

Role of the Fc ϵ RI β -chain ITAM as a signal regulator for mast cell activation with monomeric IgE

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Abstract

The β -chain of the high-affinity receptor for IgE (Fc ϵ RI) plays a crucial role for amplification of the intracellular signaling in mast cells upon Fc ϵ RI cross-linking by IgE•antigen complexes (IgE•Ag). Some monomeric IgE as well as IgE•Ag stimulate Fc ϵ RI-signaling pathways, leading to cell activation, whereas the biological functions of the β -chain in the monomeric IgE-mediated mast cell signaling and responses are largely unknown. In the present study, Fc ϵ RI is reconstituted with either wild-type β -chain or mutated β -chain immunoreceptor tyrosine-based activation motif (ITAM) employing retrovirus-mediated gene transfer into the Fc ϵ RI β -chain $^{-/-}$ mast cells. We demonstrated that the transfectants with mutated β -chain ITAM stimulated with monomeric IgE sufficiently produce inflammatory cytokines, although degranulation, intracellular Ca²⁺ mobilization and leukotriene C₄ synthesis are significantly reduced. Furthermore, analyses of molecular mechanisms of the signaling revealed that the expression of cytokine genes and activation of extracellular signal-regulated kinase 1/2 and protein kinase C were significantly delayed in the β -chain ITAM mutant cells stimulated with monomeric IgE, suggesting that the β -chain ITAM regulates kinetics of gene transcriptions and signaling pathways for cytokine production. These findings for the first time revealed the unique functions of the β -chain ITAM in both chemical mediator release and cytokine production of mast cells upon monomeric IgE stimulation.

Introduction

Mast cells express the high-affinity receptor for IgE (Fc ϵ RI) on the cell surface, which plays a central role in the development of allergic inflammation. Aggregation of the Fc ϵ RI on mast cells by bound IgE and multivalent antigens induces release of inflammatory mediators from the granules and production of inflammatory cytokines (1).

Fc ϵ RI is mainly expressed on mast cells and basophils as a tetramer of α -, β - and γ -chain homodimers (2). The α -chain binds IgE, while β - and γ -chains mediate intracellular signaling through immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic domains. The β -chain has been revealed to play an important role for amplifying the γ -chain-mediated intracellular signals through enhancing Syk kinase activation and/or α -chain maturation (3–6). In general, Fc ϵ RI cross-linking by IgE•antigen complexes (IgE•Ag) is believed

to cause tyrosine phosphorylation of the β -chain ITAM via activated Lyn, and subsequent phosphorylation of γ -chain ITAM and Syk, which then leads to activation of downstream signals such as intracellular Ca²⁺ mobilization, and activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) family (7, 8). Thus, tyrosine phosphorylation of the β -chain ITAM is a crucial event for initiation of a series of responses in mast cell activation. In addition, the β -chain ITAM possesses a non-canonical tyrosine residue between the canonical two-tyrosine residues in the C-terminal intracellular portion, although ITAM of the γ -chain and other receptors have only the canonical two-tyrosine residues. We have recently shown that phosphorylation of the non-canonical tyrosine residue of the β -chain ITAM mediates suppressive signals for cytokine production upon IgE•Ag stimulation (9). Therefore, it

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is likely that physiological functions of the β-chain ITAM in mast cells are cleverly regulated by phosphorylation of non-canonical and canonical tyrosine residues.

Previous reports revealed that binding of monomeric IgE to the FcεRI enhances mast cell survival (10, 11) and surface expression of FcεRI (12). Recently, these monomeric IgEs have been divided into two categories, highly cytokinergic and poor cytokinergic IgEs. The highly cytokinergic IgEs, e.g. SPE7 and H1-ε-26, can elicit not only the production and secretion of various cytokines but also other activation events, including receptor internalization, degranulation and histamine synthesis (13–16).

For these responses induced by IgE loading, activation of the γ-chain ITAM is crucial and loss of its function results in suppression of monomeric IgE-mediated IL-6 production and cell survival (17). However, little is known about the biological significance and functions of the β-chain ITAM in mast cell activation with the monomeric IgE stimulation. In the present study, we investigated the biological roles of the β-chain ITAM in the monomeric IgE-mediated mast cell activation employing the β-chain ITAM mutant, in which canonical and non-canonical tyrosine residues are replaced with phenylalanine. We demonstrated that normal tyrosine phosphorylation of the wild-type β-chain ITAM is indispensable for the monomeric IgE-mediated degranulation, leukotriene (LT) C₄ secretion and Ca²⁺ influx and that it contributes to initiation of cytokine gene transcription, activation of PKC and extracellular signal-regulated kinase (ERK) 1/2 *in vitro*. This is the first report that revealed functions of the β-chain ITAM in mast cell activation upon monomeric IgE stimulation.

Methods

Reagents

Anti-β-chain mAb [clone JRK: the hybridoma (JRK) was kindly gifted by Juan Rivera at National Institutes Health (NIH), USA] was prepared in our laboratory. Anti-trinitrophenol (TNP) IgE (clone IgE3) was purchased from Pharmingen (San Diego, CA, USA) and was labeled with FITC. Anti-dinitrophenyl (DNP) IgE (clone SPE7) was purchased from Sigma (St Louis, MO, USA). Anti-DNP H1-ε-26 was purified as described previously (18). DNP BSA was purchased from LSL (Tokyo, Japan). Recombinant stem cell factor (SCF) was kindly gifted from Kirin Brewery Co. (Tokyo, Japan). Polyclonal antibody to Lyn was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All anti-phospho-specific, anti-ERK1/2, anti-Akt antibodies and wortmannin were purchased from Cell Signaling Technology (Beverly, MA, USA).

Mice

FcεRI β-chain^{−/−} mice (5) were bred in the animal faculty at Nihon University School of Medicine under specific pathogen-free conditions. All experiments were performed according to the guidelines of Nihon University.

