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FcγRIIB Ile232Thr transmembrane polymorphism associated with human systemic lupus erythematosus decreases affinity to lipid rafts and attenuates inhibitory effects on B cell receptor signaling

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The B cell inhibitory receptor FcγRIIB plays crucial roles in the maintenance of self-tolerance. We have identified a polymorphism *FCGR2B* c.695T>C that results in the non-conservative replacement of 232Ile at the transmembrane helix to Thr and demonstrated the association of the polymorphism with susceptibility to systemic lupus erythematosus (SLE) in Asians. In this study, we examined the impact of *FCGR2B* c.695T>C on the functional properties of FcγRIIB by expressing each allele product in a human B cell line ST486 lacking endogenous FcγRIIB. FcγRIIB 232Thr was found to be significantly less potent than wild-type 232Ile in inhibiting B cell receptor (BCR)-mediated phosphatidylinositol-3,4,5-trisphosphate accumulation, Akt and PLCγ2 activation and calcium mobilization, and to display decreased levels of tyrosine phosphorylation and SH2-containing 5′-inositolphosphate phosphatase recruitment compared with 232Ile after IgG Fc-mediated coligation with BCR. Notably, a quantitative analysis of the subcellular distribution of FcγRIIB using ^{125}I-labeled anti-FcγRIIB revealed that FcγRIIB 232Thr is less effectively distributed to detergent-insoluble lipid rafts than 232Ile, findings in accordance with the importance of the transmembrane amino acid residues, in particular large hydrophobic amino acids including Ile, in the association of membrane proteins with lipid rafts. Given the crucial roles of lipid rafts in integrating BCR signaling, decreased association of FcγRIIB 232Thr could contribute to its impaired inhibitory potential. Collectively, the present findings indicate that the Ile232Thr substitution affects the localization and function of FcγRIIB and that the molecular mechanism may link the polymorphism and susceptibility to SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by autoantibody production, immune complex deposition and multiple organ damage. Although the etiology of SLE is still unknown, both genetic and environmental factors are involved in the pathogenesis of SLE. In humans, genetic association is known at various loci including those coding for human leukocytes antigens (HLAs), complements and low-affinity Fcγ receptors (FcγRs) (1–3). The loci for the highly homologous human FcγRs are clustered on chromosomes 1q21–24, and genome-wide linkage

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studies indicated that this area is one of the strongest candidate chromosomal regions for SLE (4,5). FcγRs are expressed in various hematopoietic cells and play crucial roles in the regulation of the immune system through the IgG immune complex-mediated signal transduction and immune complex clearance (6–8).

Among the classical FcγRs, FcγRIIB is unique in that it possesses an immunoreceptor tyrosine-based inhibitory motif (ITIM), a consensus amino acid sequence present in the cytoplasmic domain of the immune inhibitory receptor family (7). Studies in mice demonstrated that FcγRIIB plays crucial roles in preventing excessive immune reactions and autoimmunity *in vivo*. For example, FcγRIIB knockout mice display an enhanced humoral immunity (9) and spontaneously develop immune complex-mediated glomerulonephritis in a strain-specific manner (10). The molecular mechanisms underlying FcγRIIB-mediated immunosuppression have been precisely investigated. In humans, two FcγRIIB isoforms, referred to as FcγRIIB1 and FcγRIIB2, are generated by alternative splicing. The long FcγRIIB1 isoform is preferentially expressed in B cells and the short FcγRIIB2 isoform lacking the sequence encoded by the first cytoplasmic exon in cells of myeloid lineage (6). When the B cell receptor (BCR) is coligated with FcγRIIB with IgG immune complexes containing relevant antigens, the ITIM tyrosine is phosphorylated by an Src family kinase, Lyn, and recruits SH2-containing 5′-inositolphosphate phosphatase (SHIP) (11). SHIP hydrolyzes phosphatidylinositol-3,4,5-trisphosphate (PIP3) and finally attenuates the activation and survival signals in B cells (12,13).

Besides the biochemical features of the positive and negative regulations of BCR signaling, there emerge the spatio-temporal aspects of the signaling that involve a class of membrane domains, referred to as lipid rafts (14,15). Lipid rafts are enriched with glycosphingolipid and cholesterol, and several classes of upstream signal transducers including Src family kinases concentrated in lipid rafts (16–18). Upon B cell stimulation, lipid rafts promptly build up spatially compartmentalized signaling clusters, to which various signaling molecules including BCR and FcγRIIB (19,20), adaptor proteins and downstream kinases and phosphatases are recruited (18). Others and we showed that mutated BCR or Src family kinases segregated from lipid rafts can no longer transduce appropriate signaling (17,19), suggesting that molecular assembly at lipid rafts is not merely coincidental, but important for signal transduction.

Recently, we have found a non-synonymous polymorphism *FCGR2B* c.695T>C which alters 232Ile of FcγRIIB in the middle of the transmembrane helix to Thr. We detected a significant association of the polymorphism with SLE in Asian populations (21–23) and showed that this association was not caused by linkage disequilibrium with known *FCGR2A* or *FCGR3B* polymorphisms. Therefore, the FcγRIIB Ile232Thr polymorphism likely constitutes a risk factor for SLE in Asians. In this study, we examined the functional consequence of the Ile232Thr substitution and found that the amino acid substitution impairs FcγRIIB association with lipid rafts and attenuates the inhibitory effects of FcγRIIB on BCR signaling.

RESULTS

FcγRIIB Ile232Thr substitution does not alter ligand-binding affinity

As the primary structure of FcγRIIB shows only a modest homologue among species [for instance, amino acid identity between human FcγRIIB1 and the mouse homologue (24) is ~60%], we considered that the functional consequence resulting from the minimal structural alteration in FcγRIIB Ile232Thr should be examined in the context of human cells. In this study, we chose to compare the properties of FcγRIIB1 variants by expressing each of them in a human B-cell line, ST486, that is inherently lacking endogenous FcγRIIB (25). We generated multiple ST486 clones expressing human FcγRIIB1 232Ile (hereafter referred to as h-232Ile) or FcγRIIB1 232Thr (h-232Thr) (Fig. 1A) and selected four pairs of the clones expressing equivalent levels of FcγRIIB1 variants (Fig. 1B).

We first conducted ligand-binding study using ^{125}I-labeled human IgG, but the attempt was unsuccessful due to a high non-specific binding activity (data not shown). We thus developed whole cell binding assay using FITC-labeled human IgG (fluorescein/IgG molar ratio = 3.1, Sigma) as a ligand, and the specific binding activity was successfully measured by flow cytometer as described in Materials and Methods. The mean fluorescent intensity (MFI) of bound FITC-labeled human IgG was measured and the absolute ligand number was determined from the regression line obtained by the simultaneous analysis of MFIs of quantum fluorescence microbeads (Sigma), which were labeled with known numbers of FITC molecules. Data obtained from the competitive fluorescent ligand-binding experiments were analyzed using Scatchard's plot and were fitted by linear regression analysis (Fig. 1C). Dissociation constants (K_d) for h-232Ile and h-232Thr were $6.5 \pm 1.5 \times 10^{-7}$ and $5.7 \pm 1.0 \times 10^{-7}$ M, respectively, which were not significantly different, indicating that Ile232Thr substitution does not significantly affect ligand-binding affinity.

