



## Short communication

# Fc receptor- $\gamma$ subunits with both polar or non-polar amino acids at position of T22 are capable of restoring surface expression of the high-affinity IgE receptor and degranulation in $\gamma$ subunit-deficient rat basophilic leukemia cells

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## ABSTRACT

The high-affinity IgE receptor (Fc $\epsilon$ RI) is formed by the IgE-binding  $\alpha$  subunit,  $\beta$  subunit and  $\gamma$  subunits homodimer. All three subunits are required for proper expression of the receptor on the plasma membrane of mast cells and basophils. However, the exact molecular mechanism of inter-subunit interactions required for correct expression and function of the Fc $\epsilon$ RI complex remains to be identified. A recent study suggested that polar aspartate at position 194 within the transmembrane domain of the  $\alpha$  subunit could interact by hydrogen bonding with polar threonine at position 22 in the transmembrane domains of the  $\gamma$  subunits. To verify this, we used previously isolated rat basophilic leukemia (RBL)-2H3 variant cells deficient in the expression of the Fc $\epsilon$ RI- $\gamma$  subunit (FcR- $\gamma$ ), and transfected them with DNA vectors coding for FcR- $\gamma$  of the wild-type or mutants in which T22 was substituted for nonpolar alanine (T22A mutant) or polar serine (T22S mutant). Analysis of the transfectants showed that both T22A and T22S mutants were capable to restore surface expression of the Fc $\epsilon$ RI similar to wild-type FcR- $\gamma$ . Furthermore, cells transfected with wild-type, T22A or T22S FcR- $\gamma$  showed comparably enhanced Fc $\epsilon$ RI-mediated degranulation. Our data indicate that substitution of FcR- $\gamma$  T22 with non-polar amino acid does not interfere with surface expression of the Fc $\epsilon$ RI and its signaling capacity.

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## 1. Introduction

Mast cells and basophils express on their plasma membrane the high-affinity IgE receptor (Fc $\epsilon$ RI) composed of one IgE-binding  $\alpha$  subunit, one  $\beta$  subunit involved in amplification of the activation signal and a disulfide-linked  $\gamma$  subunit homodimer responsible for propagation of the activation signal. Antigen-mediated aggregation of the receptor with bound IgE results in rapid tyrosine phosphorylation of the Fc $\epsilon$ RI- $\beta$  and - $\gamma$  subunits followed by phosphorylation of other substrates by Src and Syk/Zap family protein tyrosine kinases, enhanced activity of a numerous enzymes, mobilization of cytoplasmic calcium, degranulation and production of inflammatory cytokines (Kinet, 1999; Metzger, 1991). Detailed sequence of

molecular events leading to tyrosine phosphorylation of the receptor subunits is unknown, but several models have been proposed (Bugajev et al., 2010). In all of these models precise physical and functional association of the Fc $\epsilon$ RI subunits is an essential prerequisite for Fc $\epsilon$ RI signaling. In rodents, surface expression of the receptor is dependent on the presence of all three subunits. Transmembrane region of the  $\alpha$  subunit containing an invariant motif of eight amino acids, LFAVDTGL, with a polar aspartate (at position 194; underlined) surrounded by a predominantly non-polar amino acids plays an important function in assembly of the Fc $\epsilon$ RI subunits (Farber and Sears, 1991). Our recent study, based on site-directed mutagenesis revealed a key role of D194 in formation of a functional rat/human chimeric receptor complex in transfected rat basophilic leukemia (RBL)-2H3 cells (Rashid et al., 2010). When aspartate was substituted with polar asparagine (D194N) or polar threonine (N194T) the chimeric Fc $\epsilon$ RI was functional and capable to induce calcium mobilization and degranulation. However substitution of aspartate with non-polar isoleucine (D194I) or some other amino acid residues differing in size and/or charge, resulted in failure of surface expression of the chimeric receptor. These and other data suggested the importance of the size and charge of amino acid residue at position 194 of the Fc $\epsilon$ RI- $\alpha$

**Abbreviations:** DNP, dinitrophenol; Fc $\epsilon$ RI, high-affinity IgE receptor; FcR- $\gamma$ , Fc $\epsilon$ RI- $\gamma$  subunit; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HSA, human serum albumin; PBS, phosphate buffered saline; RBL, rat basophilic leukemia; TAE, tris-acetate-EDTA; TCR, T cell receptor.