Cell culture

Bone marrow-derived mast cells (BMMCs) were prepared from the femur of 4- to 8-week old β-chain^{−/−} mice as

described previously (19). Cells were cultured in the RPMI 1640 (Sigma) supplemented with 20% FBS (Sigma) and 5 ng ml^{−1} of murine recombinant IL-3. BMMCs were used for experiments after 4–8 weeks of culture. For retroviral transfection, bone marrow cells were cultured in the presence of 100 ng ml^{−1} SCF for another 1 week. An ecotropic retrovirus packaging cell line, Platinum-E (PLAT-E) (kindly gifted by Toshio Kitamura at Tokyo University, Japan) was maintained in DMEM supplemented with 10% FBS, 1 μg ml^{−1} puromycin (Clontech, San Jose, CA, USA) and 10 μg ml^{−1} blasticidin S (Kaken Pharmaceutical Co., Tokyo, Japan).

cDNA constructions and transfections

The mouse β-chain cDNA was constructed as previously described (19). Phenylalanine substitutions for tyrosine of β-chain ITAM (Y210F/Y216F/Y220F) was generated by using a PCR-based, site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Mutation of β-chain was confirmed by DNA sequencing. Then, β-chain cDNAs were subcloned into the *EcoRI* site of the retroviral vector pMx-puro (20). These plasmids were transfected into PLAT-E packaging cells with Eugene 6 (Roche Diagnostics, Basel, Switzerland) to generate retroviruses. BMMCs (5 × 10⁶) were infected with the retroviruses for 48 h in the presence of 1 μg ml^{−1} polybrene (Sigma). For the preparation of β-chain ITAM transfectants, gene-transduced cells were selected with 1.2 μg ml^{−1} puromycin for 7 days. Viable cells (10–20% of BMMCs cultured with retroviruses) were expanded for several weeks before experiments. Wild-type and mutated β-chain ITAM transfectants were named YYY wild-type and FFF mutant, respectively.

Immunoblotting

The stimulated cells were washed twice with ice-cold PBS and lysed in Tris-buffered saline containing 1% NP-40, 60 mM octyl-β-glucoside, 2 mM phenylmethylsulfonylfluoride, 10 μg ml^{−1} aprotinin, 2 μg ml^{−1} leupeptin and pepstatin A, 50 mM NaF and 1 mM sodium orthovanadate, for 30 min on ice as previously described (21). The lysates were centrifuged for 15 min at 14 000 × *g*. For immunoprecipitation, the cell lysates were incubated with antibody-bound-protein A 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 10 or 12% SDS-PAGE and transferred on to Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was incubated with primary antibody and an appropriate secondary HRP-conjugated antibody. Signals were detected by enhanced chemiluminescence (Amersham Bioscience, Little Chalfont, United Kingdom). The immunoreactive bands were scanned to produce digital images that were quantified employing NIH Image software (<http://rsb.info.nih.gov/nih-image>), and then kinase activation was calculated by the relative amount of phospho-ERK1/2, phospho-c-Jun N-terminal kinase (JNK) 1/2 protein to each non-phospho protein.

β-hexosaminidase release assay

Degranulation of mast cells was determined by β-hexosaminidase release as described previously (19). Briefly, cells

(5×10^5) were stimulated in 0.2 ml $1\times$ Tyrode's buffer at indicated concentrations of monomeric IgEs or antigen at 37°C for 30 min. The cells were lysed with 1% NP-40 in the above-mentioned medium. The supernatants and the total cell lysates were incubated with 1.3 mg ml $^{-1}$ of *p*-nitrophenyl-*N*-acetyl- β -D-galcopyranoside (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) at 37°C for 40 min. 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 evaluated by optical absorbance at 405 nm.

Measurements of cytokines and LTC $_4$

Cell culture supernatants after Fc ϵ RI stimulation were analyzed for IL-6, IL-13, tumor necrosis factor- α (TNF- α) and LTC $_4$ production by specific ELISA kits [Cayman Chemical (Ann Arbor, MI, USA) for LTC $_4$, Endogen (Rockford, IL, USA) for IL-6, IL-13 and TNF- α] according to the manufacture's instructions. In experiments using a phosphatidylinositol 3-kinase (PI3K) inhibitor, cells were pre-treated with 0.5 μ M of wortmannin for 30 min at 37°C.

Flow cytometry

For the evaluation of Fc ϵ RI expression on the cell surface, cells (5×10^5) were stained with 1 μ g ml $^{-1}$ FITC-conjugated anti-TNP IgE at 4°C for 30 min and then washed twice with PBS containing 0.1% sodium azide. The stained cells were analyzed with FACSCalibur (Becton-Dickinson, San Jose, CA, USA). β -Chain-/- BMMCs were used as negative controls. The receptor internalization was defined as the difference of Fc ϵ RI expression on the cell surface in mean fluorescence intensity ratio (before/after Fc ϵ RI stimulation).

Reverse transcription-PCR

Total RNA was isolated from BMMCs using ISOGEN (Nippon Gene, Tokyo, Japan). cDNA was reverse transcribed from 2 μ g ml $^{-1}$ total RNA by 200 U of Superscripts II, 2.5 mM dNTP and 1.0 mM oligo (dT) primer. The resultant cDNA was then employed as templates for the PCR. The open reading frames of *IL-6*, *IL-13*, *TNF- α* and β -*actin* were amplified using appropriate primers as follows: 5'-ATGAAGTTCCTCTCTG-CAAG-3' and 5'-GTACTCCAGGTAGCTATGGT-3' (*IL-6*); 5'-ATGGCGCTCTGGGTGACTGC-3' and 5'-ATGGTCTCTCTCATTAGAA-3' (*IL-13*); 5'-ATGAGCACAGAAAGCATGAT-3' and 5'-GAACCTGGGAGTAGACAAGG-3' (*TNF- α*) and 5'-ATGGATGACGATACGCTGC-3' and 5'-CCATCACAATGCCTGTGGTA-3' (β -*actin*). PCR was performed in the conditions as follows, denaturation at 94°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 2 min with 27 cycles, and then a 10 min elongation at 72°C. The PCR products were applied on to a 1% agarose gel and visualized by ethidium bromide staining. Data were quantified employing NIH Image software, and the fold induction was calculated by the relative amount of cytokine mRNA to β -*actin* mRNA (internal control).

Measurement of intracellular Ca $^{2+}$ concentration

Change of intracellular Ca $^{2+}$ concentration ([Ca $^{2+}$] $_i$) upon Fc ϵ RI stimulation was determined as previously described (22). Briefly, cell suspension (10^6 cells ml $^{-1}$ in HBSS) was

incubated with 4 μ M Fluo-3AM at 37°C for 30 min and then washed twice with HBSS and resuspended in the medium supplemented with 1 mM CaCl $_2$. Fluo-3 fluorescence was monitored by a microplate fluorometer (Fluoroskan Ascent CF; Labsystems). The cytosolic-free calcium concentration ([Ca $^{2+}$] $_i$) was calculated using the equation: [Ca $^{2+}$] $_i$ = K_d [(F - F $_{min}$)/(F $_{max}$ - F)], where K_d is the dissociation constant of the Ca $^{2+}$ -Fluo-3 complex (450 nM), F $_{max}$ represents the maximum fluorescence (obtained by treating cells with 5 mM A23187), F $_{min}$ represents the minimum fluorescence (obtained for A23187-treated cells in the presence of 1 mM EGTA) and F is the actual sample fluorescence.

Statistical analysis

Student's *t*-test was performed to determine statistical significance among the experimental groups; *P* < 0.05 was considered significant.

