

Polymorphisms in FcεRI β Chain Do Not Affect IgE-Mediated Mast Cell Activation

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Genetic polymorphisms that result in three amino acid changes in FcεRI β chain (Ile¹⁸¹→Leu, Val¹⁸³→Leu, and Glu²³⁷→Gly) have been identified as candidates that associate with allergic disorders such as atopy and asthma. To elucidate the biological significance of these polymorphisms in regulating the expression and function of FcεRI, we generated four types of transfectants that express wild-type or mutant mouse β chains corresponding to these human variants by retrovirus-mediated gene transfer into β chain-deficient mouse-derived mast cells. No significant functional differences between the wild-type β chain transfectant and any of the mutant β chain transfectants were observed in β-hexosaminidase release, intracellular calcium mobilization, or cytokine and leukotriene C₄ production in response to FcεRI crosslinking. Our results suggest that these polymorphisms in FcεRI β chain do not affect FcεRI-mediated mast cell activation at least in our mouse *in vitro* system. © 2000 Academic Press

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Previous studies on genetic background for atopy by genome-wide screening polymorphism, and linkage analyses have identified a number of candidate genes likely to contribute to the pathogenesis of allergy (1–3). Among them, a significant role for the polymorphism of high affinity IgE receptor (FcεRI) β chain in the manifestation of phenotype has been suggested. Genetic linkage studies have shown that a locus in chromosome 11q12-13 encompassing the β chain gene is linked with various allergic disorders and high levels of serum IgE (4–7). Moreover, three variants of the β chain gene

have been identified which result in amino acid changes of Ile¹⁸¹→Leu or Val¹⁸³→Leu in the fourth transmembrane domain and of Glu²³⁷→Gly in the C-terminal intracellular domain (8–10), and strong association was found between these polymorphisms and atopy in various populations (9, 11).

The FcεRI has a tetrameric structure consisting of three distinct polypeptides including the IgE-binding α chain, the four-fold membrane-spanning β chain, and the disulfide-linked γ-γ homodimer (12–14). The α chain binds the Fc portion of IgE with high affinity, while both β and γ chains contain the immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic domain and contribute to signal transduction (15). In humans, it has been demonstrated that the γ-homodimer can act as an autonomous signaling molecule, while the β subunit amplifies early activation signals through FcεRI by five- to sevenfold (16). Expression of the β chain is restricted to mast cells and basophils that have an important effector function in allergic reactions. Aggregation of FcεRI on mast cells by bound IgE and multivalent allergens induces the release of histamine, leukotrienes inflammatory cytokines, resulting in the recruitment and activation of circulating leukocytes in the inflammatory sites. These substances then cause the characteristic symptoms of type I allergic reactions such as pruritis, edema, increased mucous production, and bronchoconstriction. Therefore, the variant forms of β chain may be functionally implicated in the pathogenesis of allergic diseases by modulating the FcεRI-mediated mast cell activation. However, the functional abnormality of these variant β chains has not been determined so far.

We recently generated β chain-deficient (β^{-/-}) mice whose bone marrow-derived cultured mast cells (BMMC) lacked cell surface expression of FcεRI in spite of normal expression of FcεRI α and γ chain mRNA (17). Therefore, the BMMC derived from β^{-/-}

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mice was useful for reconstituting cell surface Fc ϵ RI with mutant β chains. To explore the possible effects of the polymorphisms in human β chain on Fc ϵ RI function, we generated four types of $\beta^{-/-}$ mast cell transfectants which expressed the reconstituted cell surface Fc ϵ RI with wild-type mouse β chain or mutant mouse β chains corresponding to the human variants (Leu¹⁷²→Ile, Leu¹⁷⁴→Val, or Glu²²⁸→Gly) (18). Biological responses of these transfectants to Fc ϵ RI-mediated stimulation were examined, including degranulation, intracellular calcium mobilization, cytokine production, and leukotriene secretion.

