RI expression (Fig. 1B, left panels). Preincubation of the transfectants with human IgE before the culture with BFA almost completely inhibited this reduction (data not shown), as observed previously for mouse $\alpha\beta\gamma$ 2-type Fc ϵ RI on mast cells (17). This indicated that the half-life of human $\alpha\gamma$ 2-type Fc ϵ RI on the cell surface was very short unless it bound IgE. In contrast, treatment with BFA did not significantly change the surface expression of human $\alpha\gamma$ 2-type Fc γ RIIIA reconstituted in NIH3T3 during a 12-h culture (Fig. 1B, right panels), indicating that the $\alpha\gamma$ 2-type human Fc γ RIIIA was stably expressed on the cell surface in the absence of ligand binding, as in the case of $\alpha\beta\gamma$ 2-type mouse Fc γ RIII.

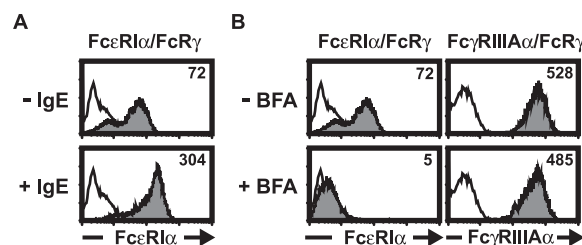


FIGURE 1. Difference between Fc ϵ RI α and Fc γ RIIIA α in their stability on the cell surface when expressed in NIH3T3 together with Fc γ R. **A**, Retroviral vectors encoding human Fc ϵ RI α together with GFP were introduced into NIH3T3 cells that had been engineered to stably produce human Fc γ R. Transfected cells were cultured without (upper) or with (lower) human IgE for 12 h and then stained with anti-Fc ϵ RI α mAb (shaded histogram) or isotype-matched control (open histogram). **B**, Retroviral vectors encoding Fc ϵ RI α (left panels) or Fc γ RIIIA α (right panels) were introduced into NIH3T3-Fc γ R cells. Transfected cells were cultured without IgE in the absence (upper panels) or presence (lower panels) of BFA for 12 h and then stained with anti-Fc ϵ RI α mAb (left panels, shaded histogram), anti-Fc γ RIII mAb (right panels, shaded histogram) or isotype-matched control (open histogram). In **A** and **B**, GFP⁺ cells (60–90%) were gated for analysis; data are representative of three repeated experiments. The mean fluorescence intensity in the stained cells is indicated in each panel.

³ Abbreviations used in this paper: BFA, brefeldin A; rST, stalk/cytoplasmic region; WT, wild type.

The stalk region of FcεRIα is a major determinant of FcεRI instability on the cell surface

Human FcεRI and FcγRIIIA reconstituted in NIH3T3 cells carried different α-chains but shared γ-chains. Therefore, the α-chains should account for their different stabilities on the cell surface. To determine the region of FcεRIα responsible for its instability on the cell surface, we generated a panel of chimeric human FcεRIα/FcγRIIIAα chains in which all or part of the non-IgE-binding portion of FcεRIα was replaced with the corresponding portion of FcγRIIIAα (Fig. 2A). Each chimeric α-chain was expressed in NIH3T3 transfectants that stably produced human Fcγ. All the chimeric α-chains were detected on the cell surface both by staining with an anti-FcεRIα mAb and by IgE binding (Fig. 2, B and C).

When the entire non-IgE-binding portion of FcεRIα, encompassing both the stalk region (membrane-proximal and transmembrane portion) and the cytoplasmic tail, was replaced with the corresponding portion of FcγRIIIAα, the basal level of the chimeric α-chain (stalk/cytoplasmic tail region; rST as shown in Fig. 2A) on NIH3T3 cells cultured without IgE was 8 times higher than that of wild-type (WT) FcεRIα (Fig. 2B and summarized in Fig. 2D). Culturing with IgE for 12 h had no significant effect on the level of rST expression, even though it induced a 4-fold up-regulation of WT FcεRIα expression (Fig. 2, B and D). Because rST was able to bind IgE (Fig. 2C), the failure of IgE to up-regulate rST on the cell surface was not due to a defect in IgE binding. Culturing with BFA did not have any significant effect on the level of rST expression, whereas it reduced WT FcεRIα >90% (Fig. 3A and summarized in Fig. 3B). Thus, rST behaved like FcγRIIIAα, indicating that the stalk region and/or cytoplasmic tail of FcεRIα was responsible for the instability of FcεRI.