Results

β -chain ITAM is indispensable for mediator release of mast cells stimulated with the monomeric IgE

Before analyses for functions of the β -chain ITAM, we confirmed expression of the reconstituted Fc ϵ RI in the β -chain transfectants using flow cytometry. Fig. 1(A) displays construction of the substituted β -chain ITAM. As shown in Fig. 1(B), Fc ϵ RI expression was at almost same levels in the YYY wild-type cells and the FFF mutant cells, and BMMCs of β -chain-/- mice expressed no Fc ϵ RI on the cell surface. These results are consistent with our recent study in which mutation of β -chain ITAM had no effects on Fc ϵ RI expression on the cell surface (9).

To clarify biological functions of the β -chain ITAM for inflammatory mediator release of mast cells, we first examined β -hexosaminidase (one of the intragranular enzymes) release from the monomeric IgE-stimulated (SPE7, H1- ϵ -26) β -chain transfectants (Fig. 1C and D). Interestingly, in the monomeric IgE-stimulated FFF mutant cells, β -hexosaminidase release was severely impaired, and no release was observed even when the concentration of the monomeric IgEs was increased to 50 μ g ml $^{-1}$ (data not shown). In contrast to monomeric IgE stimulation, upon IgE•Ag stimulation, β -hexosaminidase release of the FFF mutant cells was induced to 50% level of the YYY wild-type cells (Fig. 1E). Next, we evaluated LTC $_4$ release which is one of the newly generated inflammatory mediators. As expected, LTC $_4$ secretion as well as β -hexosaminidase release was also impaired in the monomeric IgE-stimulated FFF mutant cells. It is thereby concluded that LTC $_4$ secretion and degranulation in response to the monomeric IgE require the β -chain ITAM as a critical element.

Since intracellular Ca $^{2+}$ is an essential second messenger for chemical mediator release (23, 24), we evaluated extracellular Ca $^{2+}$ influx into these transfectants employing a calcium indicator, Fluo-3AM. As displayed in Fig. 2(B), rapid elevation of intracellular Ca $^{2+}$ concentration ([Ca $^{2+}$] $_i$), which reaches to the level of 1000 nM within 50 s after stimulation, was observed only in the YYY wild-type cells stimulated with the IgE•Ag. In the YYY wild-type cells stimulated with the SPE7, [Ca $^{2+}$] $_i$ elevated gradually upon IgE loading. On the

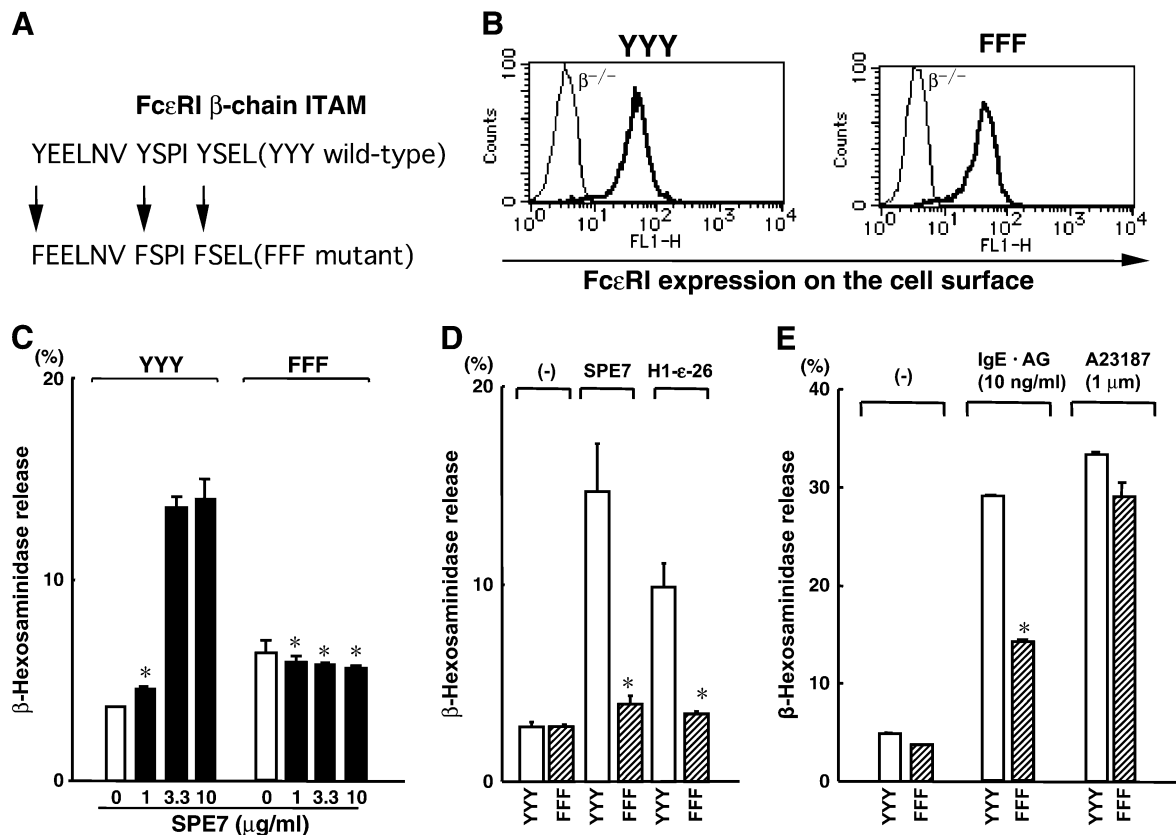


Fig. 1. Role of the FcεRI β-chain ITAM for the monomeric IgE-mediated degranulation. (A) Construction of the wild-type and mutant β-chain ITAM. (B) Reconstituted FcεRI expressions on the YYY and FFF transfectants. Cells ($5 \times 10^5 \text{ ml}^{-1}$) were incubated with FITC-labeled IgE (IgE3; monomeric IgE3 itself cannot activate FcεRI) and cell surface fluorescence intensity was evaluated by flow cytometry. β-chain (−/−) BMMC (host cells for transfection) were employed as negative controls (11). (C) β-hexosaminidase release is severely reduced in the SPE7-activated FFF mutant cells. Primary YYY and FFF transfectants were stimulated with SPE7 at indicated concentrations for 20 min. (D) β-Hexosaminidase release was significantly reduced in the H1-ε-26-activated FFF mutant cells. Primary YYY and FFF transfectants ($5 \times 10^5 \text{ ml}^{-1}$) were stimulated with 10 mg ml^{-1} of SPE7 or H1-ε-26 for 20 min. (E) Cells were stimulated with 10 ng ml^{-1} of IgE-Ag or $1 \mu\text{M}$ A23187 for 20 min. Data shown are the mean $\pm$ SD of triplicate samples from three independent experiments. Statistical analyses were performed by Student's *t*-test. **P* < 0.05 relative to maximum release of the YYY wild-type transfectants.

other hand, in the FFF mutant cells stimulated with the SPE7, $[Ca^{2+}]_i$ was slightly elevated. These results suggest that the β -chain ITAM plays a crucial role for the regulation of mediator release and intracellular Ca^{2+} mobilization in wild-type mast cells upon the monomeric IgE stimulation.

