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Letter to the Editor

Disulfide-linked dimerization of the Fc γ R chain is required for positive and negative regulation of mast cell activation via Fc ϵ RI

Dear Editor,

Mast cells (MCs) express the high-affinity IgE receptor (Fc ϵ RI) as a tetramer consisting of the IgE-binding Fc ϵ RI α chain and two signaling subunits: the Fc γ R β chain and a disulfide-linked homodimer of Fc γ R chains. The Fc γ R chain homodimer, a signal transducer in rodent and human MCs, is a critical component of Fc ϵ RI. Previous studies using chimeric antigen receptors with the Fc γ R chain intracellular domain have suggested that Fc γ R chain homodimerization is required to induce sufficient antigen-dependent cellular responses in MCs.^{1,2} Such a finding suggests that disulfide-mediated homodimerization of Fc γ R chains may play an important role in MC activation via the Fc ϵ RI complex containing Fc ϵ RI α and Fc γ R β chains. Unlike intact Fc ϵ RI, however, such chimeric receptors cannot assemble Fc ϵ RI α and Fc γ R β chains. Thus, the biological significance of the disulfide bond between Fc γ R chains in Fc ϵ RI-mediated cellular responses is poorly understood. In this study, we prepared murine bone marrow-derived MCs (BMMCs) with or without a serine substitution at the cysteine7 residue (C7S) of the Fc γ R transmembrane (TM) region and used retroviral gene transfer to introduce the Fc γ R TM constructs into Fc γ R^{-/-} MCs (Fig. 1A). Wild-type and mutated Fc γ R BMMCs were named $\alpha\beta\gamma^{\text{WT}}$ and $\alpha\beta\gamma^{\text{C7S}}$, respectively. In $\alpha\beta\gamma^{\text{C7S}}$ BMMCs, disulfide-linked dimerization of the Fc γ R chain was completely abolished in the presence or absence of a reducing agent (Fig. 1B). However, cell surface Fc ϵ RI α expression was comparable between $\alpha\beta\gamma^{\text{WT}}$ and $\alpha\beta\gamma^{\text{C7S}}$ BMMCs in the presence or absence of IgE (Fig. 1C,D). These data suggest that the disulfide bond between Fc γ R chains does not contribute to the regulation of cell surface expression of Fc ϵ RI in BMMCs in the presence or absence of IgE.

Although the expression level of Fc γ R chain proteins in $\alpha\beta\gamma^{\text{C7S}}$ BMMCs was markedly reduced (Fig. 2A), the amount of Fc γ R protein co-precipitating with the Fc γ R β protein was comparable in $\alpha\beta\gamma^{\text{WT}}$ and $\alpha\beta\gamma^{\text{C7S}}$ cell lysates solubilized using the mild detergent digitonin. Moreover, Fc ϵ RI engagement-induced receptor internalization was decreased in BMMCs by introduction of the C7S mutation into the Fc γ R TM region (data not shown). Collectively, these results raise the possibility that $\alpha\beta\gamma^{\text{C7S}}$ BMMCs exhibit altered cellular responses following Fc ϵ RI cross-linking. Finally, we examined whether the C7S mutation in the Fc γ R chain TM domain affects Fc ϵ RI-triggered degranulation responses and cytokine production. Because MC activation through Fc ϵ RI leads to bell-shaped cellular response curves depending on the strength of the stimulation,^{3,4} IgE-sensitized $\alpha\beta\gamma^{\text{WT}}$ and $\alpha\beta\gamma^{\text{C7S}}$ BMMCs were stimulated with sub-optimal (2 ng/ml), optimal (20 ng/ml), and supra-

optimal (200 ng/ml) antigen concentrations. As shown in Figure 2C and D, introduction of the C7S mutation into the Fc γ R chain TM domain resulted in various alterations in Fc ϵ RI-dependent degranulation and cytokine production. Upon sub-optimal antigen stimulation, $\alpha\beta\gamma^{\text{C7S}}$ BMMCs showed lower levels of degranulation and cytokine production than $\alpha\beta\gamma^{\text{WT}}$ BMMCs. However, when stimulated with a high dose of antigen, degranulation responses and cytokine production were significantly greater in $\alpha\beta\gamma^{\text{C7S}}$ than in $\alpha\beta\gamma^{\text{WT}}$ BMMCs. These findings suggest that in MCs, Fc γ R dimerization through the Cys7 residue contributes to controlling optimal degranulation and cytokine production, depending on the strength of Fc ϵ RI stimulation. To the best of our knowledge, this report is the first to address the biological actions of the disulfide bond between Fc γ R chains in the regulation of Fc ϵ RI-dependent MC activation.

