Cross-linking of B cell antigen receptor-related structure of pre-B cell lines induces tyrosine phosphorylation of p85 and p110 subunits and activation of phosphatidylinositol 3-kinase

Kazuhiko Kuwahara, Taro Kawai, Saori Mitsuyoshi, Yoshinobu Matsuo, Hidehiko Kikuchi, Shinobu Imajoh-Ohmi, Elkiichi Hashimoto, Seiji Inui, Max D. Cooper and Nobuo Sakaguchi

Department of Immunology, Kumamoto University School of Medicine, Honjo, Kumamoto 860, Japan
Department of Immunology and Department of Pathological Biochemistry, School of Life Science, Faculty of Medicine, Tottori University, Yonago 683, Japan
Fujisaki Cell Center, Hayashibara Biochemical Industries Inc., Fujisaki, Okayama 702, Japan
Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan
Departments of Medicine, Pediatrics, Pathology and Microbiology, Howard Hughes Medical Institute, University of Alabama at Birmingham, Birmingham, AL 35294, USA

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Abstract
To understand the function of B cell antigen receptor (BCR)-related complex on pre-B cells (pre-BCR, V_{pre-B} heavy chain/lg-a/lg-P), we examined pre-BCR- and BCR-mediated signaling events in human and mouse pre-B (Nalm-6, 697, NFS-5), Immature B (lgM^+ Daudi, WEHI-231) and mature B (lgM^+lgD^+ BALL1) cell lines. Anti-μ cross-linking induced tyrosine phosphorylation of the cytoplasmic proteins in each cell type, but did not induce a detectable Ca^2+ mobilization response in pre-B cells. While the pre-B cells expressed Syk protein at levels similar to those found in B cell lines, pre-BCR cross-linkage did not induce phosphorylation of Syk tyrosine residues. Different protein kinase C isozymes were expressed by pre-B (PKC-α), Immature B (PKC-α and -β) and mature B (PKC-β) cell lines. Anti-μ cross-linking induced PKC translocation from the cytosolic to the membrane compartment in immature and mature B cells, but did not have this effect in a pre-B cell line. Anti-μ cross-linking induced tyrosine phosphorylation of the p85 and p110 subunits of phosphatidylinositol 3-kinase (PI3-kinase) in both pre-B and B cell lines, but the pre-BCR induced PI3-kinase activation was Syk independent. Ligation of the pre-BCR complex thus triggers a characteristic signaling pattern in pre-B cells.

Introduction
B cells are generated from multipotent hematopoietic stem cells in the fetal liver or the bone marrow by undergoing differentiation through immature pro-B cell and pre-B cell stages (1–3). Some of the pre-B cells express surrogate light chains composed of the 5 and V_{pre-B} proteins (4–7). A severe deficit of surface IgM (slgM)^+ B cells in 5 knock-out mice suggests that expression of the μ chain/5/V_{pre-B} complex (pre-BCR) is important for the maturation of pre-B cells (8). To understand early B lineage cell development, it is important to elucidate the signals transmitted via the pre-BCR.

The B cell receptor (BCR) is composed of antigen-specific IgM (μ chain and light chain) and the associated heterodimer component of two kinds of proteins, Ig-α encoded by the mb-1 gene (9–12) and Ig-β encoded by the B29 gene (12,13). The BCR complex involves functional components necessary for the subsequent signal transduction such as src-type tyrosine kinases, Fyn (14,15), Lyn (14–16), Lck (17,18) and Blk (14,19), non-src type tyrosine kinase Syk (20,21), phosphatidylinositol 3-kinase (PI3-kinase) (15,22,23), other phosphoprotein components, p52 (24,25), and additional...
unidentified components (15,26). Cross-linkage of the BCR on B cells induces the initial tyrosine phosphorylation probably via pre-associated src-type tyrosine kinase(s) with the subsequent association of src-type tyrosine kinase(s) and of Syk with the BCR complex through their src homology 2 (SH2) and/or src homology 3 (SH3) domains. This initial tyrosine phosphorylation cascade then activates multiple signal transduction pathways including the activation of phospholipase C (PLC)-γ that accelerates phosphatidylinositol metabolism leading to the increase of intracellular Ca^{2+} and activation of protein kinase C (PKC) (27,28). Kurosaki and co-workers reported that Syk-deficient cells do not undergo a rapid increase of intracellular Ca^{2+} mobilization after BCR cross-linking (29). PI3-kinase, another discrete second messenger molecule associated with the BCR complex, is reported to mediate an important signal for many kinds of receptors (30–32). In B cells, BCR cross-linkage induces tyrosine phosphorylation of PI3-kinase (22,23), which is composed of a p110 catalytic subunit and p85 regulatory subunit, thereby leading to the PI3-kinase activation of further downstream signal transduction.