Effects of the β -chain ITAM on Lyn recruitment to and internalization of the Fc ϵ RI

Lyn is known as a major Src kinase in mast cells and is to be activated immediately upon IgE•Ag stimulation (25). Activated Lyn then strongly binds to β -chain ITAM, leading to subsequent activation of signaling cascades (26). Additionally, it was recently reported that Lyn activation and Fc ϵ RI β -chain phosphorylation in the monomeric IgE-stimulated mast cells were very weak, when compared with those in the IgE•Ag-stimulated mast cells (10, 16). Therefore, we examined the association between Lyn and the β -chain by co-immunoprecipitation assay upon Fc ϵ RI stimulation. Western blotting analyses revealed that recruiting of Lyn to the β -chain was almost undetectable in the YYY wild-type and the FFF mutant cells upon the monomeric IgE stimulation (Fig. 3A, left panel).

although it was sufficiently detectable in the YYY wild-type cells stimulated with IgE•Ag. In the FFF mutant cells, Lyn recruitment to the β -chain was not detected even when the concentration of the antigen was up to 100 ng ml⁻¹ (Fig. 3A, right panel). These findings suggest that the monomeric IgE stimulation in the YYY wild-type and the FFF mutant cells was insufficient for activation of the β -chain ITAM by Lyn and the subsequent signaling cascades. However, it was recently reported that monomeric IgE can induce Lyn-dependent mast cell activation. For instance, Fc ϵ R1 internalization in response to the monomeric IgE loading is severely impaired in Lyn-/- mast cells (14). To evaluate the contribution of Lyn-dependent signaling in these transfectants, we examined whether Fc ϵ R1 internalization is induced by monomeric IgE stimulation. As shown in Fig. 3(B), in the YYY wild-type cells, Fc ϵ R1 internalization was immediately induced upon SPE7 stimulation. On the other hand, in the FFF mutant cells, Fc ϵ R1 internalization was not observed until at the longest 60 min after stimulation. Collectively, these results indicate that the Lyn-dependent signaling pathway is impaired in the monomeric IgE-stimulated FFF mutant cells and that the β -chain ITAM is necessary for signal transduction through Lyn.

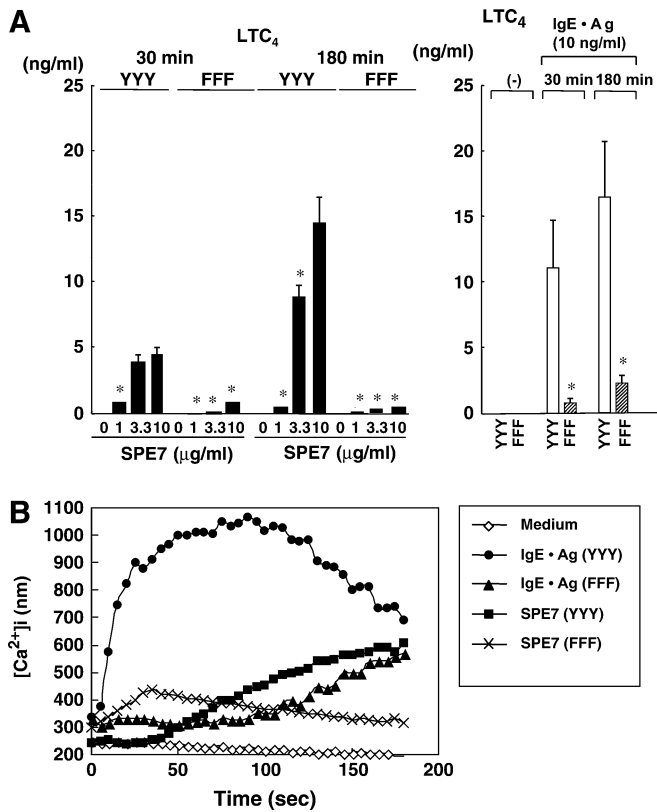


Fig. 2. Role of the β -chain ITAM in the monomeric IgE-mediated LTC₄ secretion, intracellular Ca²⁺ mobilization. (A) LTC₄ secretion was impaired in the monomeric IgE-stimulated FFF mutants. Cells ($5 \times 10^5 \text{ ml}^{-1}$) were stimulated with SPE7 at indicated concentrations (left panel) or 10 ng ml⁻¹ of IgE•Ag (right panel) for 30 and 180 min. Supernatants were collected and analyzed for LTC₄ secretion. Data shown are the mean $\pm$ SD of triplicate samples from two independent experiments. (B) Elevation of intracellular Ca²⁺ concentrations is reduced in the monomeric IgE-activated FFF transfectants. Fluo-3AM-loaded cells were analyzed using a microplate fluorometer. Data from one of the representatives of three independent experiments are demonstrated in the figure. Statistical analyses were performed by Student's *t*-test. **P* < 0.05 relative to maximum release of the YYY wild-type transfectants.

β -chain ITAM regulates transcription of cytokine genes

It was recently reported that monomeric IgE induces inflammatory cytokine production in mast cells (10). Since chemical mediator release and Lyn-dependent cellular responses were severely reduced in the activated FFF mutant cells stimulated with monomeric IgE, cytokine production was thought to be also defective in the FFF mutant cells. We therefore examined production of IL-6, IL-13 and TNF- α in the transfectants. As shown in Fig. 4(A–C), cytokine production of the SPE7-stimulated FFF mutant cells was sufficiently induced, but the levels were lower than those of the SPE7-stimulated YYY wild-type cells. In the YYY wild-type cells, H1- ϵ -26 as well as SPE7 also sufficiently induced IL-6 production, while IL-6 production of the H1- ϵ -26-stimulated FFF mutant cells was reduced and delayed (Fig. 4D). In the FFF mutant cells, significant IL-6 production was induced at 480 min after H1- ϵ -26 stimulation. These results suggest that cytokine gene

transcription is delayed in the FFF mutant cells stimulated with monomeric IgE.