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subunit for proper assembly of the receptor complex and led to the proposal of a new structural model for interaction between transmembrane domains of Fc $\epsilon$ RI- $\alpha$  and - $\gamma$  subunits. This model envisages interaction of the helical transmembrane segment of the  $\alpha$  subunit with two helical segments of disulfide linked  $\gamma$  subunits. The proposed interaction is stabilized by hydrogen-bonding between D194 of the transmembrane region of the Fc $\epsilon$ RI- $\alpha$  subunit and threonine residues at position 22 (T22) in the transmembrane region of disulfide linked Fc $\epsilon$ RI- $\gamma$  subunits (FcR- $\gamma$ ) (Rashid et al., 2010). The aim of the present study was to determine whether replacement of polar threonine at position 22 in the FcR- $\gamma$  subunit with nonpolar alanine will interfere with receptor assembly and function as was predicted by 3-helix assembly model (Call et al., 2002; Call and Wucherpfennig, 2005, 2007). To this end, we decided to transfect wild-type or mutant FcR- $\gamma$  subunits into FcR- $\gamma$ -deficient RBL-2H3 cells (Dráberová and Dráber, 1995) and analyze surface expression of the Fc $\epsilon$ RI and Fc $\epsilon$ RI-induced degranulation.

## 2. Materials and methods

### 2.1. Reagents

Dinitrophenol (DNP)-specific mouse IgE (clone SPE-7) was obtained in-house by culturing hybridoma cells. Goat anti-mouse IgE was purchased as fluorescein isothiocyanate (FITC) conjugate from Bethyl Laboratories (Montgomery, USA). All other reagents were from Sigma–Aldrich (USA), unless stated otherwise.

### 2.2. Expression vectors

cDNA for rat FcR- $\gamma$  in the pUC18 cloning vector was obtained from U. Blank and J.-P. Kinet and was used for mutagenesis and cloning into an expression vector. To generate T22A and T22S mutations in the FcR- $\gamma$ , Quickchange Site-Directed Mutagenesis Kit was used according to manufacturer's instructions (Stratagene, USA). The mutations were verified by sequencing using Cogenics kit (Takeley, UK). Wild-type and mutant cDNA was subcloned into expression vector pEE6 (Cell-tech, Slough, UK) as described (Rashid et al., 2010). Briefly, the vectors pEE6 and pUC18 with wild-type or mutant FcR- $\gamma$  were digested with *Hind*III and *Eco*RI and the DNA fragments were resolved on 1% agarose gel with Tris-acetate–EDTA (TEA) buffer, pH 8.0. The desired bands of wild-type and mutant FcR- $\gamma$  subunits (260 bps) and pEE6 vector (6200 bps) were excised from the gel, purified with the Quantum Prep™ Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, USA) and ligated with T4 DNA ligase. The ligated DNA was transformed into XL1-Blue Supercompetent Cells (Stratagene, USA). Plasmid DNA was isolated by means of Qiagen Spin Miniprep Kit (Qiagen, Germany). Test digestion of miniprep plasmid DNA was carried out using the *Eco*RI and *Hind* III restriction enzymes followed by resolving on agarose gel with TAE buffer to confirm the presence of the desired fragments. Qiagen Plasmid Midi Kit was employed for large scale plasmid purification.