To narrow down the responsible region, we analyzed a second set of chimeric FcεRIα/FcγRIIIAα chains in which either the stalk region or the cytoplasmic tail of FcεRIα was replaced with the corresponding portion of FcγRIIIAα (rS and rT, respectively, as shown in Fig. 2A). rS behaved like rST when expressed in NIH3T3 together with Fcγ, showing higher levels of basal expression than WT FcεRIα, no IgE-induced up-regulation, and no reduced surface expression when cultured with BFA (Figs. 2 and 3). In contrast, the basal level of rT expression was low, and IgE-induced up-regulation and BFA-induced down-regulation of rT were observed (Figs. 2 and 3). Thus, rT behaved like WT FcεRIα. These results indicated that the stalk region of FcεRIα and not its cytoplasmic tail was the major determinant of the instability of FcεRI on the cell surface. Given that the basal expression level of rS was lower than that of rST, the cytoplasmic tail of FcεRIα might play some role in regulating the basal level expression but not the stability of FcεRI.

In all the WT and chimeric FcεRIα transfectants that were cultured with IgE, Fcγ chains were detected in the IgE-binding complexes expressed on the cell surface, and the amount of Fcγ chains correlated with the surface FcεRIα level of each transfectant (Fig. 2E; compare with the filled bars in Fig. 2D). Thus, all the functional chimeric FcεRIα chains were expressed on the cell surface in association with Fcγ chains as in the case of WT FcεRIα chains.

The length of the stalk region of the α-chain is an important determinant of FcεRI and FcγRIIIA stability on the cell surface

When we compared the stalk region of the FcεRIα and FcγRIIIAα chains from different species, we noticed that the stalk region of FcγRIIIAα was 1.5–1.8 times longer than that of FcεRIα, by 7, 5, and 5 aa in humans, mice, and rats, respectively (Fig. 4). When the