We next analyzed expression of IL-6, IL-13 and TNF- α genes employing reverse transcription-PCR (Fig. 5A), and the results of their densitometric analyses were shown in Fig. 5(B). It was of interest that expression of IL-6 and IL-13 genes in the SPE7-stimulated FFF mutant cells did not reach maximum levels until 60 min, while expression of these genes in the SPE7-stimulated YYY wild-type cells reached maximum levels within 60 min and decreased thereafter. On the other hand, kinetics of TNF- α gene transcription was delayed in the SPE7-stimulated FFF mutant cells. These results clearly indicate that loss of function of the β -chain ITAM (changes of Y to F) makes a delay in the monomeric IgE-initiated transcription for IL-6, IL-13 and TNF- α genes.

Akt activity is severely impaired in the FFF mutant cells stimulated with the monomeric IgE

As demonstrated in Fig. 5, cytokine gene expression was obviously delayed in the monomeric IgE-stimulated FFF mutant cells. Since Krystal and colleagues reported that LY294002, one of PI3K inhibitors, significantly suppressed monomeric IgE-induced IL-6 production (10), we examined IL-6 and IL-13 production in the late phase response of the transfectants to the monomeric IgE in the presence or absence of a PI3K inhibitor, wortmannin. A concentration of 0.5 μ M wortmannin effectively suppressed IL-6 and IL-13 production in the SPE7-stimulated FFF mutant and YYY wild-type cells (Fig. 6A). These findings indicate that production of IL-6 and IL-13 may be dependent on PI3K pathway. Since Akt, a member of PI3K cascades, is known to contribute to cytokine production in mast cells (27), we next examined activation of Akt in the monomeric IgE-stimulated mast cells. Unexpectedly, Akt activation was almost undetectable in the SPE7-stimulated FFF mutant cells, while it was clearly detected in the SPE7-stimulated YYY wild-type cells (Fig. 6B).

Delayed activation of ERK in the FFF mutant cells stimulated with the monomeric IgE

Because specific inhibitors of MAPK family were reported to significantly suppress IL-6 and IL-13 production in activated mast cells (28–30), we examined activities of ERK1/2 and JNK1/2 in activated mast cells. As demonstrated in Fig. 6(C), the prolonged phosphorylation of ERK1/2 and JNK1/2 was observed in both the SPE7-stimulated YYY wild-type and FFF mutant cells. However, it is noteworthy that initiation of ERK1/2 phosphorylation in the SPE7-stimulated FFF mutant cells was significantly delayed, compared with that of the SPE7-stimulated YYY wild-type cells. These findings seem to be in accordance with the results of cytokine production in the monomeric IgE-activated YYY wild-type and FFF mutant cells.

Effects of the β -chain ITAM on SH2 domain containing inositol 5'-phosphatase and PKC activation

SH2 domain containing inositol 5'-phosphatase (SHIP)-1 is known to act as a negative regulator for mast cell activation through FcεRI, and its phosphorylation requires activation of Lyn (31). In SHIP-1^{-/-} mast cells, production of IL-6, IL-13 and TNF- α induced by IgE•Ag is significantly enhanced

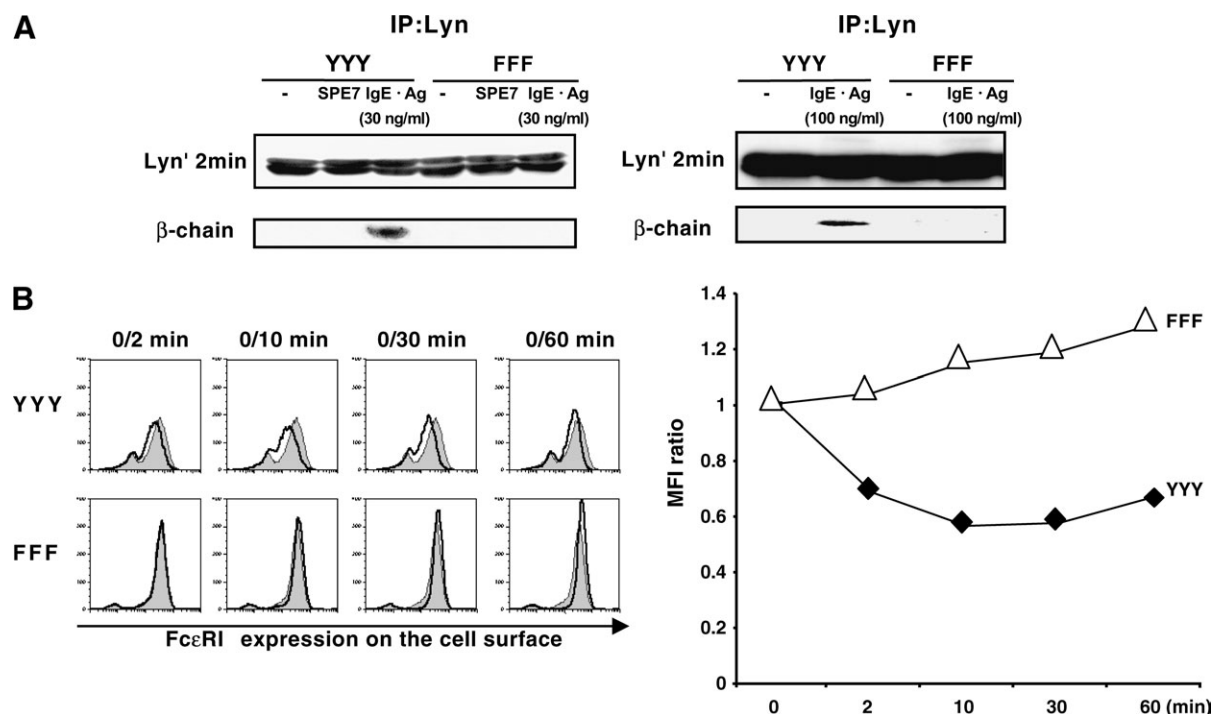


Fig. 3. Effects of the β -chain ITAM on Lyn recruitment to and internalization of the Fc ϵ RI. (A) Rapid recruitment of Lyn to the β -chain is undetectable in the monomeric IgE-activated YYY and FFF transfectants. Cells ($3 \times 10^7 \text{ ml}^{-1}$) were stimulated with 30 or 100 ng ml^{-1} of IgE $\cdot$ Ag or 10 $\mu\text{g ml}^{-1}$ of SPE7 for 2 min and lysed with the lysis buffer. After immunoprecipitation using anti-Lyn polyclonal antibody, the co-precipitated β -chain was detected by the anti- β -chain mAb (JRK). Results from one of the representatives of three independent experiments are displayed. (B) Cells ($5 \times 10^5 \text{ ml}^{-1}$) were stimulated with 10 $\mu\text{g ml}^{-1}$ of SPE7 at the indicated time points, and then the Fc ϵ RI expression on the cell surface was analyzed by flow cytometry (left panel). Histograms represent pre-stimulated cells (gray area) or SPE7-stimulated cells (white area). The receptor internalization was estimated by ratios of mean fluorescence intensities (right panel).