MATERIALS AND METHODS

Antibodies and cytokines. Anti rat β chain monoclonal Ab (clone JRK) which also recognizes mouse β chain (19) was kindly gifted from Dr. Juan Rivera at National Institute of Health (Bethesda, MD). Mouse monoclonal IgE Ab (anti-TNP) was purchased from Pharmingen (San Diego, CA) and was labeled with FITC. Recombinant murine stem cell factor (SCF) was kindly gifted from Kirin Brewery Co. (Tokyo, Japan).

Mice. The β chain-deficient ($\beta^{-/-}$) mice on the mixed C57BL/6 $\times$ 129 background have been described previously (17), and bred in the animal facility under the specific pathogen free condition at Jun-tendo University School of Medicine. The $\beta^{+/+}$ mice on the same background as $\beta^{-/-}$ mice were employed as controls. All mice were maintained within a filter-air laminar flow enclosure and provided with a standard laboratory food and water *ad libitum*.

Cell culture. Bone marrow-derived cultured mast cells (BMMC) were generated from mice femurs as described previously (20). Cells were incubated in the enriched RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated FCS (Biological Industries, Haemek, Israel), 100 U/ml penicillin and streptomycin (Gibco, Gland Island, NY), 10^{-4} M 2-mercaptoethanol (Wako, Tokyo, Japan), 110 μ g/ml sodium pyruvate (Wako, Tokyo, Japan), 1% of MEM nonessential amino acids solution (Gibco), and 10% pokeweed mitogen-stimulated spleen-conditioned medium (PWM-SCM) as a source of mast cell growth factors (21). Cells were grown in 5% CO₂-containing atmosphere at 37°C. After 4-week culture, cells were confirmed for cell surface expression of c-kit and Fc ϵ RI, and used for experiments (>95% mast cells). For retroviral transfection, bone marrow cells were cultured in the presence of 10% PWM-SCM and 100 ng/ml SCF for another 1 week. A ecotropic retrovirus packaging cell line, Phoenix-Eco (American Type Culture Collection, SD3444, Manassas, VA) was maintained in DMEM (Sigma, St. Louis, MO) supplemented 10% heat-inactivated FCS, 100 U/ml penicillin and streptomycin (Gibco).

Flow cytometry. The cells (3×10^5) were incubated with 5 μ g/ml of FITC-labeled IgE on ice for 30 min in 200 μ l of phosphate-buffered saline (PBS) with 0.1% BSA and 0.05% sodium azide. The cell surface fluorescence intensity was measured using a flow cytometer (FACScan, Becton-Dickinson, Mountain View, CA). The dead cells were excluded by propidium iodide staining.

Retroviral vectors, constructs, and transfections. The PCR-based mutagenesis described by Weiner *et al.* (22) was employed to create single nucleotide mutagenized constructs. Briefly, the 800 bp of *Eco*RI fragment containing the mouse β chain coding sequence (12) was isolated and subcloned into the *Eco*RI site of pBluescript (Stratagene, La Jolla, CA). Each two sets of PCR primers including the mutation site was designed on opposite direction from mutation site. The sequences of oligonucleotide primers for each mutagenesis were as follows: for L172I, 5'-CTG TTT CTC ACC ATC CTG GCC-3', 5'-CAT CAT CAA CAC AAT TTC TGT GG-3'; for L174V 5'-CTG TTT