Rodent Fc ϵ RI requires all three types of subunits to assemble as a tetramer ($\alpha\beta\gamma_2$).⁵ In this study, basal expression of Fc ϵ RI on the cell surface and the amount of Fc γ R protein assembled with the Fc γ R β protein were comparable in $\alpha\beta\gamma^{\text{WT}}$ and $\alpha\beta\gamma^{\text{C7S}}$ BMMCs. These findings suggest that the Fc γ R^{C7S} chain also interacts with the Fc ϵ RI α and Fc γ R β subunits, even though $\alpha\beta\gamma^{\text{C7S}}$ BMMCs lack a disulfide bond between the Fc γ R chains. However, it is unclear whether Fc ϵ RI in $\alpha\beta\gamma^{\text{C7S}}$ BMMCs is actually expressed as a tetramer ($\alpha\beta\gamma_2$) or a trimer ($\alpha\beta\gamma$). Future biochemical analysis will be required to determine the composition of the nascent Fc ϵ RI complex in $\alpha\beta\gamma^{\text{C7S}}$ BMMCs.

In the present study, we could not elucidate why abolishing disulfide bonds in Fc γ R chains results in altered Fc ϵ RI-mediated degranulation and cytokine production in response to the strength of Fc ϵ RI stimulation. In our previous study,⁶ the strength of Fc ϵ RI cross-linking was found to determine whether Fc γ R acts as a dual regulator (positive and negative) for MC activation. Fc γ R β enhances degranulation and cytokine production in the presence of sub-optimal stimulation, whereas Fc γ R β reduces degranulation and cytokine production in the presence of supra-optimal stimulus. At present, we believe that in $\alpha\beta\gamma^{\text{C7S}}$ BMMCs, engagement of Fc ϵ RI with IgE and an antigen is insufficient to elicit the dual functions of Fc γ R for the following reasons: (i) the stability of the Fc ϵ RI complex is essential for optimal aggregation of Fc ϵ RI, which induces tyrosine phosphorylation of Fc γ R β relative to positive and negative regulation of MC activation^{4,7}; and (ii) the stability of the Fc ϵ RI complex was actually decreased in $\alpha\beta\gamma^{\text{C7S}}$ BMMCs compared to that in $\alpha\beta\gamma^{\text{WT}}$ BMMCs (Fig. 2B). As demonstrated in Figure 2A, however, Fc γ R chain protein expression was partially reduced in $\alpha\beta\gamma^{\text{C7S}}$ BMMCs. Thus, we cannot exclude the possibility that reduction of intracellular Fc γ R proteins uncoupled with Fc ϵ RI may result in dysregulated cellular responses in $\alpha\beta\gamma^{\text{C7S}}$ BMMCs.

Many missense mutations in the FCER1G gene have been reported in the public human gene database GeneCards, although