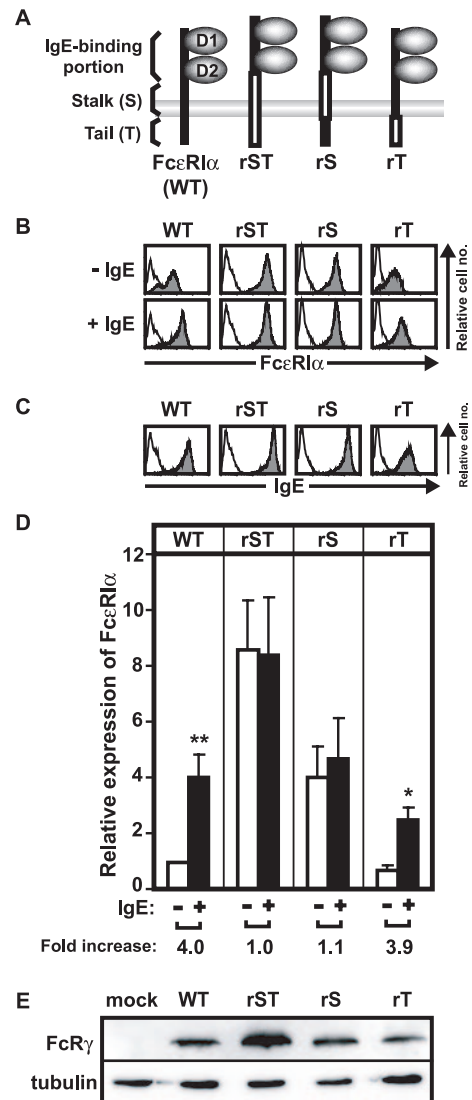


FIGURE 2. Basal and IgE-induced expression of WT and chimeric FcεRIα on the surface of NIH3T3-Fcγ transfectants. *A*, Chimeric human FcεRIα/FcγRIIIAα chains in which all or part of the non-IgE-binding portion of FcεRIα (■) was replaced with the corresponding part of FcγRIIIAα (□) were generated. *B*, Retroviral vectors encoding WT or a chimeric FcεRIα were introduced into NIH3T3-Fcγ cells. Transfected cells were cultured without (*upper*) or with (*lower*) human IgE for 12 h and then stained with anti-FcεRIα mAb as in Fig. 1A. Data are representative of three repeated experiments. *C*, Transfectants cultured with IgE were stained with anti-IgE mAb (*shaded histogram*) or isotype-matched control (*open histogram*). Analyses of GFP⁺ cells are shown; data are representative of three repeated experiments. *D*, Relative expression of WT and chimeric FcεRIα on NIH3T3 transfectants cultured without (□) or with (■) IgE in three repeated experiments was calculated from the mean fluorescence intensity of histograms as shown in *B*. The level of WT FcεRIα on the transfectants cultured without IgE was set as 1. The data are expressed as means ± SEM (*n* = 3, each). Statistical significance is calculated between the absence and presence of IgE (*, *p* < 0.05; **, *p* < 0.01). The relative increase in expression induced by IgE is shown at the bottom. *E*, The NIH3T3 transfectants were cultured with IgE for 12 h, and cell lysates were prepared from each transfectant. The FcεRI/IgE complexes were immunoprecipitated with anti-IgE mAb and resolved by SDS-PAGE, followed by immunoblotting with anti-Fcγ Ab. In parallel, aliquots of cell lysates were blotted with anti-α-tubulin Ab, as a loading control. A mock transfectant was included as a negative control that expressed Fcγ, but not FcεRIα, chains and therefore displayed no IgE-binding FcεRI on the cell surface.

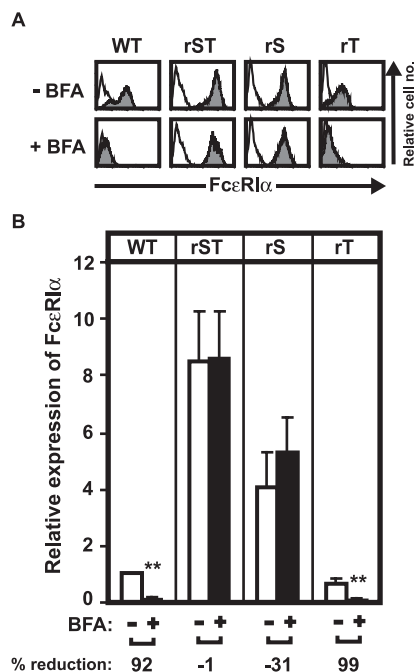


FIGURE 3. Stability of WT and chimeric FcεRIα on the surface of NIH3T3 transfectants. *A*, The NIH3T3 transfectants expressing WT or chimeric FcεRIα were cultured without IgE in the absence (*upper panels*) or presence (*lower panels*) of BFA for 12 h and then stained with anti-FcεRIα mAb (shaded histogram) or isotype-matched control (open histogram). Analyses of GFP⁺ cells are shown; data are representative of three repeated experiments. *B*, Relative expression of WT and chimeric FcεRIα on the NIH3T3 transfectants cultured without (□) or with (■) BFA in three repeated experiments was calculated from the mean fluorescence of the histograms, as in *A*. The level of WT FcεRIα on the transfectants cultured without BFA was set as 1. Data are expressed as means ± SEM (*n* = 3, each). Statistical significance is calculated between the absence and presence of BFA (**, *p* < 0.01). The percent reduction in expression induced by BFA is shown at the bottom.

extra 7 aa in the human FcγRIIIα sequence adjacent to the IgE-binding D2 domain were deleted from rST and rS, both mutant α-chains (rSΔ7T and rSΔ7, respectively in Fig. 5*A*) behaved like WT FcεRIα, in contrast to rST and rS, in terms of the basal expression level, IgE-mediated up-regulation, and BFA-induced down-regulation on the cell surface (Fig. 5, *B* and *C*). Consistent with this observation, deletion of the same 7 aa from human FcγRIIIα (FcγRIIIαΔ7) rendered it unstable on the cell surface