Ile232Thr substitution decreases association of chimeric FcγRIIB with lipid rafts

Previous studies have shown the importance of transmembrane domain of membrane proteins for their association with lipid rafts (19,26,27), suggesting the possibility that Ile232Thr substitution results in altered subcellular distribution of FcγRIIB. We attempted to examine this hypothesis by using density gradient centrifugation assay (27,28). For the quantitative assessment of FcγRIIB redistribution, we first tried to prepare ^{125}I-labeled anti-human FcγRIIB Fab fragment, because the use of whole IgG as a probe potentially leads to protein polymerization and misinterpretation of its localization (29). However, it was unsuccessful to prepare Fab fragment with a sufficiently high affinity for the assay from available anti-human CD32 monoclonal Abs (mAbs), FLI8.26 and AT-10 (data not shown). As an alternative approach, we prepared chimeric human FcγRIIB1 232Ile and FcγRIIB1 232Thr whose extracellular domains were replaced with that of mouse FcγRIIB (referred to as c-232Ile and c-232Thr, respectively; Fig. 1A), because we

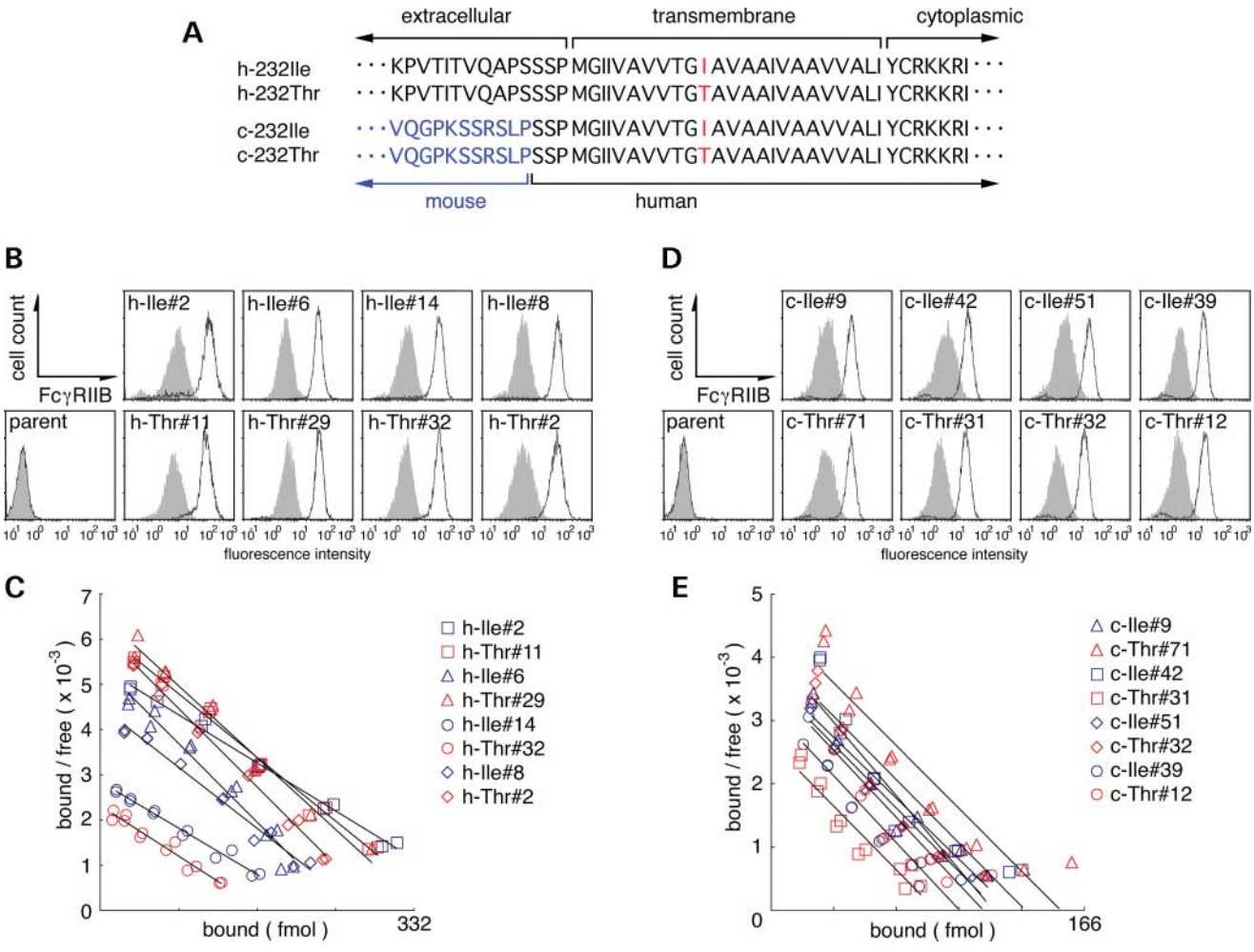

Figure 1. Ile232Thr substitution does not affect ligand affinity. (**A**) Membrane-spanning segments of human full-length FcγRIIB232Ile and 232Thr (designated as h-232Ile and h-232Thr) and mouse–human chimera FcγRIIB232Ile and 232Thr (c-232Ile and c-232Thr). Polymorphic 232Ile and 232Thr are shown in red. The transmembrane domain was determined using the SOSUI program. (**B**) Flow cytometry of parent cells and four clone pairs expressing equivalent expression levels of h-232Ile (denoted as h-Ile) or h-232Thr (h-Thr). Open peak: FITC-conjugated anti-human FcγRIIB mAb (FLI8.26); shaded peak: isotype control. (**C**) Scatchard analysis of human IgG binding to h-232Ile (h-Ile) or h-232Thr (h-Thr). Results from experiments in the four clone pairs are presented. (**D**) Flow cytometry of parent cells and four clone pairs expressing equivalent expression levels of c-232Ile (denoted as c-Ile) or c-232Thr (c-Thr). Open peak: FITC-conjugated anti-murine FcγRIIB mAb (2.4G2); shaded peak: isotype control. (**E**) Scatchard analysis of human IgG binding to c-232Ile (c-Ile) or c-232Thr (c-Thr). Results from experiments in the four clone pairs are presented.

have successfully employed Fab prepared from 2.4G2 anti-mouse FcγRII/III mAb for the density gradient centrifugation assay (27). We established multiple ST486 clones expressing c-232Ile or c-232Thr, selected four clone pairs with equivalent expression levels (Fig. 1D), and the clones were subjected to the fluorescent ligand-binding assay as described above. The K_d values for c-232Ile and c-232Thr were $3.0 \pm 0.24 \times 10^{-7}$ and $2.8 \pm 0.17 \times 10^{-7}$ M, respectively, and there was again no significant difference between the K_d values.

To analyze the association of FcγRIIB variants with lipid rafts, c-232Ile and c-232Thr were probed with ^{125}I-2.4G2 Fab and BCR with DA4-4 anti-human IgM mAbs, and these Abs were left untreated or treated with a secondary Ab to induce BCR-FcγRIIB coligation. Cells were solubilized with Brji58 and subjected to density gradient centrifugation assay as described previously (27,28). Figure 2A shows a representative profile of ^{125}I-2.4G2 Fab distribution. Fractions 8–10, which reproducibly contain the doubly palmitoylated Csk-binding protein (Cbp) (30), were identified as lipid raft fractions. Limited amounts of c-232Ile and c-232Thr were recovered from lipid rafts under resting conditions and BCR-FcγRIIB coligation noticeably induced their redistribution to lipid rafts. The amount of c-232Thr recovered from lipid rafts was consistently smaller than that of c-232Ile both under resting conditions and after BCR-FcγRIIB coligation in all the three clone pairs tested and the differences were statistically significant (Fig. 2B). These findings indicate that Ile232Thr substitution decreased the association of FcγRIIB with lipid rafts under resting conditions and after the coligation with BCR.