CTC ACC ATC CTG GCC-3', 5'-CAT CAT CAC CAC CAG TTC TGT GG-3'; for G228E 5'-TCT CCA GTT GAT TCA TAA GAA TCA-3', 5'-AGA TGT TCC CCC TTT GTC TTC C-3' (mutation sites were underlined). PCR was carried out by using 2.5 units of KOD polymerase (Toyobo, Tokyo, Japan) in a 100- μ l reaction mixture containing 10 ng of pBluescript as template, 50 pM of each phosphorylated primer, 2 mM MgCl₂, and 0.2 mM dNTPs, 120 mM Tris-HCl (pH 8.8), 10 mM KCl, 6 mM (NH₄)₂SO₄, 0.1% Triton X-100 and 10 μ g/ml BSA). Amplified samples were denatured at 94°C for 4 min before 25 cycles of amplification (94°C for 1 min; 55°C for 1 min; 72°C for 4 min), followed by a final extension at 72°C for 10 min. The PCR products were treated with *Dpn*I (New England Biolabs, Beverly, MA) to digest the template DNA followed by self-ligation. The integrity of all mutants were confirmed by sequence analysis using capillary sequencer (ABI Prism 310 Genetic Analyzer, ABI, Foster City, CA). The mutant β chain cDNAs were subcloned into the *Eco*RI site of the Moloney murine leukemia virus-based retroviral vector, pMX-puro (23). To generate retroviruses, retroviral vectors were transfected transiently using Lipofectamine (Gibco) to Phoenix-eco. After 24 h incubation, viral supernatants were harvested and concentrated by centrifugation. The pellets were resuspended in 1/10 volume of BMMC culture medium with 100 ng/ml SCF. BMMC derived from $\beta^{-/-}$ mice were incubated with infectious supernatants for 2 days in the presence of 10 μ g/ml polybrene (Sigma). Cells expressing marker gene were selected by 1.5 μ g/ml puromycin (Sigma). Empty vector (pMX-puro) was used as negative control for the transfection.

Immunoblot analysis. The mouse β chain transfectants (5×10^4) were lysed with 1% NP-40 containing buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA). The lysates were electrophoretically resolved on a 12% SDS-polyacrylamide gel and transferred to the PVDF membrane (Pall Fluorotrance, Pall Co., NY). The β chain was probed by anti-rat β chain mAb (JRK). The membranes were detected by a HRP-conjugated rabbit anti-mouse IgG using the enhanced chemiluminescence detection system (ECL plus, Amersham, England).

β -Hexosaminidase release assay. Degranulation of the transfectants via Fc ϵ RI stimulation was determined by β -hexosaminidase release assay. Cells (5×10^5 cells/ml) were incubated with anti-TNP IgE (2 μ g/ml) in Mg²⁺ and Ca²⁺-free Tyrode's buffer (10 mM Hepes buffer, pH 7.4, 130 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.1% BSA) for 30 min at 4°C. The sensitized cells were then washed and resuspended at a concentration of 5×10^5 cells/ml in Tyrode's buffer containing 1 mM CaCl₂ and 0.6 mM MgCl₂. TNP-OVA (1 ng/ml) was used for stimulation. Phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (100 ng/ml) were employed for maximum stimulation as positive control. After 1 h incubation at 37°C, the reaction was terminated by centrifugation at 4°C. Total cell lysates were obtained by 1% NP-40. The supernatants and total cell lysates were incubated with 1.3 mg/ml of *p*-nitrophenyl-*N*-acetyl- β -D-galcopyranoside (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37°C. The reaction was terminated by the addition of 0.2 M glycine buffer (pH 10.7). The release of the product 4-*p*-nitrophenol was monitored by optical absorbance at 405 nm. Percentage of β -hexosaminidase release was calculated as follows: (supernatant OD value of the stimulated cells - supernatant OD value of the unstimulated cells) $\times$ 100/(the total cell lysate OD value - supernatant OD value of the unstimulated cells). Percentage release of background usually ranged below 5%.

Intracellular Ca²⁺ mobilization. Transfectants were incubated in dark place with 2 μ g/ml of anti-TNP-IgE and loaded with 2 μ M of fura-2 AM (Dojin, Tokyo, Japan) for 30 min at 37°C in enriched RPMI 1640, and were washed and resuspended at a concentration of 5×10^5 cells/ml in Tyrode's buffer containing 1 mM CaCl₂ and 0.6 mM MgCl₂, and then stimulated with 1 ng/ml of TNP-OVA at 37°C. Intracellular Ca²⁺ mobilization was monitored at a 510-nm emission wavelength excited by 340 and 360 nm using a fluorescence spectrophotometer model F2000 (Hitachi, Tokyo, Japan).