through enhanced PKC activation and prolongation of ERK1/2, JNK1/2 and Akt activation (28). Fig. 3(A and B) suggests that SHIP-1 phosphorylation would be reduced in the monomeric IgE-stimulated FFF mutant cells. We therefore investigated whether SHIP-1 is activated in the β -chain transfectants upon monomeric IgE stimulation. As shown in Fig. 6(D), SHIP-1 phosphorylation in response to the SPE7 was significantly reduced in the FFF mutant cells. In contrast to the FFF mutant cells, SHIP-1 was obviously phosphorylated at 20 min after the SPE7 stimulation in the YYY wild-type cells, indicating that SHIP-1 activation depends on function of the β -chain ITAM. Based on the previous report using SHIP-1 $^{-/-}$ mice, we speculated that activation of PKCs might be characteristically regulated in the FFF mutant cells. To examine this possibility, we employed anti-phospho PKC (pan) antibody, which detects PKC α , β I, β II, δ , ϵ , η and θ isoforms when homologous regions of serine 660 of PKC β II are phosphorylated. Fig. 6(E) demonstrates that PKC activation in the monomeric IgE-stimulated FFF mutant cells was significantly delayed and reduced, suggesting that the kinetics of PKC activation may correlate with *IL-6/IL-13* gene expression.

Discussion

Mast cells respond not only to IgE $\cdot$ Ag but also to some highly cytokinergic IgEs such as SPE7 and H1- ϵ -26 and exert various cellular reactions. However, mechanisms of signal transduction in response to monomeric IgEs are largely unknown.

In this study, we found that upon monomeric IgE stimulation, loss of function of canonical and non-canonical tyrosine residues in the β -chain ITAM leads to impairment of immediate phase responses such as β -hexosaminidase release, intracellular Ca^{2+} mobilization and LTC $_4$ secretion, and delay of gene expression for inflammatory cytokines, ERK1/2 and PKCs phosphorylation. These results suggest that the β -chain ITAM regulates kinetics of gene transcriptions related to cytokine production through modulation of intracellular signaling pathways, although these signalings based on the β -chain ITAM are not sufficient for chemical mediator release of mast cells.

Elevation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is known as crucial for chemical mediator release (degranulation) of mast cells. Recently, it was reported that intracellular Ca^{2+} mobilization in the Lyn $^{-/-}$ mast cells was significantly reduced upon monomeric IgE stimulation (15), suggesting that Lyn plays an important role for monomeric IgE-mediated Ca^{2+} responses in mast cells. As shown in Fig. 2(B), $[\text{Ca}^{2+}]_i$ increase of the FFF mutant cells in response to monomeric IgE was also significantly reduced, and the pattern of $[\text{Ca}^{2+}]_i$ elevation is highly similar to that of Lyn $^{-/-}$ mast cells, suggesting that in the FFF mutant cells Lyn-mediated signaling pathways, which generates $[\text{Ca}^{2+}]_i$ increase upon monomeric IgE stimulation, cannot be activated. In agreement with this hypothesis, in the monomeric IgE-stimulated FFF mutant cells, Lyn-mediated cell responses such as Fc ϵ RI internalization and SHIP-1 phosphorylation were severely