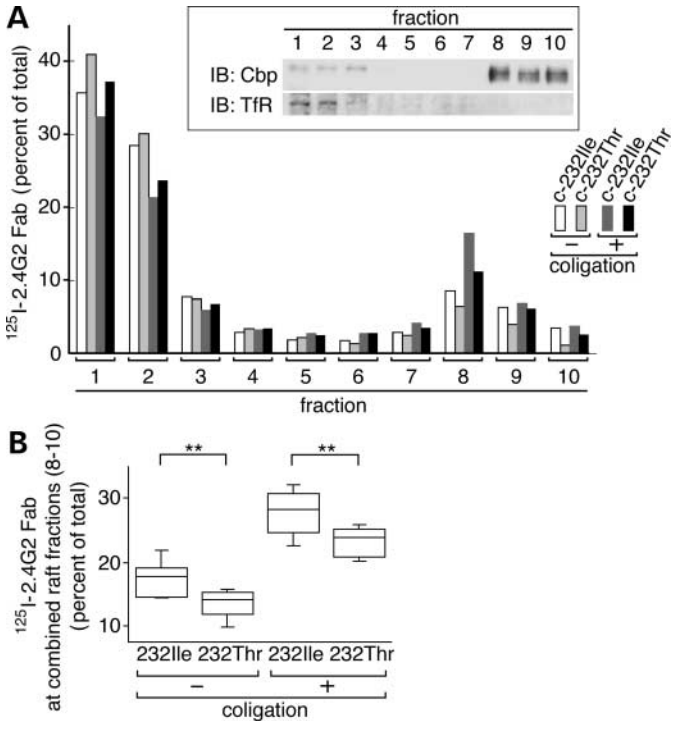

Figure 2. Ile232Thr substitution inhibits the association of FcγRIIB with lipid rafts. (**A**) Association of c-232Ile and c-232Thr with lipid rafts. c-232Ile and c-232Thr were probed with ^{125}I-2.4G2 Fab, cells were left unstimulated (coligation −) or stimulated by BCR-FcγRIIB coligation (coligation +), and subjected to the sucrose density gradient centrifugation assay as described in Materials and Methods. The ^{125}I-radioactivity of each fraction (numbered from the bottom (1) to the top (10)) was measured, and normalized by the total radioactivity of the corresponding gradient. The graph shows representative ^{125}I-2.4G2 Fab distributions in a pair of c-232Ile- and c-232Thr-expressing cells. The fractions were also subjected to immunoblotting (IB) with anti-Cbp and anti-transferrin receptor (TfR) Abs to determine the lipid rafts and non-raft fractions, respectively (upper inset). (**B**) ^{125}I-2.4G2 Fab recovered from lipid rafts (fractions 8–10) before (coligation −) and after BCR-FcγRIIB coligation (coligation +) were measured in two independent experiments using three different clone pairs ($n = 6$, $^{**}P < 0.01$). Results are represented by boxplots showing the minimum, 25th percentile, median, 75th percentile and maximum values.

BCR-mediated calcium mobilization was less inhibited by c-232Thr than by c-232Ile

Next we compared FcγRIIB variants for their impact on key events in the BCR-mediated signal transduction pathway. BCR-mediated calcium mobilization was first examined in the ST486 clones expressing c-232Ile or c-232Thr. BCR self-ligation and BCR-FcγRIIB coligation were performed by the stimulation of cells with anti-human IgM $F(ab')_2$ and anti-human IgM whole IgG (Jackson Immunoresearch), respectively. To exclude the contamination of $F(ab')_2$ with whole IgG, purity of the Abs was routinely checked before use by gel electrophoresis. Calcium mobilization in the presence of extracellular calcium (1.8 mM) was measured as described previously (27). We used suboptimal concentrations of whole IgG and $F(ab')_2$ [8 μg/ml whole IgG and 13 μg/ml $F(ab')_2$] that induce comparable calcium mobilization in parent ST486 cells.

One of the evident findings was that the magnitudes of calcium mobilization were appreciably smaller in c-232Ile-expressing cells than in parent cells and c-232Thr-expressing cells even when BCR was ligated separately from FcγRIIB (Fig. 3A and B), raising the possibility that c-232Ile and c-232Thr exert constitutive, or indirect, inhibition to different degrees even without coligation with BCR at least under the experimental conditions. The differential constitutive inhibition was not due to the chimeric constructs, because such effect was reproduced in the experiments using full-length human FcγRIIB variants (Fig. 6). In view of the difference in the magnitude of the constitutive inhibitions, we decided to evaluate the inhibitory potentials of FcγRIIB variants by comparing the net magnitudes of calcium mobilization both under BCR self-ligation and BCR-FcγRIIB coligation conditions, but not by the decrease in the magnitude of calcium mobilization by FcγRIIB coligation. Traces in Figure 3A show the average magnitude of calcium mobilization from 16 experiments using four clone pairs. Cells expressing c-232Thr reproducibly displayed a higher magnitude of calcium mobilization than cells expressing c-232Ile both after BCR self-ligation and after BCR-FcγRIIB coligation, and the differences at the peak (120 s after the addition of Abs) and at the sustained phase (400 s) were statistically significant (Fig. 3B). These observations indicate that BCR-mediated calcium mobilization is less inhibited by c-232Thr than by c-232Ile.

Cells expressing c-232Thr displayed greater magnitudes of BCR-mediated biochemical signaling than those expressing c-232Ile

BCR-mediated tyrosine phosphorylation of FcγRIIB and SHIP recruitment to FcγRIIB are the hallmarks of FcγRIIB-mediated signal attenuation and these events are closely related to FcγRIIB redistribution to lipid rafts in the vicinity of Lyn. To compare the influence of Ile232Thr substitution on these events, cells were stimulated via BCR, solubilized with *n*-octyl-β-D-glucoside, and c-232Ile and c-232Thr were subjected to immunoprecipitation with 2.4G2 and a secondary Ab, followed by immunoblotting with anti-phosphotyrosine mAb and anti-SHIP Ab. As shown in a representative immunoblot (Fig. 4A), tyrosine phosphorylation of c-232Ile and c-232Thr was marginally detectable under resting conditions, the magnitudes were noticeably increased to comparable levels by BCR self-ligation and substantially enhanced after BCR-FcγRIIB coligation with clear decrease in that of c-232Thr relative to c-232Ile. The association of SHIP with c-232Ile and c-232Thr was found to be detectable even under resting and BCR self-ligation conditions as well as after BCR-FcγRIIB coligation, and the amount of SHIP associated with c-232Thr was noticeably smaller than that associated with c-232Ile after BCR-FcγRIIB coligation, which is in good agreement with the inefficient c-232Thr tyrosine phosphorylation compared with c-232Ile under the coligation conditions. The coligation-mediated tyrosine phosphorylation of FcγRIIB and SHIP recruitment to FcγRIIB were abrogated by the pretreatment of cells with 2.4G2, showing the specificity of BCR-FcγRIIB coligation-mediated effects. These results were reproducible in three different pairs