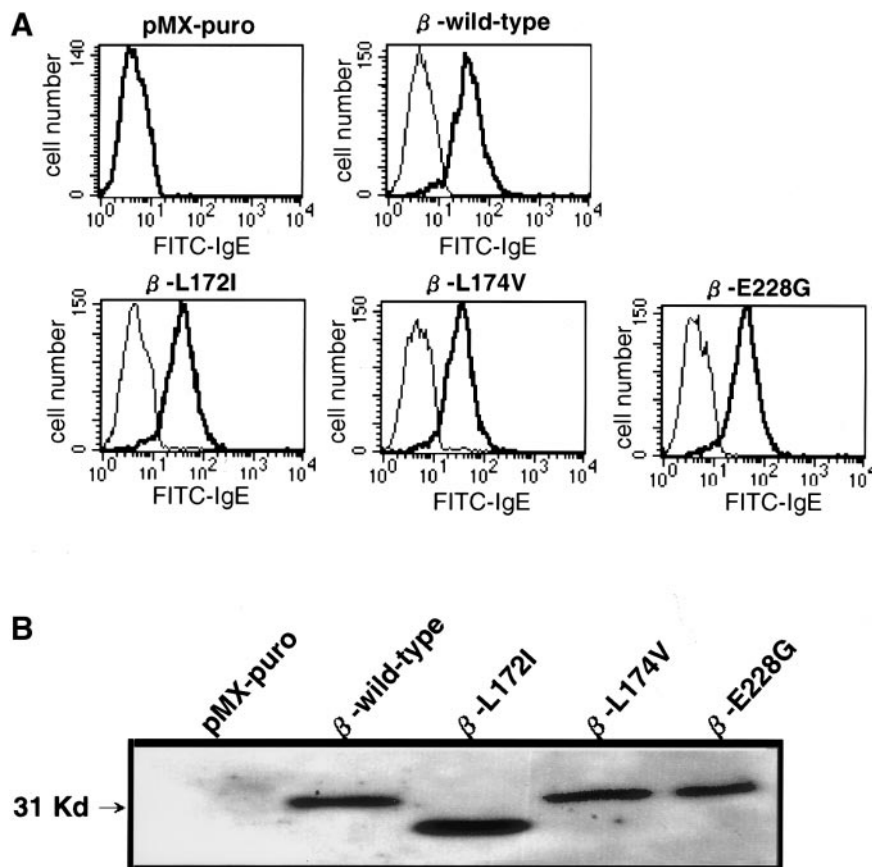


FIG. 1. Expression of wild-type or mutant FcεRI β chains in transfectants. (A) Cell surface expression of FcεRI reconstituted with wild-type or mutant β chains on transfectants. Transfectants (3×10^5 cells) were incubated with 5 μg/ml of FITC-labeled IgE. Cell surface fluorescence intensity was measured by flow cytometry. Dead cells were excluded by propidium iodide staining. Representative histograms from five independent experiments with similar results are shown. (B) Immunoblot analysis of β chains in the transfectants. Transfectants (5×10^4 cells) were lysed in buffer containing 1% NP-40. Lysates were resolved on a 12% SDS-polyacrylamide gel and transferred to PVDF membrane. β chains were probed by anti-rat FcR β moAb (JRK). Representative data from three independent experiments with similar results are shown.

Cytokine and LTC₄ ELISA. Cell culture supernatants after stimulation were analyzed for IL-6, TNF-α and LTC₄ by specific ELISA kits according to the manufacturer's instructions (Genzyme Techné, Minneapolis, MN for IL-6 and TNF-α, Cayman CHEMICAL, Ann Arbor, MI, for LTC₄). Cells (2×10^5 cells/ml) sensitized with anti-TNP IgE (2 μg/ml) were stimulated with TNP-OVA (1 ng/ml) for 3 h at 37°C.

Statistical analysis. Student's *t* test was employed to determine statistical significance among the experimental groups; $P < 0.05$ was considered significant. The data represented are pooled from three experiments.