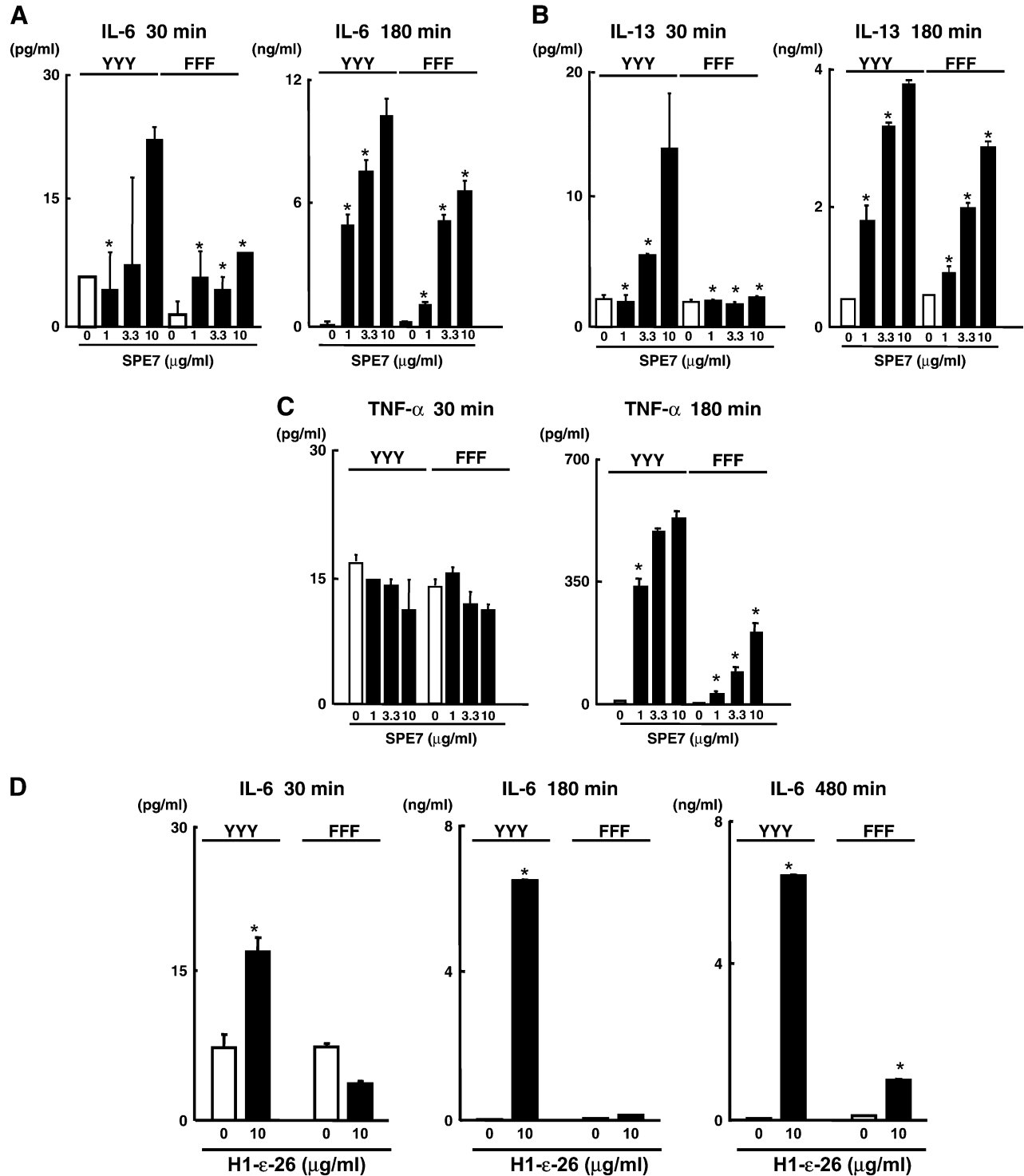


Fig. 4. Role of the β -chain ITAM in monomeric IgE-mediated cytokine synthesis. Cells ($5 \times 10^5 \text{ ml}^{-1}$) were stimulated with SPE7 at indicated concentrations for 30 and 180 min. After incubation, the supernatants were evaluated for IL-6 (A), IL-13 (B) and TNF- α (C) by specific ELISA. Statistical analyses were performed by Student's *t*-test. * $P < 0.05$ relative to maximum release of the YYY wild-type transfectants. (D) Cells were stimulated with $10 \mu\text{g ml}^{-1}$ of H1- ϵ -26 for 30, 180 and 480 min. After incubation, IL-6 production was measured by specific ELISA. Statistical analyses were performed by Student's *t*-test. * $P < 0.05$ relative to IL-6 production of each non-stimulated transfectant.

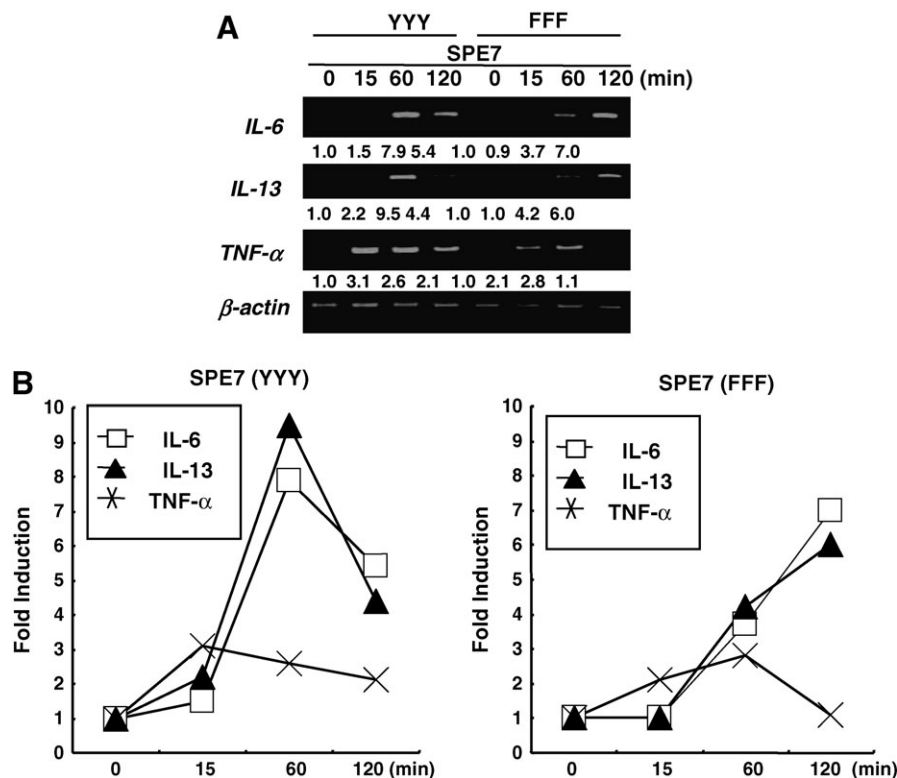


Fig. 5. Expression of cytokine genes is delayed in the monomeric IgE-activated FFF mutant cells. Cells ($1 \times 10^6 \text{ ml}^{-1}$) were stimulated with $10 \mu\text{g ml}^{-1}$ of SPE7 and then harvested at the indicated time points. (A) Total cellular RNA was analyzed for transcription levels of *IL-6*, *IL-13* and *TNF-α* genes by reverse transcription-PCR. The housekeeping gene encoding *β-actin* was employed as internal control. Data were quantified employing NIH Image software, and the fold induction was calculated by the relative amount of cytokine cDNA to *β-actin* cDNA. (B) Graphs of densitometric analyses are demonstrated.

impaired. In addition to these findings, recruitment of Lyn to the β -chain was actually undetectable upon the monomeric IgE stimulation. Collectively, these results indicate that the FFF mutant cells lack the signaling which evokes $[\text{Ca}^{2+}]_i$ increase leading to the release of inflammatory mediators in mast cells, which reveals signal regulatory roles of the β -chain ITAM for degranulation.

In this study, it is of note that monomeric IgE-stimulated FFF mutant cells can produce substantial amount of inflammatory cytokines although their intracellular signaling is deficient for chemical mediator release, which suggests that the β -chain ITAM may act as a signal amplifier (for chemical mediator release) or a signal suppressor (for cytokine production) in mast cell activation by monomeric IgE stimulation. Regarding cytokine production induced by monomeric IgE stimulation, one of the crucial roles of the β -chain ITAM is regulation of the kinetics for cytokine gene expression. As shown in Fig. 5(A), in the monomeric IgE-stimulated FFF mutant cells, transcription of *IL-6* and *IL-13* genes was obviously delayed and their production was considerably suppressed by wortmannin, which inhibits PI3K pathway including Akt (Fig. 6A). However, inhibitory effects of wortmannin were not correlated with Akt activities in mast cells. Since wortmannin broadly inhibits class I PI3K, class II PI3K-C2 α , 2 β and 2 γ , class III PI3K and class IV PI3K activity (32), even if this compound affects a PI3K activity in the process of cytokine synthesis, it will be difficult to dissect the involvement of individual PI3K members. In addition, the

roles of individual PI3K isoforms in Akt activation are largely unknown, although Akt is one of the downstream molecules of PI3K cascades. These findings may suggest that cytokine production of the monomeric IgE-stimulated FFF mutant cells may be regulated by other PI3K signalings, except PI3K-Akt pathway. Alternatively, this discrepancy might be explained by other pharmacological actions of wortmannin such as suppression of translational process through inhibition of p70 S6 kinase activation (33, 34). Although we cannot exclude the possibility that wortmannin may affect protein synthesis of *IL-6* and/or *IL-13*, it is clear that Akt activation does not contribute to at least kinetics of *IL-6/IL-13* gene expression in the monomeric IgE-stimulated FFF mutant cells. We conclude that ERK1/2 and/or PKCs are candidates involved in common pathways for *IL-6/IL-13* gene transcription because phosphorylation of these kinases was specifically delayed in the FFF mutant cells (Fig. 6F). Since induction of β -hexosaminidase release and *IL-6* production showed almost the same kinetics in the YYY wild-type and the FFF mutant cells upon monomeric IgE stimulation (in preparation to be submitted elsewhere), we speculate that delayed mast cell activation may be caused by abrogation of phosphorylation on non-canonical and canonical tyrosine residues in the β -chain ITAM. Currently, the signaling pathways of the mutated β -chain ITAM to these kinases are not clarified. However, previous studies revealed that inflammatory cytokine production in response to the monomeric IgE entirely depends on Fc ϵ RI γ -chain (13) or Syk