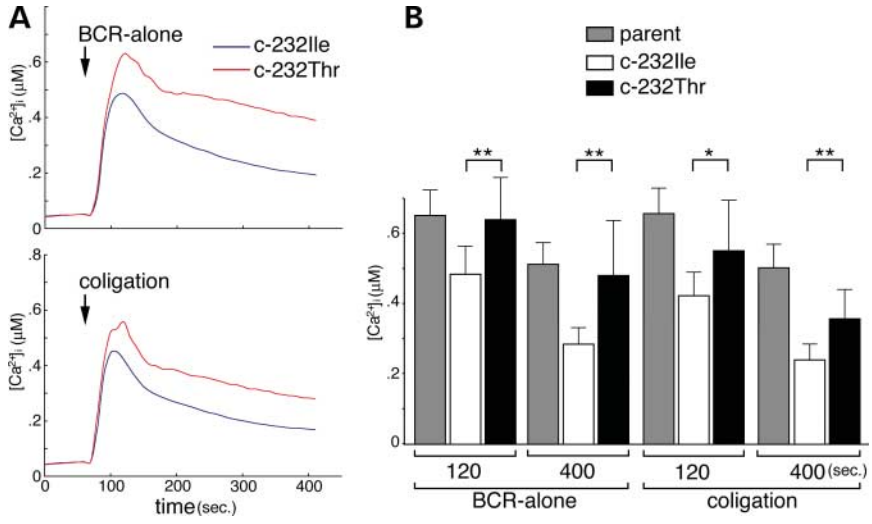

Figure 3. BCR-mediated calcium mobilization was less inhibited by c-232Thr than by c-232Ile. (**A**) Time-dependent changes in $[Ca^{2+}]_i$ in c-232Ile- and c-232Thr-expressing cells stimulated with anti-IgM $F(ab')_2$ (BCR-alone, upper panel) or with whole IgG (coligation, lower panel). The pair of traces at each panel represents the average of four independent experiments of the four different clones pairs ($n = 16$). (**B**) $[Ca^{2+}]_i$ values at the peak (120 s after the stimulation) and plateau (400 s) phases in six experiments of parent cells ($n = 6$), and four experiments using the four different clone pairs expressing c-232Ile ($n = 16$) or c-232Thr ($n = 16$). To avoid complexity, statistical comparison between the data from the c-232Ile- and c-232Thr-expressing cells is presented (mean $\pm$ SD, $^{**}P < 0.01$, $^{*}P < 0.05$).

of c-232Ile- and c-232Thr-expressing ST486 clones. These findings strongly suggest that Ile232Thr substitution decreases FcγRIIB tyrosine phosphorylation level and attenuates SHIP association with FcγRIIB after BCR-FcγRIIB coligation.

FcγRIIB coligation with BCR attenuates BCR-mediated CD19 tyrosine phosphorylation which leads to impaired intracellular calcium mobilization (31). To compare the effects of c-232Ile and c-232Thr on this event, cells were stimulated via BCR, solubilized with 1% NP-40 and 0.5% deoxycholate, and CD19 was immunoprecipitated and subjected to immunoblotting with anti-phosphotyrosine mAbs. As shown in Figure 4B, BCR self-ligation augmented CD19 tyrosine phosphorylation, and FcγRIIB-BCR coligation decreased the magnitude of the phosphorylation both in c-232Ile and c-232Thr cells. The pretreatment of cells with 2.4G2 mAbs prevented the dephosphorylation of CD19, confirming the roles of FcγRIIB in the dephosphorylation of CD19. Notably, the magnitudes of CD19 tyrosine phosphorylation was reproducibly greater in c-232Thr-expressing cells than those in c-232Ile-expressing cells both after BCR self-ligation and BCR-FcγRIIB. These results were reproducible in three independent experiments.

We next compared the effects of c-232Ile and c-232Thr on BCR-mediated early biochemical events by focusing on the signaling induced by BCR-FcγRIIB coligation, because the attenuated inhibitory effect of c-232Thr compared with that of c-232Ile on BCR-mediated calcium mobilization was consistently observed regardless of the BCR ligation conditions (Fig. 3). The most upstream event examined was time-dependent changes in PIP3 accumulation. Cells were labeled with ^{32}P-phosphorus, ^{32}P-labeled phospholipids were extracted and the changes in the distribution of radioactivity among phospholipids were analyzed by thin-layer chromatography. As shown in Figure 4C, PIP3 levels in c-232Thr-expressing cells were found to be significantly higher than those in c-232Ile-expressing cells under resting conditions and at an early period after BCR-FcγRIIB coligation (0.5 min). The activations of Akt and PLCγ2, which are in part dependent on PIP3 levels and the determinants of B cell survival and calcium signaling, respectively (32), were next examined by immunoblotting using phosphorylation site-specific Abs. As shown in Figure 4D, the magnitudes of Akt and PLCγ2 activations induced by BCR-FcγRIIB coligation were reproducibly greater in c-232Thr-expressing cells than those in c-232Ile-expressing cells. To further examine the impact of c-232Ile and c-232Thr on the signaling at lipid rafts, we prepared combined raft and non-raft fractions before and 4 min after BCR-FcγRIIB coligation as indicated in Figure 2, and PLCγ2 activation was assessed in the separated fractions using phosphorylation site-specific Abs. As shown in Figure 4E, the magnitude of PLCγ2 activation at lipid rafts was greater in c-232Thr-expressing cells than that in c-232Ile-expressing cells, thereby suggesting that the augmented signaling at lipid rafts in c-232Thr cells likely explain the net increase in PLCγ2 activation in the whole cell lysates (Fig. 4D) as well as enhanced calcium mobilization in c-232Thr cells (Fig. 3).

Ile232Thr substitution-mediated functional alterations of FcγRIIB in the context of full-length human FcγRIIB constructs

Because it is theoretically possible that the functional differences between c-232Ile and c-232Thr are dependent on the chimeric structure, we attempted to examine the effects of Ile232Thr substitution on the functions of human full-length FcγRIIB1. To this end, we prepared a recombinant retrovirus encoding h-232Ile or h-232Thr to avoid possible clonal variations. ST486 cells were infected with the viruses, and the cell populations were subjected to selection with