RESULTS

Generation of stable transfectants expressing mutant forms of FcεRI β chain. The amino acid residues located in the fourth transmembrane domain of the human β chain, Ile¹⁸¹, Val¹⁸³ and in the C-terminal intracellular domain, Glu²³⁷, correspond to Leu¹⁷², Leu¹⁷⁴, and Glu²²⁸ of the mouse β chain, respectively (19). To investigate the significance of these amino acids in mast cell functions, we generated three types of con-

structs of retrovirus vector with mutant β chains in which Leu¹⁷²→Ile (β-L172I), Leu¹⁷⁴→Val (β-L174V), Glu²²⁸→Gly (β-G228E) and wild-type (β-wild-type), and reconstituted FcεRI by retroviral transfection of them in β^{-/-} mouse mast cells. First, we analyzed the cell surface expression of FcεRI on these transfectants by flow cytometry. The cells expressing the β-L172I, β-L174V, β-G228E and β-wild-type were all FcεRI positive on their cell surface (>95%), while pMX-puro did not express FcεRI as same as β^{-/-} mast cells (Fig. 1A). These results indicate that these mutations of the β chain did not affect the cell surface expression of FcεRI. The fluorescence intensities of FcεRI on each transfectant were almost the same. Further by immunoblotting with moAb to β chain, we confirmed that β chain transfectants actually expressed FcεRI β protein. The 31-kDa bands, which correspond to the β chain in β-L172I, β-L174V, β-G228E and β-wild-type transfectants were detected while no band was observed around the same position in the pMX-puro transfectant. The expression

levels of β chain appeared equal in β -L172I, β -L174V and β -G228E transfectants as well as β -wild-type transfectant, while in pMX-puro transfectant no β chain was detected (Fig. 1B). Although the reason is unknown, the product of β -L172I showed different molecular weight on SDS-PAGE compared to other transfectants. It is known that the mobility of protein on SDS-PAGE is influenced by their structure, isoelectric point, and hydrophilicity; thus mutation of β -L172I may affect these factors.

β -Hexosaminidase release. Degranulation of mast cells is a critical reaction in allergic inflammations such as immediate hypersensitivity, atopic dermatitis and asthma. We examined whether the transfectants could degranulate upon receptor crosslinking with IgE-antigen stimulation. The cells were sensitized with anti-TNP IgE and stimulated with 1 ng/ml of TNP-OVA to induce receptor crosslinking. The degree of mast cell degranulation was determined by measuring the release of β -hexosaminidase. All cells expressing surface Fc ϵ RI with wild-type or mutant β chains but not pMX-puro transfectant were capable of degranulation in response to surface IgE cross-linking. As demonstrated in Fig. 2A, the percentage of β -hexosaminidase release were β -L172I, $48.2 \pm 0.38\%$; β -L174V, $49.4 \pm 0.39\%$; β -G228E, $46.7 \pm 2.31\%$; and β -wild-type, $49.3 \pm 0.17\%$, respectively. There were no significant differences in the degree of degranulation among these transfectants. Moreover, we examined the dose response for TNP-OVA in IgE-sensitized transfectants. The degrees of degranulation in the β mutants were similar compared to these of wild-type transfectants (Fig. 2B).

Intracellular Ca^{2+} mobilization. Since calcium influx is an early event induced upon Fc ϵ RI crosslinking, we investigated whether or not Ca^{2+} mobilization was affected by the mutation of β chain. Each transfectants were incubated with anti-TNP IgE and loaded with fura-2/AM. In β -wild-type transfectants, the concentration of intracellular Ca^{2+} reached the maximum level within 50 s after stimulation of specific antigen, and we observed no significant differences in the pattern of Ca^{2+} mobilization among β -L172I, β -L174V, β -G228E (Fig. 3). No Ca^{2+} mobilization was detected in pMX-puro transfectant.

Cytokine secretion. Activation of mast cells upon aggregation of Fc ϵ RI leads to degranulation and secretion of preformed and *de novo* synthesized cytokines such as IL-4, IL-5, IL-6, and TNF- α . Next, we investigated whether mutation of the β chain would influence the cytokine production and secretion from mast cells. Since IL-6 and TNF- α are major cytokines that are produced and secreted from mast cells, we measured their release with specific ELISA. Transfectants were incubated with an anti-TNP IgE, stimulated with 1 ng/ml of TNP-OVA for 3 h and supernatants were corrected. There were no significant differences in the