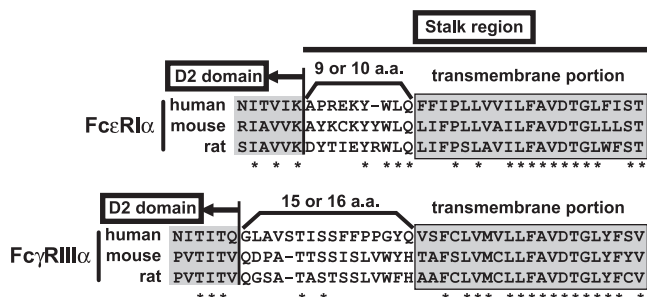


FIGURE 4. The membrane-proximal region of the FcεRIα ectodomain is shorter than the corresponding region of FcγRIIIα in humans, mice, and rats. Amino acid sequences of the C terminus of the IgE-binding D2 domain and the stalk region, including the membrane-proximal and transmembrane portions in human, mouse, and rat FcεRIα and FcγRIIIα, are aligned. *, Amino acid residues conserved among the three species. Gaps (–) were created to make the best match of alignments.

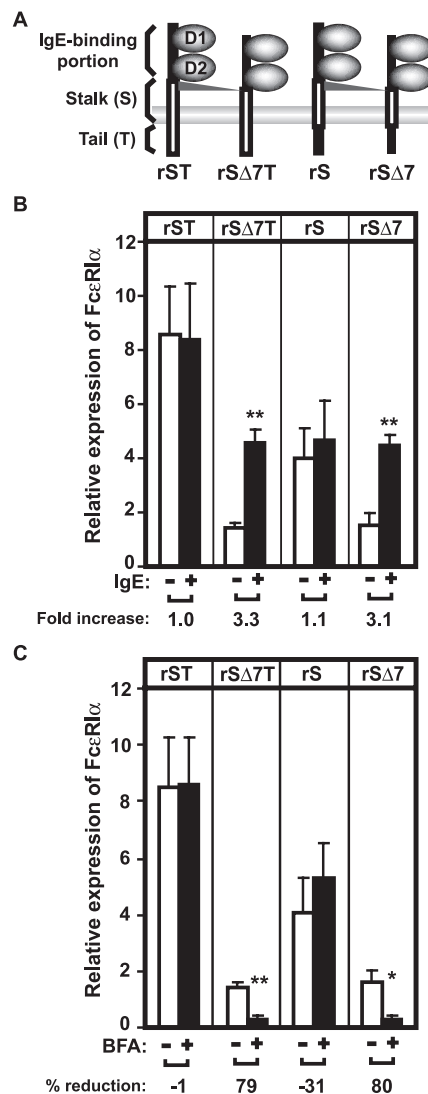


FIGURE 5. Deletion of the 7 N-terminal amino acids from the stalk region of chimeric FcεRIα/FcγRIIIα chains renders them unstable on the cell surface. *A*, Mutant α-chains, rSΔ7T and rSΔ7, were created, in which the 7 aa (GLAVSTI) in the FcγRIIIα sequence adjacent to the IgE-binding D2 domain were deleted from rST and rS, respectively. NIH3T3-Fcγ cells were then infected by retroviral vectors encoding these mutants as well as rST and rS. *B*, Relative expression of each mutant FcεRIα on NIH3T3 transfectants cultured without (□) or with (■) IgE in three repeated experiments is shown as in Fig. 2*D*. The level of WT FcεRIα on the transfectants cultured without IgE was set as 1. *C*, Relative expression of each mutant on the NIH3T3 transfectants cultured without (□) or with (■) BFA in three repeated experiments is shown as in Fig. 3*B*. The level of WT FcεRIα on the transfectants cultured without BFA was set as 1.

when expressed with Fcγ in NIH3T3 (Fig. 6). The basal level of FcγRIIIαΔ7 was ~40% that of WT FcγRIIIα. Culturing with BFA reduced FcγRIIIαΔ7 to <20% of its basal level, although it had no significant effect on the expression of WT FcγRIIIα. Thus, the presence or the lack of these 7 aa had a great impact on the stability of the Fc receptors.