Previously, we and others characterized the BCR-related structure of human pre-B cell lines as a complex of μ chain, 22 kDa λ5, 16 kDa V_{pre-B}, Ig-α and Ig-β proteins on the surface (33–36). Cross-linking of the pre-BCR on pre-B cell lines activates src-type tyrosine kinases, Fyn and Lyn, which can then induce a rapid increase in tyrosine phosphorylation of other cellular proteins (33). Here, we found that while the pre-BCR on pre-B cell lines is capable of inducing signal transduction, the signaling pathways differ from those triggered by the BCR on IgM^+ B cell lines.

Methods

Cells and cell culture

The human pre-B cell lines, Nalm-6 and 697, the immature B cell line, Daudi (IgM^+), and the mature B cell line, BALL1 (IgM^+IgD^+), were described previously (33,37,38). The murine pre-B cell line, NFS-5, and the immature B cell line WEHI-231 were described previously (39). All cell lines were maintained in RPMI 1640 culture medium (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) containing 10% heat-inactivated FCS (Gibco/BRL, Grand Island, NY), 2 mM L-glutamine (Biowhittaker, Walkersville, MD), streptomycin (100 μg/ml), penicillin (100 U/ml) and 2-mercaptoethanol (5×10^{-5} M) in a 5% CO_2 incubator at 37°C.

Antibodies

Rabbit anti-porcine p72^{59K} antibody, which cross-reacts with human and mouse Syk molecules, a mouse anti-phosphotyrosine mAb (4G10) and a mouse anti-human p85 subunit of PI3-kinase mAb were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-human p72^{59K} antibody, used for Western blot analysis, was kindly provided by Dr Edward A. Clark (University of Washington, Seattle, WA) (40). Mouse anti-human p72^{59K} mAb, used for immunoprecipitation studies, was purchased from Upstate Biotechnology, was purchased from Wako Pure Chemical Industries (Osaka, Japan). Goat F(ab')_2 anti-human IgM (μ chain specific) antibody was obtained from Antibodies Incorporated (Davis, CA). Goat F(ab')_2 anti-mouse IgM (μ chain specific) antibody was purchased from Organo Teknica Cappell Products (West Chester, PA). Goat non-immune IgG was purchased from Biopool (Umeå, Sweden). Rabbit anti-PKC antibodies (CTα...
and CTβ2) were produced by immunizing with synthetic oligopeptides corresponding to human PKC isozymes as described (41,42).

**Cell lysis, immunoprecipitation and SDS–PAGE**

After incubation, cells were quickly spun down in an Eppendorf tube and lysed with lysis buffer containing 1% Nonidet P-40 as described previously (43). Cell lysates, being precleared with Protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden), were processed for the immunoprecipitation with the reagents indicated in the figure legends. After washing, samples were boiled for 5 min in the sample buffer containing 5% of 2-mercaptoethanol and separated on SDS–PAGE.

**Western blot analysis**

Western blot analysis was performed as described previously (43). Briefly, whole cell lysates or immunoprecipitates were separated on SDS–PAGE and transferred electrophoretically to a nitrocellulose filter membrane (Schleicher & Schuell, Dassel, Germany). The filter was blocked with 3% BSA/PBS/0.1% Tween 20 and incubated with anti-phosphotyrosine mAb (4G10). The filter was washed and followed by incubation with peroxidase-labeled Protein A (Zymed Laboratories, San Francisco, CA). After washing with PBS/0.1% Tween 20, the specific bands were detected by the ECL system (Amersham International, Amersham, UK).