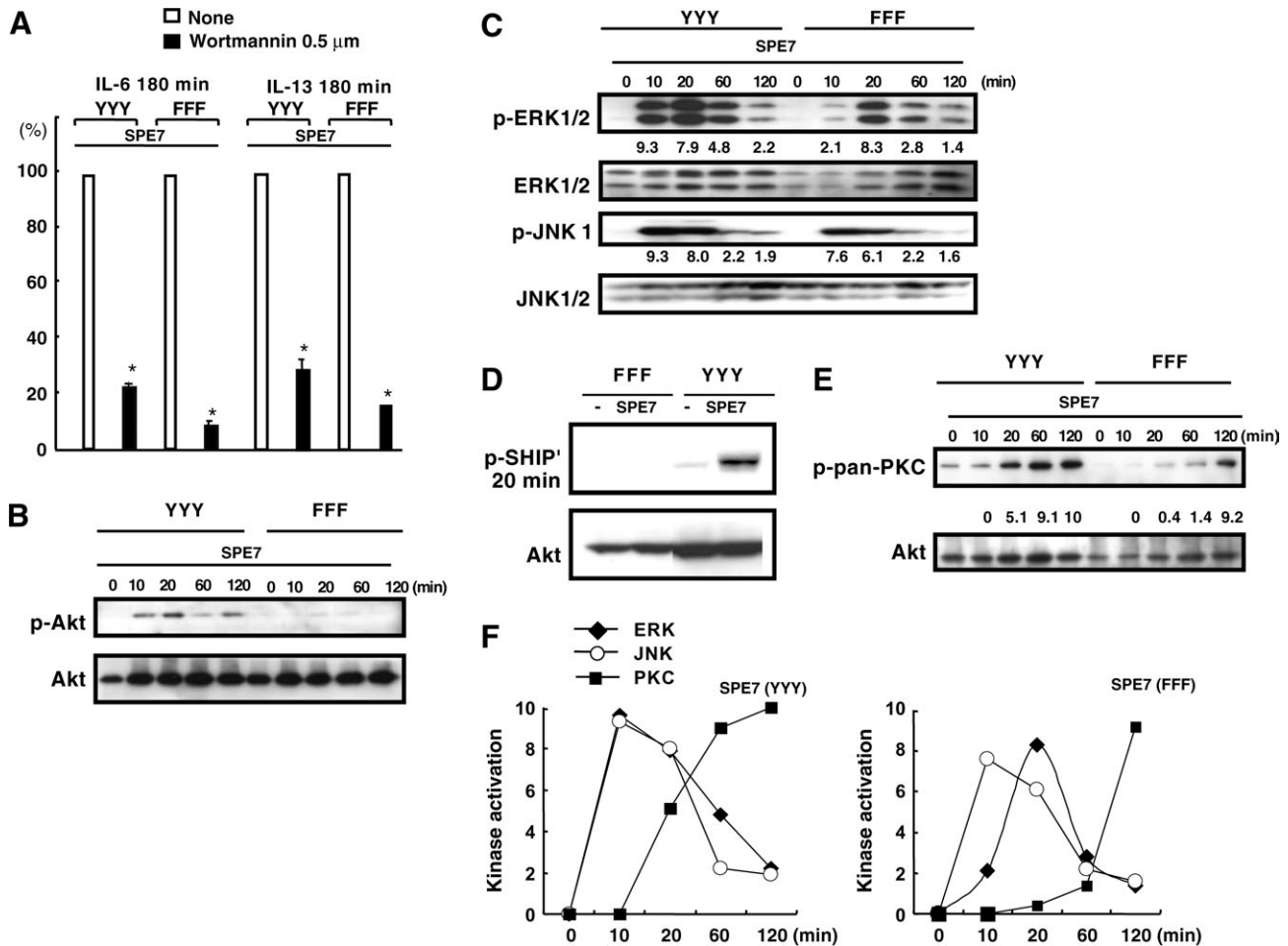


Fig. 6. Effects of the β -chain ITAM on signal transduction pathways of Fc ϵ RI. (A) Wortmannin significantly inhibited IL-6 and IL-13 production. Cells ($5 \times 10^5 \text{ ml}^{-1}$) were stimulated with $10 \mu\text{g ml}^{-1}$ of SPE7 with/without pre-treatment of 0.5 μM wortmannin. After 180 min, the supernatants were collected and evaluated for IL-6 and IL-13 production by specific ELISA. Cytokine production was expressed as percentage of the maximal production of each non-treated transfectant. Data shown are the mean $\pm$ SD of triplicate samples from two independent experiments. Statistical analyses were performed by Student's *t*-test. **P* < 0.01 relative to maximum production of each non-treated transfectant. Cells ($1 \times 10^6 \text{ ml}^{-1}$) were stimulated with $10 \mu\text{g ml}^{-1}$ of SPE7 for indicated time periods and lysed. Whole proteins were analyzed by immunoblotting with phospho-specific antibodies to Akt (B), ERK1/2 and JNK1/2 (C), SHIP-1 (D) and pan-PKC (E). ERK1/2, JNK1/2 or Akt (each bottom panel) were used as loading controls of protein. One of representative of three independent experiments is demonstrated. (F) Graphs for densitometric analyses of ERK, JNK and PKCs activation are displayed.

activation (13, 17), which are downstream events of the β -chain ITAM. Thus, β -chain ITAM is likely to affect kinetics and/or strength of these proximal signaling pathways.

For future study, we are focusing on how kinetics of signaling pathways are regulated through the function of β -chain ITAM, and will examine changes of co-localization of proximal signaling molecules and the β -chain in the lipid raft of the transfectants in response to the monomeric IgEs. We believe that our findings in this study will give useful informations for elucidation of Fc ϵ RI signaling.

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Abbreviations

BMMC	bone marrow-derived mast cell
DNP	Anti-dinitrophenyl
ERK	extracellular signal-regulated kinase
Fc ϵ RI	high-affinity receptor for IgE
IgE•Ag	IgE•Ag complexes
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
LT	leukotriene
MAPK	mitogen-activated protein kinase
NIH	National Institutes of Health
PI3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
PLAT-E	Platinum-E
SCF	stem cell factor

SHIP SH2 domain containing inositol 5'-phosphatase
 TNF tumor necrosis factor
 TNP trinitrophenol

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