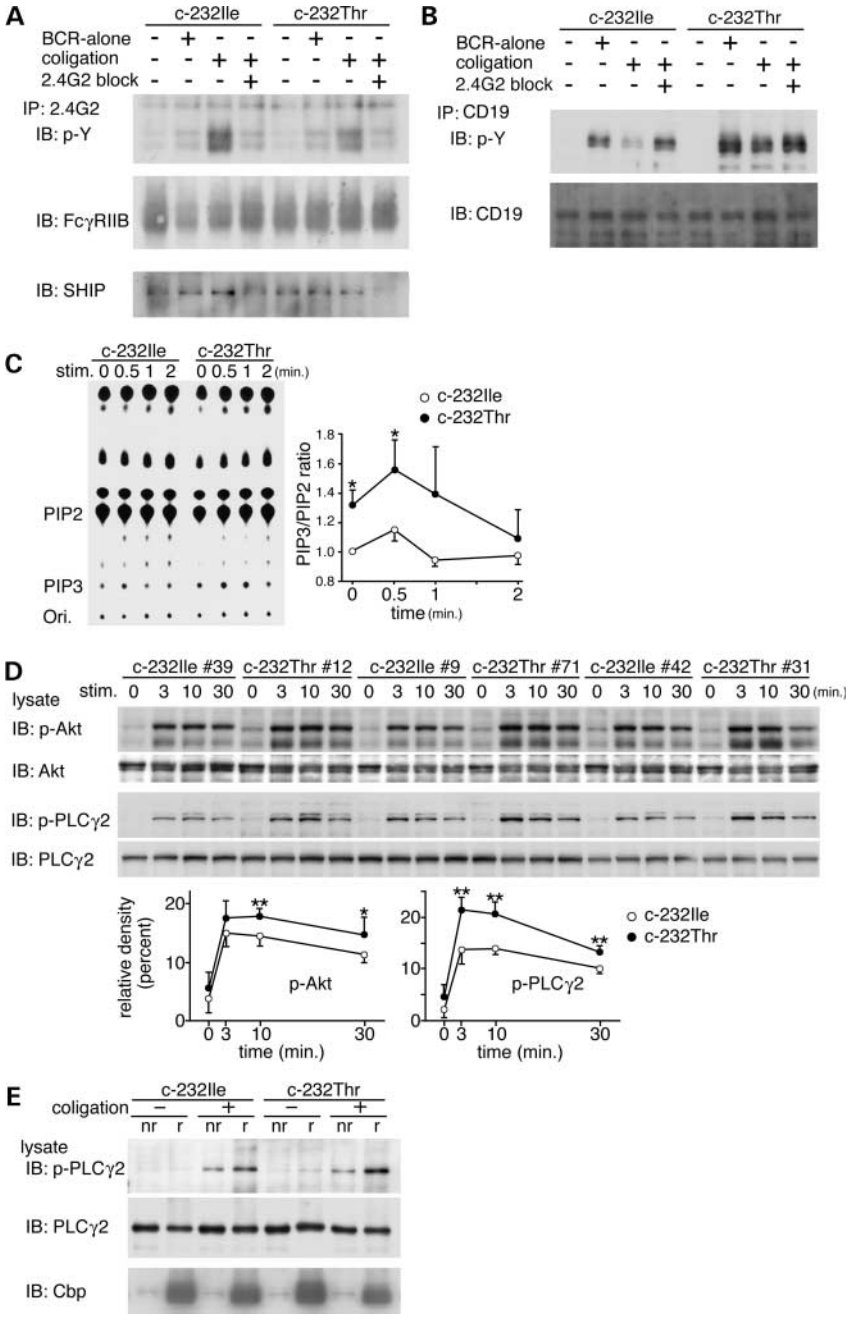

Figure 4. Ile232Thr substitution impairs FcγRIIB tyrosine phosphorylation and SHIP recruitment, and ability of FcγRIIB to inhibit BCR-mediated biochemical signaling. (**A**) c-232Ile and c-232Thr tyrosine phosphorylation, and SHIP recruitment to FcγRIIB variants. Cells were stimulated with anti-IgM $F(ab')_2$ (BCR alone) or with whole IgG (coligation) in the presence or absence of the pretreatment with blocking Abs (2.4G2 block) for 1 min at 37°C. c-232Ile and c-232Thr were immunoprecipitated with 2.4G2, and the immunoprecipitates were subjected to immunoblotting (IB) for the analysis of FcγRIIB tyrosine phosphorylation (p-Y) and for that of SHIP co-precipitation (SHIP). After stripping, the blotted membrane was reprobed with anti-FcγRIIB polyclonal Abs for loading control. (**B**) Tyrosine phosphorylation of CD19 in c-232Ile- and c-232Thr-expressing cells. Cells were stimulated and processed as described in (A) and CD19 was immunoprecipitated. The immunoprecipitates were divided into two halves, one was subjected to anti-phosphotyrosine immunoblotting (IB) and the other to anti-CD19 immunoblotting for loading control. (**C**) Time-dependent changes in PIP3 accumulation in c-232Ile and c-232Thr clones after BCR-FcγRIIB coligation. Phospholipids extracted from ^{32}P-labeled cells were analyzed by thin-layer chromatography followed by BAS2000 (Fuji Photo Film Co., Ltd) imaging. Changes in PIP3/PIP2 ratio (normalized by the basal value (time 0) in c-232Ile cells) in three different clone pairs are shown (mean ± SD, $n = 3$, $^{*}P < 0.05$). (**D**) Time-dependent changes in the phosphorylation of Akt Ser473 and PLCγ2 Tyr1217. c-232Ile and c-232Thr clones solubilized with *n*-octyl-β-D-glucoside were subjected to the immunoblotting with phosphorylation-site-specific Abs. Membranes were reprobed with Abs against cognate molecules for loading control. Graphs show changes in the density of phosphorylation signals in two independent experiments using three different clone pairs. Signal density at each time point is normalized by the total signal density in the respective c-232Ile and c-232Thr pairs (mean ± SD, $n = 6$, $^{**}P < 0.01$, $^{*}P < 0.05$). (**E**) PLCγ2 Tyr1217 phosphorylation at lipid rafts. c-232Ile and c-232Thr clones stimulated by BCR-FcγRIIB coligation were subjected to sucrose density gradient centrifugation assay and non-raft fractions (nr) and raft fractions (r) were subjected to immunoblotting with PLCγ2 phosphorylation-site-specific Abs. Cbp and PLCγ2 stainings were performed for the verification of raft fractions and for loading control, respectively. This blot is representative of two experiments using different clone pairs.

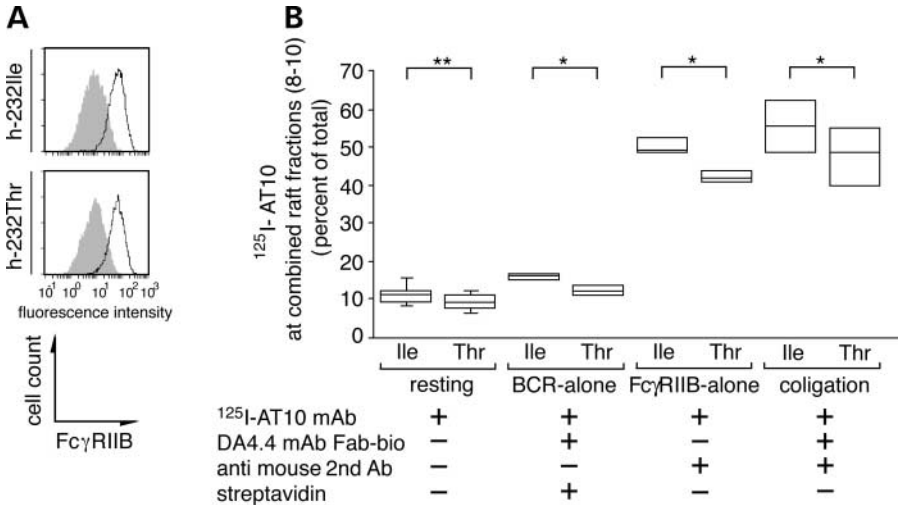

Figure 5. Decreased association of h-232Thr with lipid rafts compared with h-232Ile under various stimulation conditions. (**A**) Expression of human FcγRIIB transduced with retrovirus infection. Open peak: FITC-conjugated FcγRIIB mAbs (FLI8.26); shaded peak: isotype control. (**B**) Association of h-232Ile and h-232Thr with lipid rafts under various stimulation conditions. h-232Ile and h-232Thr were probed with ^{125}I-AT10, BCR with or without biotinylated DA4-4 Fab and the Abs were subjected to ligation with a secondary antibody or with streptavidin to achieve the respective ligation conditions as indicated. Radioactivity in raft fractions was analyzed and normalized as described in the legend for Figure 2. Data shown were obtained from more than four independent experiments ($n = 4–6$, $^{**}P < 0.01$, $^{*}P < 0.05$). Results are represented by boxplots showing the minimum, 25th percentile, median, 75th percentile and maximum values.

puromycin as described in Materials and Methods. As shown in Figure 5A, the levels of h-232Ile and h-232Thr expressions in the cell populations were equivalent, suggesting that the Ile232Thr substitution does not critically impair protein stability or the membrane sorting of FcγRIIB.

We first sought to compare the distributions of h-232Ile and h-232Thr to lipid rafts. Owing to the insufficient affinity of Fab fragments prepared from FLI8.26 and AT-10 for density gradient centrifugation assay (see above), we tested the use of ^{125}I-AT10 whole IgG as the probe of human FcγRIIB and found that ^{125}I-AT10 does not in itself induce such unacceptable levels of FcγRIIB redistribution to lipid rafts as previously noted in the case of using 2.4G2 as the probe of mouse FcγRIIB [(27) and data not shown]. We thus decided to use ^{125}I-AT10 as the probe and analyzed h-232Ile and h-232Thr distribution to lipid rafts under various stimulation conditions, including BCR self-ligation, FcγRIIB self-ligation and BCR-FcγRIIB coligation. To this end, h-232Ile and h-232Thr were probed with ^{125}I-AT10, and BCR with or without biotinylated DA4-4 Fab, and the Abs were ligated with a secondary Ab or with streptavidin, as indicated in Figure 5B. Cells were subjected to density gradient centrifugation assay, and ^{125}I radioactivities recovered from lipid raft and non-raft fractions were measured as described above (Fig. 2). As shown in Figure 5B, a small amount of FcγRIIB was associated with lipid rafts under resting conditions, and BCR self-ligation increased the association to ~1.5 times the basal level. FcγRIIB self-ligation and BCR-FcγRIIB coligation markedly enhanced FcγRIIB distribution to lipid raft fractions four to five times the basal level. The amount of h-232Thr associated with lipid rafts was reproducibly smaller than that of h-232Ile under resting conditions after BCR self-ligation, after FcγRIIB self-ligation and after BCR-FcγRIIB coligation. These results unequivocally showed that Ile232Thr substitution attenuates the association of human FcγRIIB with lipid rafts.