**Ca\(^{2+}\) mobilization assay**

Intracellular levels of Ca\(^{2+}\) were measured after BCR cross-linkage of various lymphoid cells by a method previously described (44). Twenty million cells were washed and resuspended in 1 ml of RPMI 1640 medium containing 10% FCS, and incubated with 4 μM of Fura 2-AM (Dojin, Kumamoto, Japan) for 15 min at 37°C. After the addition of RPMI 1640

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**Fig. 2.** Measurement of Ca\(^{2+}\) mobilization in human B cell lines. Cells were loaded with Fura 2-AM as described in Methods. Goat F(ab')\(^2\) anti-human \(\mu\) antibody (20 μg/ml) was added to the cuvette at the time indicated by the arrow. Goat non-immune IgG (20 μg/ml) and calcium ionophore A23187 (0.1 μg/ml) were used as negative and positive controls respectively.
Fig. 3. Expression of Syk protein in B cell lines. Whole cell lysates were separated on 8% SDS-PAGE, then transferred to a nitrocellulose filter membrane by electroblotting. Syk protein was detected by rabbit anti-human p72<sup>syk</sup> antibody in combination with peroxidase-labeled Protein A as described in Methods. The same filter was re-blotted with control antibody (preimmune rabbit serum).

Fig. 4. Tyrosine phosphorylation of Syk protein upon anti-μ stimulation. Human B cell lines (A) and murine B cell lines (B) were incubated at 37°C with goat F(ab′)<sub>2</sub> anti-human μ antibody or goat F(ab′)<sub>2</sub> anti-mouse μ antibody respectively for various periods of time. The Nonidet P-40 lysates, immunoprecipitated with rabbit anti-Syk serum or mouse anti-human Syk mAb, were separated on 7.5% SDS-PAGE, then transferred onto a nitrocellulose filter by electroblotting. Tyrosine phosphorylated proteins were detected with anti-phosphotyrosine mAb (4G10) followed by peroxidase-labeled Protein A.
Fig. 5. Induction of Syk activity upon anti-μ stimulation Human B cell lines (A) and murine B cell lines (B) were stimulated with goat F(ab')2 anti-human μ antibody or goat F(ab')2 anti-mouse μ antibody respectively. Cell lysates were immunoprecipitated with rabbit anti-Syk serum or mouse anti-human Syk mAb in combination with Protein A-Sepharose. After in vitro kinase reaction with [γ-32P]ATP, phosphorylated proteins were separated on 7.5% SDS-PAGE, then visualized by autoradiography.

In vitro kinase assay
Phosphorylation activities in the specific immunoprecipitates were measured as described previously (45). Briefly, immunoprecipitates were washed four times with lysis buffer containing 1% Nonidet P-40, once with kinase reaction buffer and resuspended in kinase reaction buffer (50 mM Tris-HCl, pH 7.2, 10 mM MgCl₂ and 3 mM MnCl₂). The samples were incubated for 10 min at 30°C with 10 μCi [γ-32P]ATP (Amersham). The reaction was terminated by adding stop solution (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 2 mM EDTA). Phosphorylated proteins were separated on SDS–PAGE.

Subcellular fractionation
This procedure was performed as described by Yamanashi et al. (46). In brief, after stimulation with the anti-μ antibody

medium (9 ml), the cells were incubated for another 15 min at 37°C, and then centrifuged and resuspended in 10 ml of working buffer (145 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 0.5 mM MgSO₄, 5 mM glucose, 20 mM HEPES, pH 7.3 and 1 mM CaCl₂). The intracellular calcium concentration was measured by an F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Calcium ionophore (A23187), purchased from Sigma (St Louis, MO), was used as a positive control.
for 10 min, cells were suspended in hypotonic buffer (20 mM HEPES, pH 7.4, 10 mM EDTA, 2 mM dithiothreitol and 10 μg/ml of aprotinin) and were left on ice for 15 min. Cells were homogenized with a Dounce homogenizer. Nuclei were separated by centrifugation at 800 g for 1 min. The supernatant was centrifuged at 30,000 g for 30 min to separate the membrane (pellet) and cytosolic (supernatant) fractions. Each fraction was mixed with SDS-sample buffer, boiled and examined by Western blot analysis with the specific antibody to human PKC-α or PKC-β (41, 42).