### 2.3. Cells and DNA transfection

RBL-2H3 cells were maintained in culture medium [minimum essential medium, Life Technologies, USA, supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin] as described (Bingham et al., 1994). RBL-2H3-derived cells deficient in the expression of FcR- $\gamma$  were isolated from chemically mutagenized cells by 5 successive cycles of negative immunomagnetic separation followed by cloning of Fc $\epsilon$ RI-deficient cells by limiting dilution. These cells exhibited decreased expression

of surface Fc $\epsilon$ RI, which could be restored by transfection of FcR- $\gamma$  (Bocek et al., 1995; Dráberová and Dráber, 1995). Transfections were carried out using electroporation apparatus (Bio-Rad, USA). The cells ( $10^7$  in 0.8 ml) were mixed with 20  $\mu$ g of plasmid DNA and electroporated (250 V, 960  $\mu$ F) according to the manufacturer's instructions. An empty vector (sham transfection) was used as a negative control. The cells were incubated for 24 h in culture media, followed by 5 days in culture media supplemented with 800  $\mu$ g/ml G-418 sulfate (Invitrogen, USA) and subsequent maintenance in culture media supplemented with 400  $\mu$ g/ml G-418 sulfate.

### 2.4. Flow cytometry

Cells were washed in phosphate buffered saline (PBS), pH 7.4, harvested with a non-enzymatic cell dissociation solution (Sigma–Aldrich) and resuspended in PBS supplemented with 1% FCS. Surface Fc $\epsilon$ RI was detected by two-step immunofluorescence procedure with IgE (1  $\mu$ g/ml) in the first step and FITC-labeled goat anti-mouse IgE in the second step. As a negative control, the cells were exposed to PBS-FCS only in the first step. Samples were analyzed using FACSCalibur (BD Biosciences, USA).

### 2.5. $\beta$ -Hexosaminidase release assays

Cells were harvested, resuspended at a concentration of  $0.5 \times 10^6$ /ml in culture medium supplemented with DNP-specific IgE and plated in 96-well flat bottomed tissue culture plates. Next day the cells were washed with a buffer containing 120 mM NaCl, 5 mM KCl, 0.04 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 25 mM PIPES, pH 7.4, and activated with increasing concentrations of DNP-human serum albumin (HSA) conjugate for 20 min. To determine amount of  $\beta$ -hexosaminidase released, 50  $\mu$ l of the cell supernatant was mixed with 50  $\mu$ l of  $\beta$ -hexosaminidase substrate (2 mM *p*-nitrophenyl *N*-acetyl  $\beta$ -D-glucosaminide) in citrate buffer, pH 4.5. After incubation for 2 h at 37 °C the reaction was stopped by adding 150  $\mu$ l 1 M Tris–HCl, pH 9.0 and absorption was analyzed in ELISA plate reader at 405 nm wavelength. Total amount of cellular  $\beta$ -hexosaminidase was determined after solubilization of the cells with a buffer containing 1% Triton X-100.

## 3. Results and discussion

### 3.1. Restoration of Fc $\epsilon$ RI surface expression in FcR- $\gamma$ -deficient cells transfected with mutant FcR- $\gamma$

In an attempt to determine the role of polar T22 of the FcR- $\gamma$  in the surface expression of Fc $\epsilon$ RI complex capable to bind IgE, we introduced into  $\gamma$ -deficient RBL cells constructs containing cDNA for FcR- $\gamma$  subunit of the wild-type, FcR- $\gamma$ -T22A, FcR- $\gamma$ -T22S or empty vector. Transfected cells were selected and analyzed together with parental RBL-2H3 cells and RBL- $\gamma$ -deficient cell for surface expression of Fc $\epsilon$ RI. Data presented in Fig. 1A–D indicate that  $\gamma$ -deficient RBL cells, untransfected (Fig. 1C) or transfected with empty vector (Fig. 1D), exhibited lower expression of Fc $\epsilon$ RI than parental RBL cells (Fig. 1B). When compared to RBL cells with second antibody alone (Fig. 1A), some binding of IgE to  $\gamma$ -deficient cells was detected; this was apparent especially at later passages, which is in accord with previous studies (Bocek et al., 1995; Swann et al., 1999), suggesting that these cells possess a defect in regulatory elements rather than in the structural gene for FcR- $\gamma$ . After transfection of  $\gamma$ -deficient cells with a vector containing cDNA for wild-type FcR- $\gamma$ , expression of Fc $\epsilon$ RI was restored (Fig. 1E). Similar restoration of Fc $\epsilon$ RI expression was also observed after transfection of the vector coding for FcR- $\gamma$  mutant T22S (Fig. 1G). Thus,