We next asked whether the length of the additional amino acid region or a particular residue(s) among the 7 aa adjacent to the D2 domain was important for determining the stability of the Fc receptors. To address this question, we generated a panel of mutant FcεRIα chains, 197A1, 197A2, 197A3, 197A4, and 197A7, in which 1, 2, 3, 4, or 7 alanine residues, respectively, were inserted at aa 197 of FcεRIα, between the D2 domain and the stalk region,

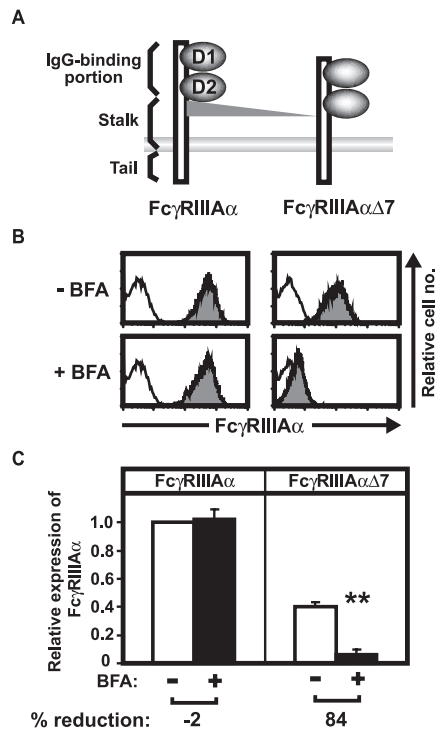


FIGURE 6. Deletion of the 7 N-terminal amino acids from the stalk region of FcγRIIIAα renders FcγRIIIAα unstable on the cell surface. **A**, A mutant FcγRIIIAα (FcγRIIIAαΔ7) was created in which the N-terminal 7 aa (GLAVSTI) in the stalk region were deleted. NIH3T3-Fcγ cells were then infected by retroviral vectors encoding WT FcγRIIIAα or FcγRIIIAαΔ7. **B**, The transfectants were cultured in the absence (upper) or presence (lower) of BFA for 12 h and then stained with anti-FcγRIII mAb as in Fig. 1B. Analyses of GFP⁺ cells are shown; data are representative of three repeated experiments. **C**, Relative expression of FcγRIIIAα and FcγRIIIAαΔ7 on NIH3T3 transfectants cultured without (□) or with (■) BFA in three repeated experiments was calculated from the mean fluorescence of the histograms, as in **B**. The level of WT FcγRIIIAα on the transfectants cultured without BFA was set as 1. Data are expressed as means ± SEM (n = 3, each). Statistical significance is calculated between the absence and presence of BFA (**, p < 0.01). The percent reduction in expression induced by BFA is shown at the bottom.

as shown in Fig. 7A. When expressed in NIH3T3 together with FcεRγ, all the mutant α-chains were detected on the cell surface both by IgE binding (Fig. 7B) and by staining with anti-FcεRIα mAb (Fig. 7C). 197A1 behaved like WT FcεRIα in terms of its basal expression level, IgE-mediated up-regulation, and BFA-induced down-regulation (Fig. 7, C and D). In contrast, the basal expression of 197A2 was 5 times higher than that of WT FcεRIα. 197A3, 197A4, and 197A7 showed 14–16 times higher basal expression levels. In these three mutants, neither the IgE-mediated up-regulation nor BFA-induced down-regulation was observed (Fig. 7, C and D) even though they could bind IgE (Fig. 7B), as in the case of rST and rS. As the basal expression level and BFA-induced down-regulation of each mutant were examined under conditions without IgE binding, the observed differences among the mutants did not seem to be attributed to the difference in their affinity to IgE, if one exists. Thus, the length rather than a particular amino acid sequence of the stalk region appeared to be a major factor in determining the fate of the Fc receptors.