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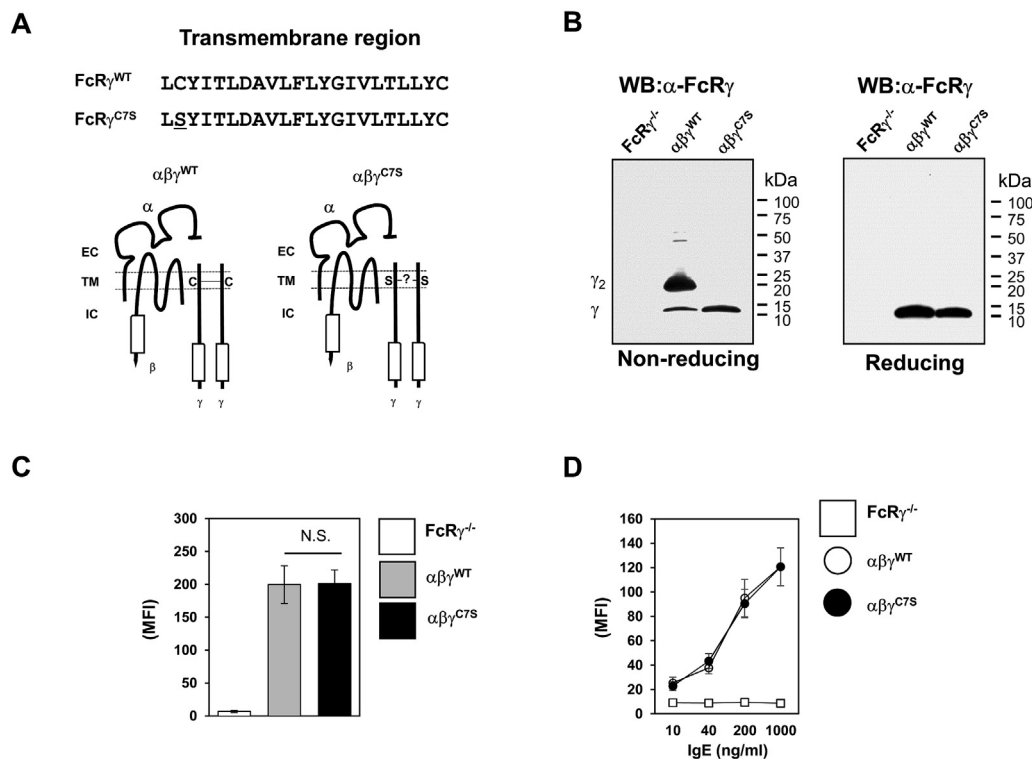


Fig. 1. A single mutation in the FcR γ TM domain does not alter Fc ϵ RI expression in MCs. (A) Wild-type FcR γ (FcR γ^{WT}) and mutant FcR γ (FcR γ^{C7S}) with a cysteine residue in the TM domain replaced with a serine residue. (B) The introduction of FcR γ genes into FcR γ -null MCs resulted in defective disulfide bonds. SDS-PAGE was performed in the presence or absence of a reducing reagent and analyzed by immunoblotting with an anti-FcR γ polyclonal antibody as described in Supplementary Methods. The results shown are representative of three independent experiments with similar results. (C) Comparable expression of Fc ϵ RI α in $\alpha\beta\gamma^{WT}$ and $\alpha\beta\gamma^{C7S}$ BMMCs. (D) IgE-mediated up-regulation of Fc ϵ RI on the cell surface. In (C) and (D), the data shown represent the means $\pm$ SD of samples ($n = 4$) obtained from four independent experiments.