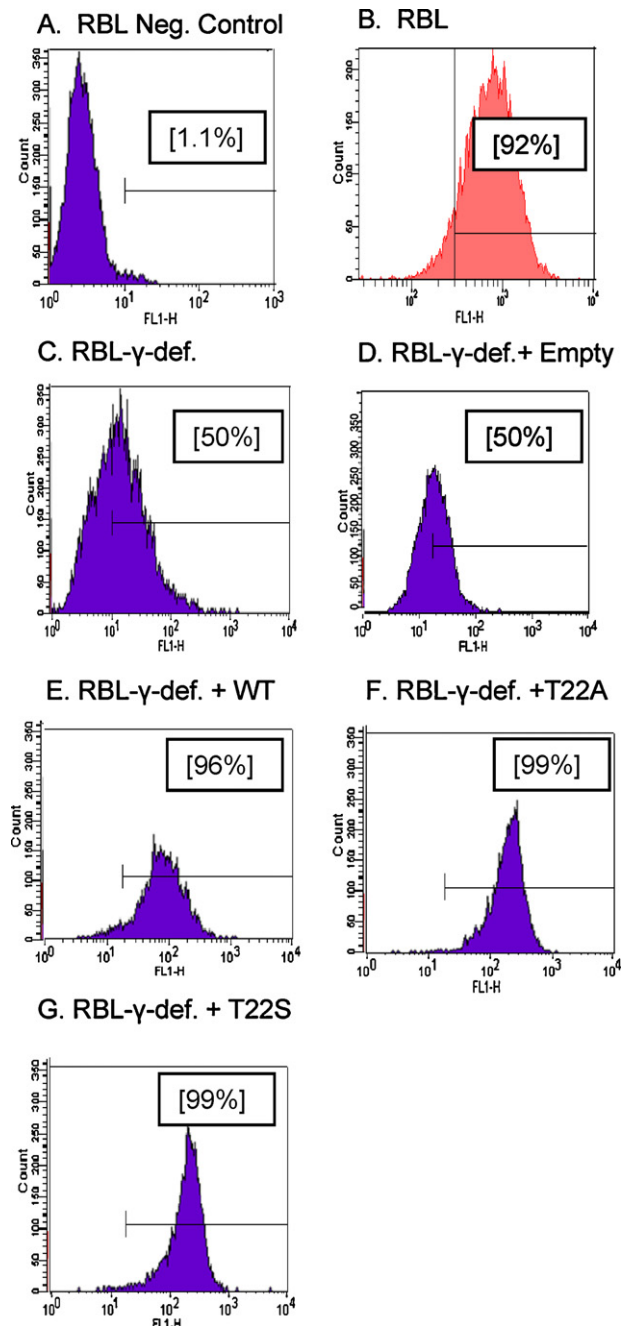


**Fig. 1.** Restoration of the Fc $\epsilon$ RI surface expression in FcR- $\gamma$ -deficient cells transfected with mutated FcR- $\gamma$  subunit T22A or T22S. RBL cells (A, B), RBL- $\gamma$ -deficient cells (C) or RBL- $\gamma$ -deficient cells transfected with empty vector (D) or a vector coding for FcR- $\gamma$  subunit of the wild-type (E) or with mutation T22A (F) or T22S (G) were analyzed for surface expression of Fc $\epsilon$ RI using 2-step immunofluorescence procedure combined with flow cytometry as described in Section 2.4. In negative control (A), RBL cells were incubated during the first step in PBS-BSA alone (without IgE). Numbers in square parentheses represent percentage of positive cells. The results are typical examples of at least 3 experiments performed.

substitution of polar threonine by another polar amino acid, serine, also supported expression of Fc $\epsilon$ RI on the plasma membrane. Interestingly, restoration of Fc $\epsilon$ RI expression was also observed in  $\gamma$ -deficient cells transfected with a vector coding for FcR- $\gamma$  mutant T22A (Fig. 1F). This unexpected finding indicates that polar amino acid at position 22 of the FcR- $\gamma$  is not required for proper assembly and surface expression of the Fc $\epsilon$ RI. Thus, 3-helix model based on transmembrane interaction of A194 in Fc $\epsilon$ RI- $\alpha$  subunit with T22 in