To examine the positional effect of the alanine insertion, we generated two additional mutants of FcεRIα chains, 202A7 and 206A7, in which 7 consecutive alanine residues were inserted into the middle and the C terminus, respectively, of the membrane-

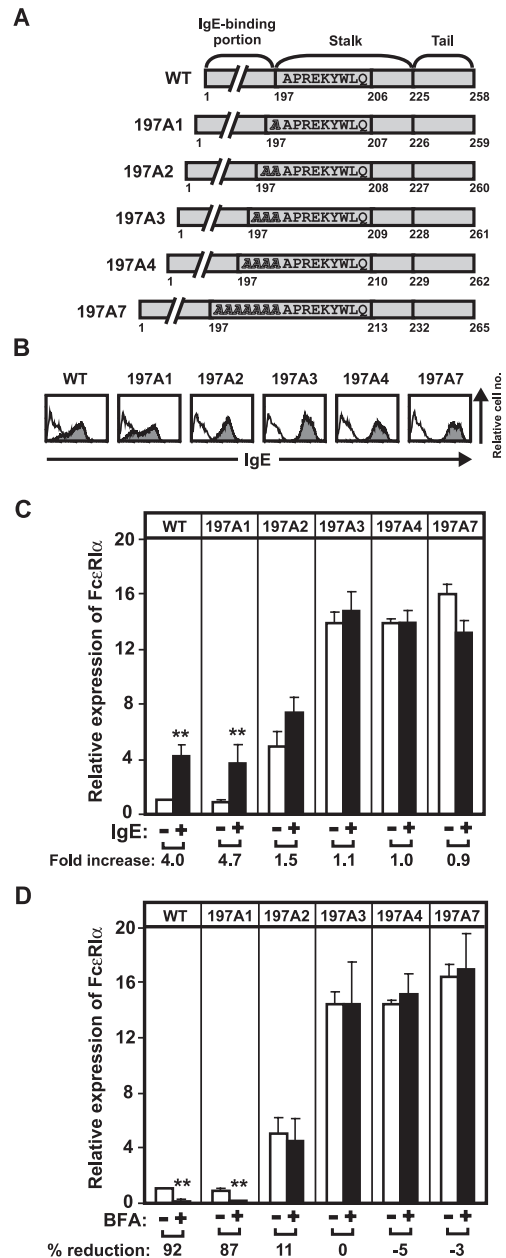


FIGURE 7. Elongation of the N terminus of the stalk region in FcεRIα renders FcεRIα stable on the cell surface. **A**, Mutant FcεRIα chains were created with 1, 2, 3, 4, or 7 alanine residues inserted at aa 197 of FcεRIα, between the IgE-binding D2 domain and the stalk region. **B**, NIH3T3-FcεRγ cells were infected by retroviral vectors encoding WT or a mutant FcεRIα, cultured with IgE for 12 h, and then stained with anti-IgE mAb (shaded histogram) or isotype-matched control (open histogram). Analyses of GFP⁺ cells are shown; data are representative of three repeated experiments. **C**, The NIH3T3 transfectants were cultured with or without IgE for 12 h and then stained with anti-FcεRIα mAb. The relative expression of WT and chimeric FcεRIα on NIH3T3 transfectants cultured without (open bar) or with (filled bar) IgE in three repeated experiments is shown as in Fig. 2D. The level of WT FcεRIα on the transfectants cultured without IgE was set as 1. **D**, The NIH3T3 transfectants shown in **B** were cultured without IgE in the absence or presence of BFA for 12 h and then stained with anti-FcεRIα mAb. The relative expression of WT and mutant FcεRIα on NIH3T3 transfectants cultured without (□) or with (■) BFA in three repeated experiments is shown as in Fig. 3B. The level of WT FcεRIα on the transfectants cultured without BFA was set as 1.

proximal region between the IgE-binding D2 domain and the transmembrane region of FcεRIα (Fig. 8A). The basal expression of 202A7 on the cell surface was 8 times higher than that of WT