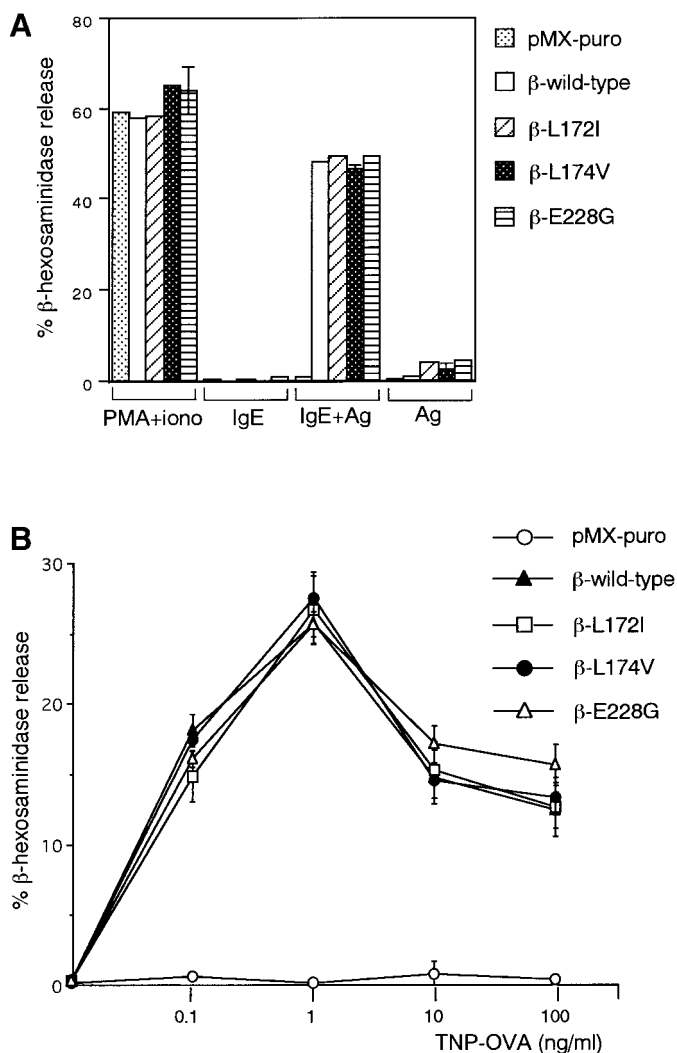


FIG. 2. Fc ϵ RI-mediated β -hexosaminidase release from β chain transfectants. Transfectants (5×10^5 cells/ml) were incubated with 2 μ g/ml of anti-TNP mouse IgE for 30 min, and subsequently stimulated with 1 ng/ml of TNP-OVA (Ag). After 1 h incubation at 37°C, supernatants were collected and the cells were lysed in 1% NP-40. Cell-free supernatants and cell lysates were incubated with 1.3 mg/ml of *p*-nitrophenyl-*N*-acetyl- β -D-galcopyranoside for 90 min at 37°C. Percentage of β -hexosaminidase release was calculated as described under Materials and Methods. (A) PMA (10 ng/ml) and ionomycin (100 ng/ml) were used for maximal stimulation as a positive control. (B) Dose response is analyzed with various concentration of TNP-OVA. Data are represented as means $\pm$ SD of triplicate samples. Similar results were obtained in three independent experiments.

secretion of the cytokines among these transfectants (Table 1). No secretion of IL-6 and TNF- α was detected in pMX-puro transfectants. Moreover, their abilities to release LTC $_4$ were similar among these transfectants.

DISCUSSION

Among multimeric receptors, the Fc ϵ RI β chain is an unique component because of its function as an intrin-