**PI3-kinase assay**

The PI3-kinase assay was performed by the method described by Yanagi et al. (47). Cell lysates were prepared from 1x10^7 cells, either unstimulated or stimulated with goat anti-human μ antibody. Specific immunoprecipitates with anti-phosphotyrosine mAb (4G10) and Protein A-Sepharose were washed twice with lysis buffer and three times with a 10 mM Tris-HCl buffer, pH 7.1. PI3-kinase assay was performed in the buffer (50 μl) containing 0.2 mg/ml of sonicated phosphatidylinositol (Serdary Research, Englewood Cliffs, NJ), 20 mM HEPES, pH 7.1, 0.4 mM EGTA, 0.4 mM Na_2HPO_4, 10 mM MgCl_2 and 0.1 μCi/μl [γ-^32P]ATP. The reaction was carried out for 5 min at room temperature and terminated by the addition of 15 μl of 4 N HCl and 130 μl of chloroform:methanol (1:1). The samples were mixed vigorously by vortexing for 30 s. The lower phase solution (30 μl) was spotted onto a silica gel plate (Merck, Darmstadt, Germany), which had been pre-treated with a solution of 1% potassium oxalate and activated at 100°C. The lipids on the plate were extended in a chamber with chloroform:methanol:4 M NH_4OH (9:7:2) and the plate was dried for the exposure.
Signaling via pre-B cell receptor

Results

Cross-linkage of pre-BCR on human pre-B cell lines induces tyrosine phosphorylation of cellular proteins

First, we confirmed that the Nalm-6 and 697 cell lines express the pre-BCR complex of μ chain, 22 kDa λ5 and 16 kDa V_{pre-B}, and the Ig-α/Ig-β heterodimer as reported (33–36). We could not detect κ chain protein and λ chain protein by surface staining, by Western blot analysis or by immunoprecipitation and diagonal SDS-PAGE analysis (data not shown). Here, we compared the signal transduction events mediated via the pre-BCR and BCR on the pre-B (Nalm-6 and 697), immature B (Daudi) and mature B (BALL1) cell lines. All of these cell lines responded to receptor cross-linkage by anti-μ antibody with an increase in tyrosine phosphorylation of intracellular proteins. The rapid increase in tyrosine phosphorylation of intracellular proteins is illustrated in the Western blot analysis shown in Fig. 1(A). Daudi showed strong tyrosine phosphorylation. Nalm-6 and BALL1 showed a weaker but similar level of tyrosine phosphorylation at the 70, 56 and 53 kDa bands. These results indicated that pre-BCR expressed on the surface of Nalm-6 mediates the signal into cells by inducing rapid tyrosine phosphorylation (Fig. 1A). Parallel experiments indicated that anti-μ cross-linking induces the tyrosine phosphorylation of Lyn through the pre-BCR of Nalm-6 levels comparable with those induced by cross-linkage of BCR on Daudi and BALL1 cells (data not shown and 48). To generalize this evidence of pre-BCR signaling, we extended the study to other human, 697, and murine, NFS-5, pre-B cell lines that express the μ chain/λ5/V_{pre-B} pre-BCR complex on the cell surface (36,39). These pre-B cell lines also responded to pre-BCR cross-linkage by enhanced tyrosine phosphorylation of intracellular proteins (Fig. 1A and B).

Cross-linkage of pre-BCR did not induce a demonstrable increase in intracellular Ca^{2+} mobilization or the activation of Syk

We next compared the effects of pre-BCR and BCR cross-linkage on the intracellular Ca^{2+} mobilization response. While anti-μ cross-linking of the pre-BCR on Nalm-6 and 697 cell lines did not induce a detectable Ca^{2+} mobilization response, both immature Daudi and mature BALL1 B cell lines...
Signaling via pre-B cell receptor

Anti-μ stimulation

The results indicate that cross-linking of the BCR-related structure on Nalm-6 induces tyrosine phosphorylation of Lyn but does not cause Ca$^{2+}$ response. Syk activation has also been implicated in the induction of intracellular Ca$^{2+}$ mobilization in B cells (29). Northern blot analysis using a syk-specific probe indicated that syk mRNA is expressed at a comparable level in Nalm-6 pre-B cells as in the Daudi and BALL1 B cells (data not shown). Western blot analysis with an anti-Syk antibody clearly shows that 697 and Nalm-6 express 72 kDa Syk as well as seen in Daudi and BALL1 (Fig. 3, left panel). Mouse pre-B, NFS-5, and B, WEHI-231, cell lines also express p72$^{syk}$ protein (Fig. 3, right panel).