We next examined BCR-induced calcium mobilization. In this study, we frequently experienced difficulty in detecting IgG Fc-mediated inhibition by human and chimeric FcγRIIB (Fig. 3B), in agreement with a previous indication by Van Den Herik-Oudijk *et al.* (33) that BCR signaling is less efficiently inhibited by coligation with human FcγRIIB than by that with mouse FcγRIIB. To circumvent the difficulty, we designed additional procedures that coligate FcγRIIB and BCR in a more stringent and polyvalent manner via biotin–streptavidin interaction. Cells were loaded with Fluo-3, and BCR and FcγRIIB were probed with a fixed concentration (1 μg/ml) of biotinylated DA4-4 Fab and with increasing concentrations of biotinylated AT10 (0–1 μg/ml) to allow the variation of number of FcγRIIB molecules coligated to BCR. These Abs were ligated with an excess amount of streptavidin (100 μM), and calcium mobilization was monitored with Flexstation fluorometric scanning plate reader (Molecular Devices). The average peak $[Ca^{2+}]_i$ ($n = 6$) was found to be significantly greater in h-232Thr-expressing cells than in h-232Ile-expressing cells when BCR was self-ligated (AT10 = 0) (Fig. 6), findings in good agreement with those in c-232Ile- and c-232Thr-expressing cells (Fig. 3). We also detected a similar tendency when a low concentration of biotinylated AT10 (0.04 μg/ml) was used, although the difference was not significant ($P = 0.08$). A prominent inhibition of calcium mobilization was achieved at higher concentrations of biotinylated AT10, but difference was not detected between the cells expressing FcγRIIB variants. These findings confirmed that Ile232Thr substitution decreased the inhibition of calcium mobilization by FcγRIIB when BCR is self-ligated, and suggested that under the coligation conditions, the differential inhibitory effects are evident only

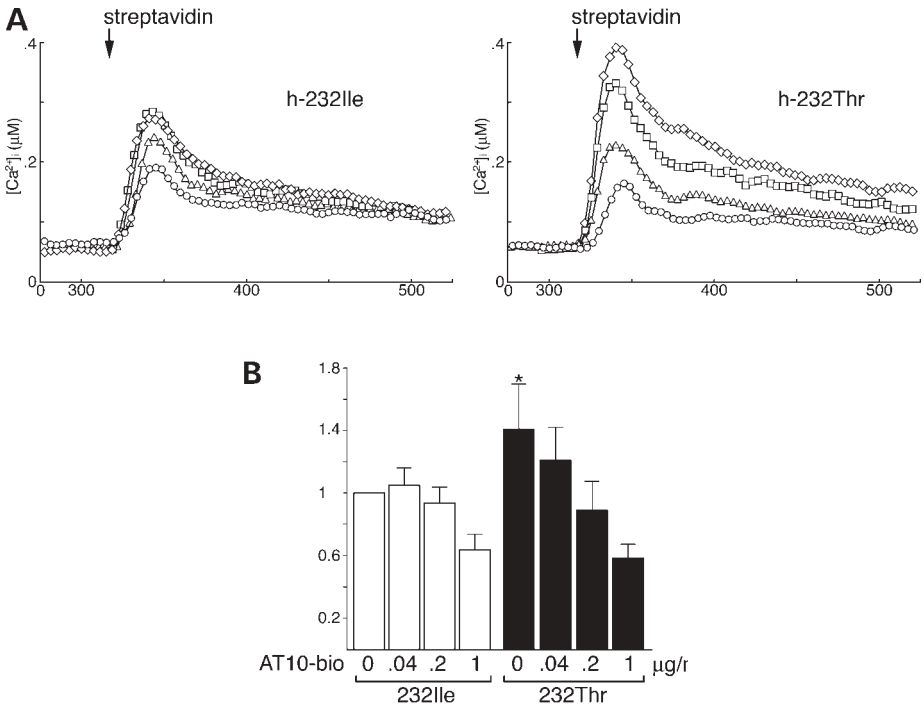

Figure 6. BCR-mediated calcium mobilization was less inhibited by h-232Thr than by h-232Ile. (**A**) The effects of the increasing levels of h-232Ile and h-232Thr coligation on BCR-mediated calcium mobilization. Fluo3-loaded cells were preincubated with 1 μg/ml of biotinylated DA4-4 mAb Fab and with various concentrations of biotinylated AT10 (0: diamond, 0.04 μg/ml: square, 0.2 μg/ml: triangle, and 1 μg/ml: circle), and subjected to BCR-FcγRIIB coligation with 100 μM streptavidin, and changes in $[Ca^{2+}]_i$ were monitored by Flexstation fluorometric scanning 96-well microplate reader (Molecular Devices) that allows the simultaneous $[Ca^{2+}]_i$ measurement in a h-232Ile and h-232Thr pair in quadruplicate. The results from a representative experiment are shown. (**B**) AT-10 concentration-dependent inhibition at maximal $[Ca^{2+}]_i$. Data obtained from six independent experiments are shown. (mean ± SD, $^*P < 0.05$).

when a small number of FcγRIIB molecules were coligated with BCR.

DISCUSSION

Previous studies using various mouse models have shown the critical roles of FcγRIIB in preventing autoimmunity *in vivo* (7,9,10). We have for the first time identified a non-synonymous polymorphism Ile232Thr in FcγRIIB and found its association with SLE susceptibility in Asians (21–23). So far, statistically significant association of the polymorphism with SLE susceptibility has not been detected in Caucasians or in African Americans (34,35). These apparent discrepancies may result from low frequency of 232Thr allele in Caucasians (34,35), or from allelic heterogeneity in other genes closely interacting with FcγRIIB with respect to SLE susceptibility. Such epistatic interaction has been shown in mouse models of SLE (10) and we recently described an example in humans in which *CD72* polymorphisms profoundly influence the association of *FCGRIIB* 232Thr with SLE susceptibility (36). To evaluate the possible contribution of 232Thr allele in the pathogenesis of SLE, precise functional studies of the allele products are required. Thus, we examined the properties of human FcγRIIB1 232Ile and 232Thr by taking advantage of ST486 human B cell line lacking endogenous FcγRIIB.