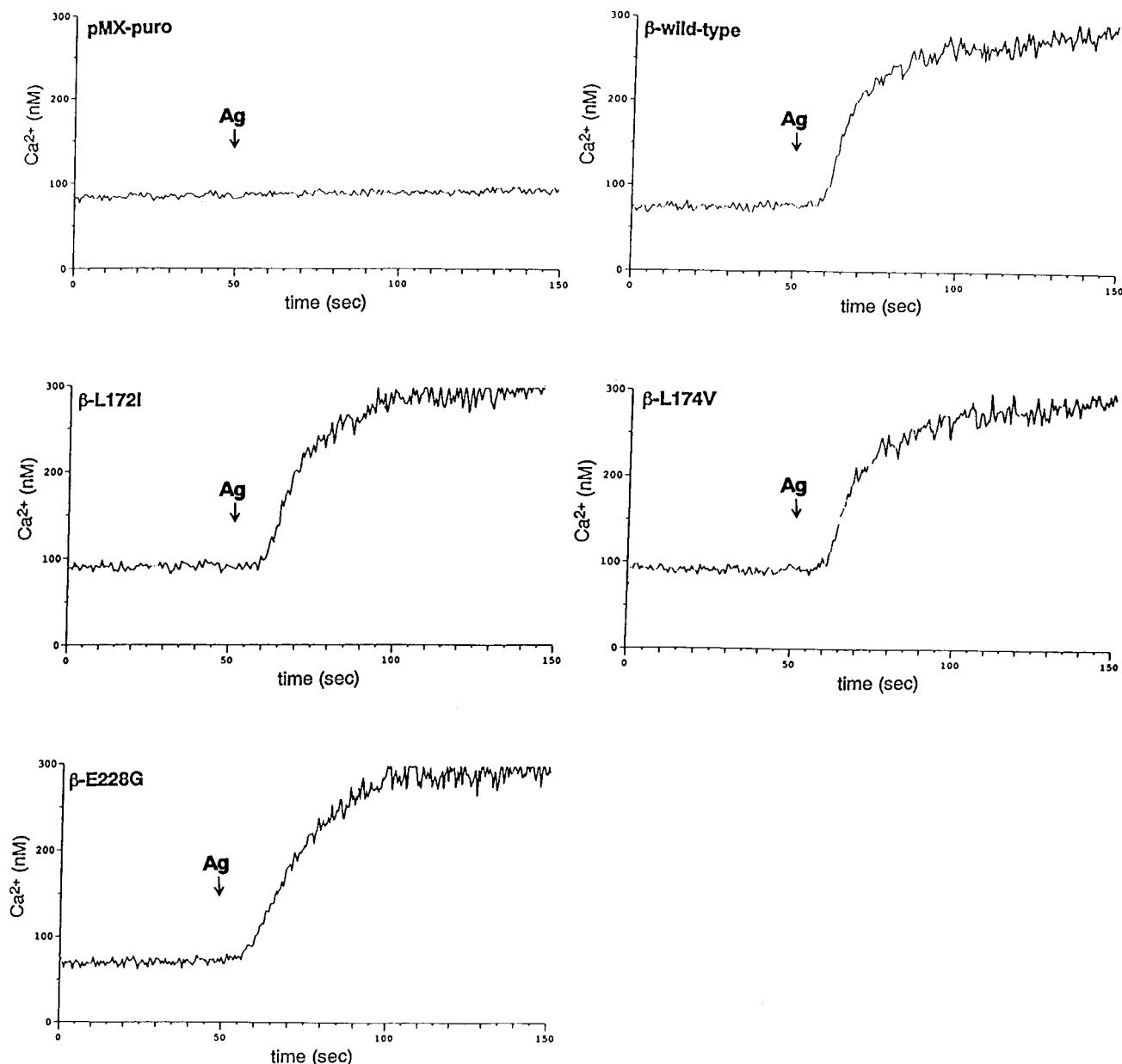


FIG. 3. Kinetics of intracellular Ca^{2+} mobilization in wild-type or mutant β chain transfectants upon $\text{Fc}\epsilon\text{RI}$ crosslinking. Transfectants (5×10^5 cells/ml) were incubated with $2 \mu\text{g/ml}$ anti-TNP IgE and with $2 \mu\text{M}$ fura-2 AM for 30 min at 37°C . The cells were washed and resuspended at 5×10^5 cells/ml in Tyrode's buffer, and then stimulated with 1 ng/ml TNP-OVA (Ag). Intracellular Ca^{2+} mobilization was monitored on a fluorescence spectrophotometer as described under Materials and Methods. Data shown are representatives of three independent experiments with similar results.