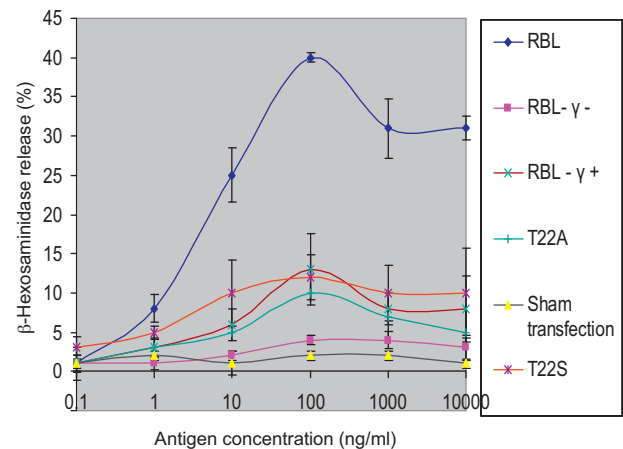


**Fig. 2.** Comparable restoration of Fc $\epsilon$ RI-induced degranulation in FcR- $\gamma$ -deficient cells transfected with FcR- $\gamma$  of the wild-type or with mutation T22A or T22S. RBL cells, RBL- $\gamma$ -deficient cells or RBL- $\gamma$ -deficient cells transfected with empty vector (Sham transfection), a vector coding for FcR- $\gamma$  of the wild-type ( $\gamma$ +) or with mutation T22A or T22S were sensitized with DNP-specific IgE. After 16 h the cells were washed and exposed to antigen at various concentrations (0.1 ng/ml–10  $\mu$ g/ml) for 20 min. Amount of  $\beta$ -hexosaminidase released from the cells was determined as described in Section 2.5. Data are presented as means  $\pm$  S.D. from three separate experiments performed in triplicates.

FcR- $\gamma$  does not fully explain how components of the Fc $\epsilon$ RI interact in the plasma membrane.

### 3.2. Restoration of Fc $\epsilon$ RI-induced degranulation in FcR- $\gamma$ -deficient cells transfected with mutant FcR- $\gamma$

Next we analyzed whether FcR- $\gamma$  mutants, T22A or T22S, could restore degranulation in  $\gamma$ -deficient RBL cells. The extent of mast cell degranulation was quantified by measuring the level of  $\beta$ -hexosaminidase released from IgE-sensitized cells by challenge with cognate antigen. Data presented in Fig. 2 indicate that exposure of IgE-sensitized RBL cells to different concentrations of antigen resulted in release of  $\beta$ -hexosaminidase peaking at 100 ng/ml of the antigen. As expected, and in agreement with previous results,  $\gamma$ -deficient cells showed no degranulation at all concentrations of antigen tested. Similarly, no response to antigen was observed in cells transfected with empty vector (Sham transfection). After transfection of  $\gamma$ -deficient cells with wild-type FcR- $\gamma$ -subunit degranulation was partially restored peaking at the same concentration of antigen as in wild-type cells (100 ng/ml). Similar increase of degranulation was observed in  $\gamma$ -deficient cell transfected with a vector coding for FcR- $\gamma$ -T22A or FcR- $\gamma$ -T22S. Thus, substitution of threonine at position T22 with polar serine or nonpolar alanine had no effect on signaling capacity of Fc $\epsilon$ RI-activated cells.