Next, we examined whether Syk could be activated by anti-μ cross-linkage of pre-BCR as well as by cross-linkage of BCR on B cells. To immunoprecipitate human Syk after pre-BCR and BCR cross-linkage, we used two kinds of anti-Syk antibodies. The rabbit anti-porcine Syk antibody cross-reacts well to the human Syk and a mouse anti-human Syk mAb also immunoprecipitates Syk of identical size from Daudi and BALL1. Western blot analysis of the anti-Syk immunoprecipitates with anti-phosphotyrosine mAb confirmed that the 72 kDa Syk is tyrosine phosphorylated following BCR cross-linkage on the Daudi and BALL1 B cell lines (Fig. 4A, upper and lower panels, p72$^{syk}$ indicated by the arrow).

However, Nalm-6 or 697 pre-B cell lines did not show any induction of Syk tyrosine phosphorylation (Fig. 4A, upper and lower panels). Anti-μ cross-linking of murine pre-B cell line, NFS-5, also did not induce tyrosine phosphorylation of p72$^{syk}$, but did elicit a Syk tyrosine phosphorylation response in WEHI-231 B cells (Fig. 4B, p72$^{syk}$ indicated by the arrow).

Anti-μ stimulation enhanced the kinase activity of p72$^{syk}$ in Daudi and BALL1 B cells, but not in Nalm-6 pre-B cells (Fig. 5A, upper panel). The mouse mAb against human Syk immunoprecipitates the p72$^{syk}$ that can be autophosphorylated in vitro (Fig. 5A, lower panel). Baseline levels of autophosphorylation were observed before anti-μ cross-linking with this assay and anti-μ stimulation induced an increase of kinase activity in the B cell lines, Daudi and BALL1, but not Nalm-6 and 697 (Fig. 5A, lower panel). When murine pre-B and B cell lines were examined in the same way, inducible autophosphorylation of p72$^{syk}$ was observed in anti-μ stimulated WEHI-231 B cells (Fig. 5B, right panel). In contrast, NFS-5 pre-B cells did not exhibit the p72$^{syk}$ autophosphorylation band after anti-μ stimulation (Fig. 5B, left panel, the expected band indicated by the arrow with p72$^{syk}$). The bands observed below the p72$^{syk}$ of NFS-5 with control antibody and anti-Syk antibody are non-specific. These results suggest that pre-BCR cross-linkage can transduce a signal leading to Lyn activation but cannot mediate an appropriate signal for Syk.

Cross-linking of pre-BCR on Nalm-6 does not induce membrane translocation of PKC

The above results did not reveal downstream signal transduction following the rapid increase of tyrosine phosphorylation in Nalm-6. We next examined the expression of PKC-α and PKC-β in Nalm-6, Daudi and BALL1. One type of PKC activation occurs on cleavage between regulatory and catalytic domains by the action of a calcium-dependent serine protease named calpain (41,42). Rabbit antibodies against
synthetic oligopeptides can differentially recognize PKC-α and PKC-β as holoenzymes of regulatory and catalytic domains (41,42). Using antibodies that recognize holoenzymes and calpain-cleaved catalytic domains, the expression of PKC-α and PKC-β was examined (Fig. 6). Daudi expresses both PKC-α and PKC-β on Western blot analysis (Fig. 6). However, Nalm-6 expresses PKC-α only but not PKC-β. The mature B cell line BALL1 only expresses PKC-β. Anti-μ stimulation of these B cell lines did not induce an increase of calpain-activated cleavage of PKC (Fig. 6) (expected size indicated by the open arrows), suggesting that the BCR-signaling pathway does not induce calpain-mediated PKC activation. The bands indicated by the asterisks are non-specific. Repeated experiments with shorter periods of anti-μ cross-linking for 5 and 10 min did not show any calpain-dependent cleavage of PKC-α and PKC-β in any of three B cell lines (data not shown). Since translocation of PKC occurs during the activation of PKC in B cells (49), we examined the location of PKC in receptor-stimulated pre-B and B cells. Figure 7 shows results of Western blot analysis in which cross-linking of pre-BCR did not show any increase of PKC proteins into the membrane fraction of Nalm-6. Daudi and BALL1, however, showed inducible translocation of PKC (Fig. 7A–C). These results do not demonstrate apparent evidence of the signal transduction through pre-BCR in Nalm-6.
Cross-linking of pre-BCR induces tyrosine phosphorylation of p85 and p110 subunits and causes induction of PI3-kinase activity