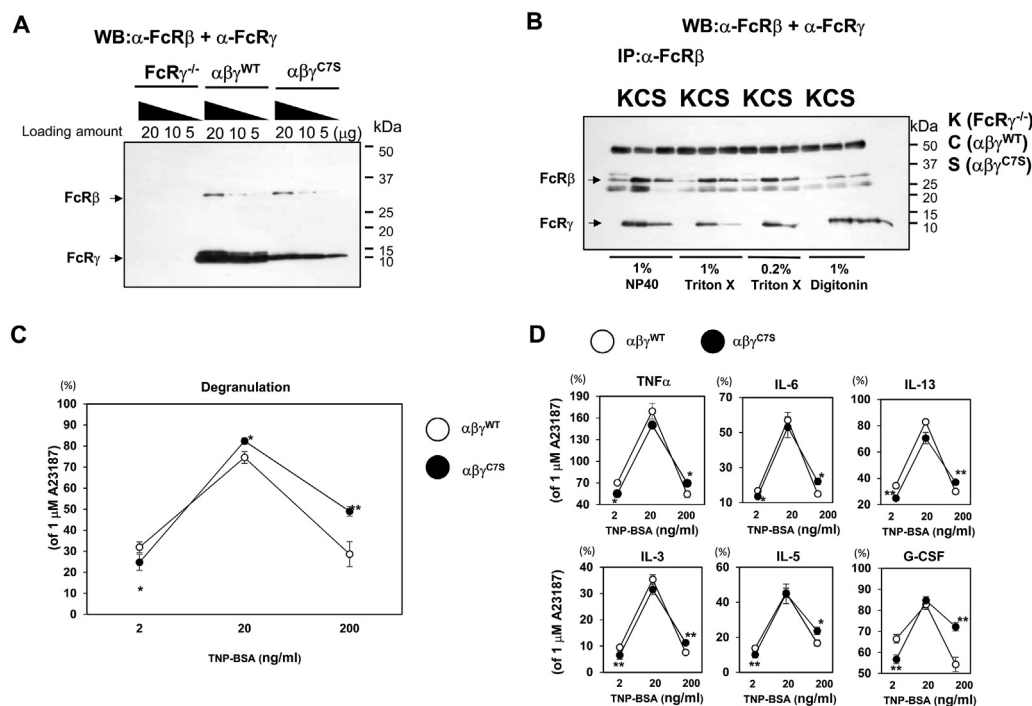


Fig. 2. $\alpha\beta\gamma^{C7S}$ BMMCs show altered degranulation responses and cytokine production in response to antigen concentrations. (A) Western blot analysis of FcR β and FcR γ expression. Black triangles indicate that a reduced amount of cell lysate was loaded. (B) Comparable association between FcR β and FcR γ in $\alpha\beta\gamma^{WT}$ and $\alpha\beta\gamma^{C7S}$ cell lysates solubilized using the mild detergent digitonin. In (A) and (B), the results are representative of three independent experiments with similar results. IgE-sensitized $\alpha\beta\gamma^{WT}$ and $\alpha\beta\gamma^{C7S}$ BMMCs were stimulated with antigen (TNP-BSA) at the indicated concentrations for 0.5 h to assess degranulation (C) or 8 h to assess cytokine production (D). The data shown represent the mean $\pm$ SE of quadruplicate experiments and are expressed as the relative percentage of degranulation and cytokine production in the presence of the calcium ionophore A23187 (1 μ M). * $p < 0.05$; ** $p < 0.01$ (Student's t -test).

individuals carrying a missense mutation at Cys7 of FCER1G have not yet been reported. We consider that Cys7 may be well conserved in humans to ensure FcR γ disulfide bond formation and Fc ϵ RI signaling in response to antigens.

In conclusion, in the present study, we reveal novel actions of the FcR γ chain during Fc ϵ RI-dependent MC activation, and our findings strengthen our understanding of antigen-specific MC function in allergic inflammation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alit.2017.04.010>.

Conflict of interest

The authors have no conflict of interest to declare.

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Supplementary Methods

Reagents

Mouse anti-TNP IgE (clone 15.3.2) and FITC-conjugated rat anti-mouse IgE monoclonal antibody (mAb) (clone R35-72) were purchased from BD Biosciences (San Diego, CA, USA). Rabbit anti-FcR γ polyclonal antibody and TNP-BSA were obtained from Millipore Corporate (Billerica, MA, USA) and BIOSEARCH Technologies (Petaluma, CA, USA), respectively. Recombinant murine IL-3 was obtained from PeproTech (Rocky Hill, NJ, USA), and the PerCP-conjugated hamster anti-mouse Fc ϵ R1 α mAb (clone MAR-1) was purchased from Affymetrix eBioscience Japan (Tokyo, Japan). The anti-FcR β mAb (clone JRK) was prepared in our laboratory.

Animals

FcR γ ^{-/-} mice were bred in the animal facility of the Nihon University School of Medicine under specific pathogen-free conditions. Following approval by the animal experimentation committee at Nihon University, all of the experiments were performed according to the guidelines for the care and use of laboratory animals of Nihon University.

Cell Culture

The packaging cell line PLAT-E, which was a kind gift from Dr. Toshio Kitamura (Tokyo University, Japan), was cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1 μ g/ml puromycin (BD Clontech, San Jose, CA, USA) and 10 μ g/ml blasticidin S (Kaken Pharmaceutical Co, Tokyo, Japan).