The molecular basis of impaired degranulation of  $\gamma$ -deficient cells transfected with wild-type FcR- $\gamma$  subunit, as compared to the parental RBL cells, is not clear and several possibilities exist. One scenario is that the transfected FcR- $\gamma$  subunit does not properly integrate into the Fc $\epsilon$ RI complex. However, this possibility is unlikely because the transfected FcR- $\gamma$  subunit was capable to restore surface expression of Fc $\epsilon$ RI as shown in Fig. 1E and in previous studies (Bocek et al., 1995; Dráberová and Dráber, 1995; Swann et al., 1999). Alternatively, chemical mutagenesis used in the course of production of  $\gamma$ -deficient cells (Dráberová and Dráber, 1995) could produce changes which interfere not only with FcR- $\gamma$  subunit expression but also with Fc $\epsilon$ RI-induced signaling events leading to degranulation. This possibility is supported by our data indicating that not only degranulation, but also Ca<sup>2+</sup> response was impaired in  $\gamma$ -deficient cells transfected with vectors coding wild-type and

mutant FcR- $\gamma$  subunit (not shown). Because signaling capacity of  $\gamma$ -deficient cells is not completely restored after re-expression of the Fc $\epsilon$ RI, the cells are suboptimal for studies on Fc $\epsilon$ RI-mediated signaling although suitable for studies on the role of FcR- $\gamma$  subunit in formation of functional IgE binding Fc $\epsilon$ RI complex on the plasma membrane.

Full restoration of IgE binding capacity in  $\gamma$ -deficient cells transfected with mutant T22A FcR- $\gamma$  subunit indicates that polar amino acid threonine can be substituted with nonpolar amino acid alanine without any effect on surface expression of the Fc $\epsilon$ RI. This is an important finding suggesting that other motifs are involved in the interactions between the Fc $\epsilon$ RI subunits. The model explaining interaction between D194 of Fc $\epsilon$ RI- $\alpha$  subunit and T22 of FcR- $\gamma$  subunits (Rashid et al., 2010) is based on 3-helix assembly principle, which seems to be valid for assembly of several activating immune receptors (Call et al., 2002; Call and Wucherpfennig, 2007). The model proposes that formation of stable receptor complex in lipid bilayer is produced by interaction of one charged polar transmembrane residue in the ligand-binding subunit and two charged transmembrane residues in the signaling dimer. Involvement of another receptor subunit, such as Fc $\epsilon$ RI- $\beta$  subunit, is not considered in the model. However, previous studies indicated that Fc $\epsilon$ RI- $\beta$  subunit is an important player in Fc $\epsilon$ RI expression and function in mast cells and basophils (Blank et al., 1989) and that the first uncharged transmembrane domain of the  $\beta$ -chain contains the interface for receptor stabilization (Singleton et al., 2009). Furthermore, other studies indicated that L21 and some other amino acids in the FcR- $\gamma$  subunit are critical for stabilization and surface expression of Fc $\epsilon$ RI- $\alpha\beta\gamma$ 2 complex (Hida et al., 2009; Wines et al., 2006).

It should be noted that the T cell receptor (TCR) and some other immunoreceptors which are assembled according to 3-helix assembly model are characterized by single membrane spanning proteins of the Type I and Type II family, which are stabilized in a series of sequential post-translational assembly events (Call et al., 2002; Call and Wucherpfennig, 2007). In contrast, several pieces of evidence support a concept that formation of Fc $\epsilon$ RI tetramer proceeds via a co-translational assembly events in endoplasmic reticulum (Fiebiger et al., 2005). This difference in assembly requirements suggests that formation and stabilization of the Fc $\epsilon$ RI complex is different from formation and stabilization of some other immunoreceptor complexes.

In conclusion, data presented in this study indicate that FcR- $\gamma$  in which polar threonine at position 22 is substituted for polar serine or nonpolar alanine is capable to facilitate surface expression of the Fc $\epsilon$ RI and its signaling capacity. The data support the concept that principles of Fc $\epsilon$ RI tetramer ( $\alpha\beta\gamma$ 2) assembly is different from TCR and some other immunoreceptors in which the assembly is based on 3-helix assembly model. Comprehension of molecular mechanisms responsible for functional assembly of the Fc $\epsilon$ RI complex components is important for rational approaches toward inhibition of the signaling cascade set in motion by the activation of the receptor complex, thus effecting modulation of Fc $\epsilon$ RI-dependent events in allergy and other diseases.

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