One of the unexpected findings of this study is that the expression of human FcγRIIB1, particularly that of 232Ile, induced a considerable level of inhibition of calcium mobilization under the BCR self-ligation conditions. Such an indirect or constitutive inhibition of BCR signaling has frequently been shown in other inhibitory co-receptors in mouse B cells including CD22 and CD72 (7,37), but has not been clearly described in mouse FcγRIIB (7,9,38,39). We also noticed moderate, but noticeable levels of FcγRIIB tyrosine phosphorylation and SHIP association with FcγRIIB after BCR self-ligation, which is reminiscent of the CD22- and CD72-SHP-1 systems (37). It indicates that under the present experimental conditions, BCR-alone signaling could transphosphorylate FcγRIIB. In addition, we experienced difficulty in detecting such distinct levels of IgG Fc-mediated inhibition of BCR-induced calcium mobilization as encountered in the studies of mouse FcγRIIB (7,9,38,39) (Fig. 3B; data not shown). The apparent inconsistency with previous studies with respect to the magnitudes of the constitutive and IgG Fc-mediated inhibition of BCR signaling may be simply due to different experimental conditions including the expression levels of FcγRIIB. Alternatively, in view of the modest homology between human FcγRIIB1 and the mouse homologue (24), it is also possible that mouse and human FcγRIIB differently transduce constitutive and IgG Fc-mediated inhibition. Although the available information on human FcγRIIB is limited, Van Den Herik-Oudijk *et al.* (33) also suggested that the coligation-mediated inhibition of BCR signaling by human FcγRIIB is less efficient than that by mouse FcγRIIB. To address the possible species

differences, studies are now being undertaken in our laboratory to precisely compare the properties of human and mouse FcγRIIBs.

In view of the significant difference in the levels of constitutive inhibition mediated by the human FcγRIIB variants, we separately compared the BCR-alone-mediated and BCR-FcγRIIB coligation-mediated calcium mobilization, and concluded that the Ile232Thr substitution attenuates the inhibitory effects of FcγRIIB. A detailed examination of BCR-mediated biochemical signaling demonstrated that the attenuated inhibitory effects of 232Thr were evident at the level of PIP3 metabolism. The differential inhibitory effects were overridden by excessive FcγRIIB coligation to BCR with a biotin–streptavidin system, indicating that the role of 232Ile is regulatory rather than obligatory for the inhibitory function of FcγRIIB.

Considering that FcγRIIB 232Thr was less preferentially distributed to lipid rafts than 232Ile both after BCR self-ligation and after BCR-FcγRIIB coligation, it is tempting to hypothesize that the Ile232Thr substitution decreases the amounts of FcγRIIB SHIP complex associated with lipid rafts, and enhances PIP3 accumulation. It was also observed that FcγRIIB 232Thr was less tyrosine-phosphorylated than FcγRIIB 232Ile after BCR-FcγRIIB coligation, thereby suggesting that Ile232Thr substitution renders the FcγRIIB less accessible to Lyn (16,17), at least under the coligation conditions. These findings strongly suggest a causal link between the level of FcγRIIB association with lipid rafts and the extent of the inhibition of BCR signaling. However, this hypothesis should be further investigated by experiments employing other FcγRIIB mutants that are excluded from lipid rafts. Our preliminary experiments utilizing alanine-scanning mutagenesis have indicated that two additional FcγRIIB mutants are partially excluded from lipid rafts, and concurrently show decreased levels of inhibition on BCR-mediated calcium mobilization (40). We are now investigating further the properties of artificial FcγRIIB mutants.

Recently, Li *et al.* (34) have reported a functional analysis of FcγRIIB Ile232Thr polymorphism using the IIA1.6 mouse B cell line. They compared the ratios of the decrease in BCR-mediated calcium mobilization and CD19 tyrosine phosphorylation by IgG Fc-mediated inhibition, and concluded that FcγRIIB 232Thr more effectively inhibits BCR signaling than 232Ile (34). The apparent discrepancy between their conclusions (34) and ours (this study) could be due to the different behavior of human protein with one amino acid substitution in the context of human and mouse B cells, but careful reading of their data reveals intriguing similarity between their observations with ours, suggesting that these are not indeed contradictory. Thus, in the study of Li *et al.*, the magnitudes of calcium mobilization and CD19 tyrosine phosphorylation, particularly those induced by BCR self-ligation, appear to be significantly higher in cells expressing FcγRIIB 232Thr than in those expressing 232Ile. Therefore, it is possible that human FcγRIIB 232Ile exerted a greater constitutive inhibition than 232Thr in IIA1.6 mouse B cells as well, and that the entire inhibitory effect of FcγRIIB 232Ile was underestimated in the study by Li *et al.*, because the constitutive inhibition of FcγRIIB was not taken into consideration in their study.

This study may provide the first example of a disease-associated receptor polymorphism that affects the distribution of the receptor to lipid rafts, and simultaneously impairs the receptor functions. Admittedly, the functional differences resulted from the transmembrane amino acid substitution are partial and appear too modest to account for disease susceptibility when compared with the often extreme outcomes observed in genetically manipulated mouse models. However, the extents of the functional alterations caused by human polymorphic genes associated with multifactorial diseases are quantitatively at the similar level of the present results (41–44). Therefore, the partial effect of Ile232Thr substitution does not seem in itself to preclude the possible contribution of the polymorphism to the disease susceptibility. From mechanistic points of view, it is the focus of considerable interests how the Ile232Thr substitution decreases the affinity of FcγRIIB to lipid rafts. In the case of the influenza HA protein, large hydrophobic amino acids including Ile and Leu seem to be critical for the association with lipid rafts (45). The potential roles of 232Ile in protein–protein or protein–lipid interaction should be further studied.

Recently, a novel human FcγRIIB promoter polymorphism has been reported to be associated with SLE in Caucasian (46,47). In contrast to the FcγRIIB promoter polymorphism in autoimmunity-prone mouse strains (48,49), the promoter haplotype associated with human SLE was found to enhance FcγRIIB expression (47). Therefore, it is of interest to analyze the linkage disequilibrium between the two polymorphisms in the promoter and in exon 5 encoding the transmembrane of FcγRIIB gene to consider potential functional interaction between the two independent functional polymorphisms. Alternatively, it may also be possible that different functional polymorphisms in other genes contribute to the susceptibility to SLE in different populations depending on their genetic background, for instance, through epistatic interaction (36). Such possibilities should be validated by further genetic and functional studies.

MATERIALS AND METHODS

Cells and expression constructs

The ST486 human B cell line was maintained in Hybridoma SFM medium (Gibco/Invitrogen) supplemented with 10% FCS and antibiotics. cDNAs encoding mouse FcγRIIB1 and human FcγRIIB1 232Ile and 232Thr (referred to as h-232Ile and h-232Thr, respectively) were amplified from the mouse cDNA library (Clonetech) and from cDNAs obtained from genotyped human donors (21), and subcloned into pBluescript (Stratagene). To prepare chimeric constructs containing the mouse FcγRIIB extracellular domain and human FcγRIIB 232Ile or 232Thr transmembrane-cytoplasmic domain, the mouse and the human full-length cDNAs were first subcloned into pBluescript in tandem in this order, and subjected to the reverse direction PCR (50) using primers 5′-TGGAACCTGCT TTTTCTTGA-3′ and 5′-TAGTCTCCCTTGGCGAATTC-3′. The resulting chimera constructs in which amino acids 1–210 of mouse FcγRIIB were fused to amino acids 219–310 of human FcγRIIB 232Ile or Thr (referred to c-233Ile or c-232Thr; Fig. 1A) were confirmed by DNA sequencing.

The cDNAs encoding the human and chimeric FcγRIIB variants were subcloned into the pCAGGS expression vector (51) and transfected by electroporation into ST486, and stable transformants were generated as described previously (27). The expression of the chimeric and human FcγRIIB variants was examined by flow cytometry (EPICS XL SYSTEM II, Coulter) using FITC-conjugated 2.4G2 anti-mouse FcγRII/III (Pharmingen) and FITC-conjugated FLI8.26 anti-human FcγRII (Pharmingen), respectively. We also introduced human FcγRIIB with the aid of an ecotropic retrovirus system. We first transfected the pSSR-α-blasticidin vector encoding the ecotropic retrovirus receptor (EcoRVR) into ST486 cells to facilitate ecotropic retrovirus infection (52). Human FcγRIIB 232Ile and 232Thr cDNAs subcloned into the pMSCVpuro retrovirus vector (Clonetech) were transfected using Lipofectamine 2000 (Invitrogen) into PLAT-E packaging cells (52), and the culture supernatants were obtained. The virus stock was used to infect ST486 cells expressing EcoRVR (52). The bulk of the infected ST486 cells were selected with 0.8 μg/ml puromycin, and stable ST486 infectants were obtained.