sic molecular amplifier for the signal through the receptor complex. Since a number of groups have independently identified $\text{Fc}\epsilon\text{RI}$ β variants associating with allergic phenotypes in various ethnic populations, this gene is considered as one of the most likely candidate genes for the development of atopy. Li *et al.* (24) demonstrated that there were no significant differences among atopic individuals with or without I181L mutation in skin prick tests and in histamine release from peripheral basophils. In their paper, some of individu-

als with I181L $\text{Fc}\epsilon\text{RI}$ β chain showed enhanced-sensitivity in skin mast cell reactions to allergen, compared with subjects without I181L mutation, however results were not statistically significant because of small sample size. This variability made interpretation of the data difficult and more refined functional experiments were required. To elucidate the effect of β chain polymorphisms on mast cell activation, we conducted an *in vitro* study of $\text{Fc}\epsilon\text{RI}$ reconstituted with mutant β chains.

TABLE 1

Cytokine and LTC₄ Secretion by Wild-Type or Mutant β Chain Transfectants upon Fc ϵ RI Crosslinking

	IL-6 (pg/ml)	TNF- α (pg/ml)	LTC ₄ (ng/ml)
pMX-puro	ND	ND	ND
β -Wild-type	207 $\pm$ 26.3	34 $\pm$ 5.8	28.6 $\pm$ 1.43
β -L172I	201 $\pm$ 13.8	21 $\pm$ 8.1	28.3 $\pm$ 2.0
β -L174V	202 $\pm$ 4.7	35 $\pm$ 10.0	24.7 $\pm$ 3.77
β -E228G	215 $\pm$ 3.0	32 $\pm$ 2.3	30.5 $\pm$ 1.89

Note. Transfectants (2×10^6 cells/ml) were sensitized with anti-TNP IgE (2 μ g/ml) and then stimulated with TNP-OVA (1 ng/ml). After 3 h, supernatants were collected, and IL-6, TNF- α , and LTC₄ concentrations were determined by ELISA. Data are shown as means $\pm$ SD of triplicate samples. Similar results were obtained in two independent experiments. ND, not detectable.

We investigated *in vitro* whether Fc ϵ RI carrying mutant β chain functioned abnormally comparing to wild type β chain; the Fc ϵ RI expression levels on the surface of transfectants, β -hexosaminidase release as degradation response, intracellular calcium mobilization, and production of major inflammatory cytokines. It was hypothesized that mutations within the transmembrane domain might influence efficient and intact Fc ϵ RI expression. On the other hand, the polymorphism of Gly²³⁷ located in the C-terminal domain of the β chain, might be gain-of-function mutation in the regulation of the signal transduction through Fc ϵ RI. But none of the β chain polymorphisms examined in this study affected IgE-mediated Fc ϵ RI functions as long as tested this time. Our results suggest that these polymorphisms in Fc ϵ RI β chain do not affect Fc ϵ RI-mediated mast cell activation at least in our mouse *in vitro* system.

Since atopic diseases are polygenic, it seems that combination with another genetic factors may increase the risk of the disease and change the effector cell responsiveness *in vivo*. Recently, Risch and Merikangas (25) have demonstrated that the future genetic studies of complex human diseases may depend, to a large extent, on applications of new linkage analyses to family-based data such as the transmission disequilibrium test for disease genes with weak to moderate effects on disease risks. There is a possibility that the polymorphisms of the β chain may be in linkage disequilibrium with another identified or unidentified variants to promote the development of atopy. By identifying and characterize those genes for atopy, it provides useful genetic markers in diagnosis and is helpful in defining therapeutic targets.

It is suggested that the β chain functioned not only as a signal amplifier but also as a stabilizer of Fc ϵ RI complex (26). It would be possible that β chain variants protect surface Fc ϵ RI from degradation more efficiently compared with wild type or that they change the bind-

ing affinity of Fc ϵ RI for IgE. Furthermore, the β chain is a common subunit for the low affinity IgG receptor, Fc γ RIII which contributes to the initiation of allergic reactions as well as Fc ϵ RI (27). It is demonstrated that the β chain also functions as an amplifier of mast cell activation via Fc γ RIII (17), suggesting the functional consequences of mutant β chain on the Fc γ R signaling. Elucidation of these points is remained for future investigation.

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