Retroviral transfection

Retroviral gene transduction was performed as previously described (1). Briefly, pMX-puro plasmids harboring FcR γ ^{WT} and FcR γ ^{C7S} cDNA were transfected into the PLAT-E cells to obtain recombinant retroviruses. Bone marrow cells were infected with the retroviruses for 24 h in the presence of 10 μ g/ml polybrene (Sigma). Gene-transduced cells were selected with 1.2 μ g/ml puromycin for 10 days. The viable FcR γ transfectants (10-20 % of the bone marrow cells cultured with retroviruses) were further grown in a medium containing IL-3 (5 ng/ml) for several weeks.

Analysis of cell surface Fc ϵ R γ expression

FcR γ transfectants and FcR γ null MCs (1×10^5) were labeled with a

PerCP-conjugated anti-mouse Fc ϵ RI α mAb at 4°C for 30 min as previously described (2). After washing with PBS, the labeled cells were analyzed using a FACSCalibur instrument (BD Biosciences). For analysis of IgE-mediated up-regulation of Fc ϵ RI, the FcR γ transfectants and FcR γ null MCs (1×10^5) were cultured with mouse IgE (15.3.2) for 24 h at the indicated concentrations. These cells were washed twice with PBS and labeled with 0.1 μ g/ml FITC-conjugated anti-mouse IgE mAb for 15 min on ice. The labeled cells were analyzed with a FACSCalibur instrument.

Analysis of Fc ϵ RI internalization

Receptor internalization was defined as changes in cell surface Fc ϵ RI expression after Fc ϵ RI cross-linking. Briefly, FcR γ transfectants (1×10^6) were sensitized with 0.5 μ g/ml mouse IgE (15.3.2) overnight. The cells were washed twice with PBS and stimulated with or without 20 ng/ml TNP-BSA for 20 min. The stimulated cells were labeled with 0.1 μ g/ml anti-mouse IgE mAb-FITC for 15 min on ice. The labeled cells were analyzed with FACSCalibur.

Degranulation and cytokine production assays

FcR γ transfectants (1×10^6) were sensitized with 0.5 μ g/ml anti-TNP IgE overnight. The IgE-sensitized MCs (2×10^5) were washed twice with PBS and then stimulated with or without antigen (TNP-BSA) at the indicated concentrations for 0.5 h to determine degranulation or 8 h to determine cytokine production. Degranulation was detected according to β -hexosaminidase net release as described previously (3). Net production of cytokines was analyzed using a Bio-plex Multiplex cytokine array (Bio-rad Laboratories, Tokyo, Japan). To normalize the data obtained from $\alpha\beta\gamma^{\text{WT}}$ and $\alpha\beta\gamma^{\text{C7S}}$ MCs in this study, A23187 (1 μ M) was used as a non-immunological positive control. The data are expressed relative to the percentage of A23187-induced net release of β -hexosaminidase and cytokines.

Immunoblotting

FcR γ transfectants (1×10^6) were lysed for 30 min on ice in a lysis buffer (Tris-buffered saline containing the indicated detergents, 2 mM PMSF, 10 μ g/ml aprotinin, 2 μ g/ml leupeptin and pepstatin A) as previously described (1). The lysates were centrifuged for 15 min at 15,000 g. For immunoprecipitation assays, the cell lysates were incubated with antibody-bound protein G-Sepharose for 3 h

on ice. The immunoprecipitates were resuspended in an equal volume of 2× Laemmli buffer. The samples were then boiled, separated on a 12 % SDS-PAGE gel under reducing or non-reducing conditions, and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated with a primary antibody and an appropriate secondary horseradish peroxidase (HRP)-conjugated antibody. The signals were detected by enhanced chemiluminescence (GE Healthcare Biosciences).

Statistical analysis

The data shown represent the mean $\pm$ SE or SD. The statistical analysis was performed using the Student's *t*-test. P values less than 0.05 were considered to indicate statistically significant differences.

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