Competitive fluorescent ligand-binding assay

Because conventional ^{125}I-labeled ligand binding could not be applicable due to a high non-specific binding, particularly that to dead cells (data not shown), we utilized FITC-conjugated human IgG (mixture of subclasses purified from human serum, Sigma) for whole cell binding assay. Cells were incubated with 10^{-7} M FITC-conjugated human IgG (fluorescein/IgG molar ratio: 3.1) in the absence or presence of various concentrations ($0-3.2 \times 10^{-6}$ M) of unlabeled human IgG in PBS for 1 h on ice, washed once with ice-cold PBS and subjected to flow cytometry. The high non-specific binding activity to dead cells could be avoided by appropriate gating. Quantum fluorescence microbeads (Sigma) conjugated with four different numbers of FITC were also analyzed as a reference, and a regression line between MFIs and absolute FITC numbers was drawn. MFI of the samples was thus converted to absolute FITC-IgG number bound to one cell, and expressed as mol of IgG/cell. The binding data are subjected to Scatchard analysis, and least-square analysis was used to form regression lines.

Antibodies used for probing or ligation of BCR and FcγRIIB

Anti-human IgM rabbit whole IgG and anti-human IgM rabbit IgG $F(ab')_2$ were obtained from Jackson Immunoresearch. Before use, these Abs were subjected to gel electrophoresis followed by Coomassie Brilliant Blue staining to exclude the contamination of $F(ab')_2$ with whole IgG. Hybridoma cell lines producing DA4-4 (anti-human IgM mouse IgG) and that producing 2.4G2 were obtained from ATCC. DA4-4 and 2.4G2 mAbs were purified from culture supernatants. The preparation of the Fab fragments of DA4-4 and 2.4G2, and the biotinylation of the Fab fragments were described previously (27). The ^{125}I labeling of 2.4G2 Fab and AT-10 (anti-human FcγRII mouse IgG, Santa Cruz) was conducted using IODO beads (Pierce) according to the manufacturer's instructions.

Calcium mobilization assay

ST486 cells were loaded with Fura-2AM (Dojindo, Tokyo) in HEPES-Tyrode's solution with 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% fatty acid-free BSA (loading buffer) as described previously (27), and changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were measured in the same buffer using a fluorescence spectrophotometer F-2000 (Hitachi, Japan). Cells at 1.5×10^6 ml^{-1} were stimulated at room temperature with anti-human IgM rabbit whole IgG at 8 μg/ml or with $F(ab')_2$ at 13 μg/ml, concentrations that induce comparable responses in parent ST486 cells. In some experiments, $[Ca^{2+}]_i$ was measured using a FlexStation 96-well fluorometric imaging plate reader (Molecular Devices). In this setting, cells were loaded with Fluo-3AM (Dojindo, Tokyo) in the loading buffer containing 1% FCS, 0.02% Pluoronic acid (Molecular probe) and 2.5 mM Probenecid (Sigma) for 30 min at room temperature, washed once and plated at 1.5×10^5 per well (100 μl) in 96-well clear-bottomed black microplates (Corning). Cells were set in the device and preincubated for 15 min at 37°C. Then cells were treated with 1 μg/ml biotinylated DA4-4 Fab and with various concentrations (0–1 μg/ml) of biotinylated AT10 for 5 min, stimulated with 100 μM streptavidin, and the changes in fluorescence intensity were measured from the bottom with excitation at 485 nm and emission at 525 nm. Relative fluorescence unit (RFU) data are exported from the FLEXstation and converted into estimates of $[Ca^{2+}]_i$ using the formula: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where the K_d for Fluo-3 is 390 nM. F_{max} was recorded in the presence of 10 μM ionomycin, 1 μM thapsigargin and 40 mM $CaCl_2$, and F_{min} in the presence of 10 μM ionomycin, 1 μM thapsigargin and 10 mM EGTA.

Analysis of subcellular distribution of FcγRIIB

ST486 cells expressing chimeric FcγRIIB variants (1.5×10^7 ml^{-1}) were incubated with 2 μg/ml ^{125}I-2.4G2 Fab and 4 μg/ml DA4-4 Fab in the loading buffer for 30 min on ice, washed once, prewarmed at 37°C for 5 min, and left untreated or treated with 10 μg/ml secondary Abs (anti-rat IgG, Cappel) reactive to both the Fabs for 4 min. Cells were solubilized with 1.0 ml of ice-cold 1% Brij58 lysis buffer (28) and subjected to sucrose density gradient separation as described previously (28). Ten fractions (1 ml) were sequentially collected from the bottom and ^{125}I radioactivity was determined by γ-ray counting. In the case of full-length human FcγRIIBs, cells were treated with 2 μg/ml ^{125}I-AT10 whole IgG and with or without 4 μg/ml biotinylated DA4-4 Fab as described above and BCR self-ligation, FcγRIIB self-ligation and BCR-FcγRIIB coligation were conducted using 10 μg/ml secondary Abs (anti-mouse IgG, Cappel) or 100 μM streptavidin for 4 min, as detailed in Figure 5. Cells were solubilized and subjected to density gradient centrifugation assay as described above.

Cell stimulation and biochemical analysis

ST486 cells expressing the chimeric or human FcγRIIB variants (1×10^7 ml^{-1}) were stimulated with anti-human IgM whole IgG (8 μg/ml) or with $F(ab')_2$ (13 μg/ml) for indicated

periods at 37°C, collected by brief centrifugation (30 s) in a microcentrifuge, solubilized with 2% *n*-octyl-β-D-glucoside lysis buffer (20 mM Tris–HCl pH 7.4, 2% *n*-octyl-β-D-glucoside, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 20 mM β-glycerophosphate and protease inhibitors), and centrifuged at 12 000 rpm (10 000 g) for 10 min at 4°C to obtain the supernatant (total cell lysate). Chimeric FcγRIIB was immunoprecipitated from the total cell lysate with 2.4G2, and subjected to immunoblotting with 4G10 antiphosphotyrosine mAbs or with anti-SHIP1 Abs (Santa Cruz), and after stripping, with anti-human FcγRIIB cytoplasmic antibody (Santa Cruz). For the analysis of CD19 tyrosine phosphorylation, cells were stimulated for 1 min as stated above, and solubilized with 1% NP-40 and 0.5% deoxycholate lysis buffer (20 mM Tris–HCl pH 7.4, 1% NP-40, 0.5% deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 20 mM β-glycerophosphate and protease inhibitors). CD19 was immunoprecipitated from the cleared supernatant (total cell lysate) with anti-CD19 polyclonal antibody (Santa Cruz). The immunoprecipitates were divided into two halves: one was subjected to immunoblotting with 4G10 anti-phosphotyrosine mAbs and the other to that with anti-CD19 Abs (Santa Cruz). The total cell lysate was also subjected to immunoblotting with anti-phospho Akt (p-Ser473) Abs and anti-phospho PLCγ2 (p-Tyr1217) Abs (Cell Signaling Technology), and reprobed with anti-Akt and anti-PLCγ2 Abs (Santa Cruz) after stripping. Signals were detected using HRP-conjugated secondary Abs and an ECL chemiluminescent system (Amersham). Cell labeling with [^{32}P]-phosphorus (Amersham) and phospholipid analysis by thin-layer chromatography were conducted as described previously (12).

Statistical analyses

Statistical analyses were performed using Student's *t*-test.

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