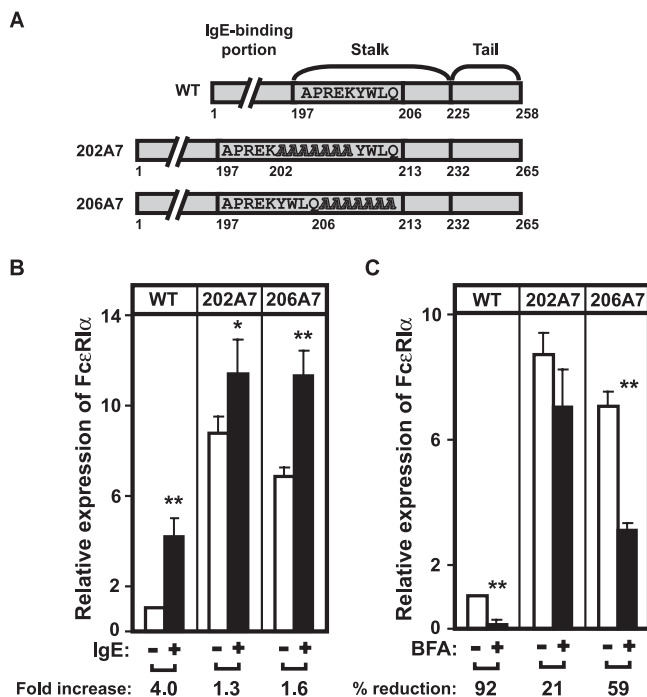


FIGURE 8. The insertion of 7 consecutive alanine residues in different positions between the IgE-binding D2 domain and the transmembrane region of FcεRIα. **A**, Mutant FcεRIα chains, 202A7 and 206A7, were generated, in which 7 consecutive alanine residues were inserted into the middle (at aa 202) and the C terminus (at aa 206), respectively, of the membrane-proximal region between the IgE-binding D2 domain and the transmembrane region of FcεRIα. **B**, NIH3T3-FcRγ cells were infected by retroviral vectors encoding WT, 202A7, or 206A7 and cultured with or without IgE and then stained with anti-FcεRIα mAb. The relative expression of WT and chimeric FcεRIα on NIH3T3 transfectants cultured without (□) or with (■) IgE in three repeated experiments is shown as in Fig. 7C. **C**, The NIH3T3 transfectants shown in **B** were cultured without IgE in the absence or presence of BFA and then stained with anti-FcεRIα mAb. The relative expression of WT and mutant FcεRIα on NIH3T3 transfectants cultured without (□) or with (■) BFA in three repeated experiments is shown as in Fig. 7D.

FcεRIα, and as little as 1.3-fold up-regulation of 202A7 was observed after the culture with IgE, compared with a 4-fold up-regulation of WT (Fig. 8B). No significant reduction in the 202A7 expression was observed when cultured with BFA (Fig. 8C). Thus, 202A7 behaved like rST, rS, and 197A7 rather than WT. This result further supported the conclusion that the length rather than the amino acid sequence of the stalk region is the major determinant of the stability of the Fc receptors. Interestingly, 206A7 behaved somewhat differently from other FcεRIα mutants. Even though the basal expression of 206A7 was 7 times as high as that of WT, a 1.6-fold up-regulation and a 59% reduction in the 206A7 expression were induced by IgE and BFA, respectively, albeit to a lesser extent than in case of WT (Fig. 8, B and C). As the WLQ sequence (aa 203–205) immediately upstream of the transmembrane region of FcεRIα is well conserved among different species (Fig. 4), the connecting portion between the membrane-proximal and transmembrane regions might play some role in regulating FcεRIα expression. Taking these facts together, we concluded that the length of the FcεRIα stalk region was of great importance among others in determining the basal expression level and stability of FcεRI on the cell surface.

Discussion

FcεRI and FcγRIIIA, which carry distinct α-chains but the same γ-chains, behave differently in terms of their stability on the cell surface. FcεRI was unstable unless it bound Ig in contrast to FcγRIIIA. An x-ray crystallography of soluble FcεRIα and FcγRIIIAα has revealed that their overall structures are remarkably similar to each other (24, 25). Furthermore, the overall pattern of the receptor-Fc interactions are also preserved well, although the FcεRIα-IgE interaction shows more extensive hydrophobic interface area as well as more prominent electrostatic interactions (26, 27). Therefore, the difference of the stability on the cell surface between FcεRIα and FcγRIIIA does not seem to be attributed to their Ig-like domains (D1 and D2). For crystallization, the cytoplasmic tail and most of the stalk region of the Fc receptors were deleted to make soluble proteins. Hence, these deleted portions might account for the difference of the stability on the cell surface in the two Fc receptors. In the present study, we demonstrated by swapping these portions between FcεRIα and FcγRIIIAα that the stalk region but not the cytoplasmic tail played a critical role in determining the instability on the cell surface and IgE-mediated up-regulation of FcεRI.

Previous studies by others showed that deletion of 7 or 11 aa from FcεRIα between the IgE-binding D2 domain and the transmembrane region drastically reduced IgE-binding capacity of FcεRIα even though this region did not appear to be directly involved in IgE binding (28, 29). The substitution of this membrane-proximal region of FcεRIα chain with the corresponding region of FcγRIIIAα had apparently no adverse effect on IgE binding (28). An mAb specific to the membrane-proximal region of FcεRIα chain, 5H5F8, did not inhibit IgE binding to FcεRI (30, 31). Furthermore, the amino acid sequence of this region does not appear to be well conserved among humans, mice, and rats (Fig. 4). Therefore, it was suggested that the membrane-proximal region acted as a spacer to ensure correct topology of the FcεRI on the cell membrane such that the binding site is available to interact appropriately with IgE (28).

The present study suggests that the length rather than the amino acid sequence of the FcεRIα stalk region is an important factor in determining the stability of FcεRI on the cell surface. Therefore, it is unlikely that a particular regulatory molecule specifically associates with the stalk region to control the surface expression of the Fc receptor. It was predicted by x-ray crystallography that the D1-D2 cleft is generated near the transmembrane anchor in FcεRI (24). If this cleft can function as a docking site for other membrane-bound protein(s), the length of the stalk region might determine the distance between the cleft and its ligand protein on the cell surface and hence influence their association. We demonstrated in this study that elongation of the stalk region of FcεRIα conferred on FcεRIα a longer half-life on the cell surface membrane. Therefore, one may assume that a putative membrane protein binds to the D1-D2 cleft of FcεRIα and functions as a destabilizer to facilitate the internalization of FcεRI. Elongation of the stalk region might create a distance between the cleft and the destabilizer, resulting in stabilization of FcεRI on the cell surface. IgE binding to FcεRI could have the same effect by changing the conformation of FcεRIα to release such a destabilizer. Shortening the stalk region of FcγRIIIAα might render its D1-D2 cleft able to associate with the same or a similar destabilizer, causing FcγRIIIA to behave like FcεRI. FcRγ is a candidate for such a destabilizer. It is also possible that a putative cleft-binding membrane-bound protein functions as a stabilizer rather than a destabilizer of the Fc receptors. In this scenario, the distance between the cleft and the cell surface is too short for the stabilizer to dock into the cleft

properly. The IgE-induced conformational change of FcεRIα or the artificial elongation of the stalk region could enable the stabilizer to interact with the cleft, leading to the stabilization of FcεRI. x-ray crystallographic analyses showed that IgE undergoes marked structural rearrangements upon receptor ligation, whereas the soluble form of FcεRIα shows little change in conformation upon ligand binding (26, 32). Thus, it remains to be determined whether IgE binding elicits the aforementioned conformational alterations in the full-length membrane-bound form of FcεRIα.

Interestingly, 5H5F8, an mAb specific to the membrane-proximal region of the FcεRIα-chain, has been shown to inhibit the Ag-induced calcium flux and chemical mediator release from IgE-sensitized mast cells and basophils, even though it does not block the interaction between IgE and FcεRI (31). The mechanism of this inhibition remains to be clarified. Taken together with the present study, the membrane-proximal region of the FcεRIα chain appears to function not merely as a spacer to lift the IgE-binding ectodomain above the membrane environment, making it accessible for IgE binding (28), but also as a regulator of FcεRI surface expression and signal transduction.

In conclusion, we defined the stalk region of the human FcεRIα chain as the portion of the molecule regulating the stability of FcεRI on the cell surface. We further identified the length rather than the amino acid sequence of the stalk region as the important determinant, providing new insights into the mechanism regulating surface FcεRI expression.

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Disclosures

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