In order to characterize further the signal transduction pathway(s) through pre-BCR, we examined the activation of PI3-kinase following receptor cross-linkage on pre-B and B cell lines. Western blot analysis indicated inducible tyrosine phosphorylation of p85 and p110 subunits of PI3-kinase in both types of cells (Fig. 8A), although the tyrosine phosphorylation responses of p85 and p110 subunits were not uniform among cell lines. The results presented here are similar using two kinds of anti-PI3-kinase antibodies (data not shown). Daudi shows a strong tyrosine phosphorylation in the p85 subunit but has slight inducible tyrosine phosphorylation in the p110 subunit. Pre-BCR cross-linkage on Nalm-6 resulted in a response pattern similar to that observed for BALL1, except that Nalm-6 had higher background levels of p85 and p110 tyrosine phosphorylation. Interestingly, unstimulated Nalm-6 had a prominent tyrosine phosphorylated 200 kDa band that disappeared after pre-BCR cross-linkage. Anti-μ stimulation of pre-BCR on the human 697 and the murine NFS-S pre-B cell lines also induced the increase of tyrosine phosphorylation of the p110 subunit, although tyrosine phosphorylation of the p85 subunit was not clearly evident in either of the cell lines [Fig. 8A (lower panel) and B]. Tyrosine phosphorylation of the p110 subunit was also induced by anti-μ stimulation of the WEHI-231 murine B cell line, albeit at a relatively low level (Fig. 8B). Next, we examined whether this inducible tyrosine phosphorylation of PI3-kinase in pre-B cells is involved in the signal transduction through the catalytic action of p110 of PI3-kinase. Anti-tyrosine immunoprecipitates showed an increase of PI3-kinase activity of B cells stimulated with anti-μ antibody (23). PI3-kinase activity detected by thin layer chromatography of anti-tyrosine immunoprecipitates showed an increase of PI3-kinase activity in two human pre-B cell lines (Nalm-6 and 697) as well as Daudi and BALL1 (Fig. 9A). Anti-μ cross-linkage of a murine pre-B cell line (NFS-S) also induces the PI3-kinase activity as well as in WEHI-231 (Fig. 9B). These results indicated that cross-linking of pre-BCR induces PI3-kinase activity.

Discussion

This comparative analysis of the signal pathways initiated via the pre-BCR and the conventional BCR expressed by mature B cells indicates significant difference. First we confirm that pre-BCR cross-linkage induces tyrosine phosphorylation of cytoplasmic proteins in pre-B cells, albeit to a lesser extent than the tyrosine phosphorylation cascade initiated by cross-linkage of the BCR on B cells. In addition, triggering through the pre-BCR induces the tyrosine phosphorylation of the p85 and p110 subunits of PI3-kinase and results in the enhancement of PI3-kinase activity. In contrast to the important Syk activation following BCR triggering of B cells, Syk is not activated or tyrosine phosphorylated by pre-BCR stimulation, although the Syk protein is easily demonstrated in the pre-B cell lines used in this study. Moreover the failure to activate Syk by pre-BCR cross-linkage is correlated with the failure to mobilize Ca$^{2+}$ in the pre-B cell response. The well-known difference in the level of pre-BCR and BCR expression could be one reason for the differences in signal transduction in pre-B cells and mature B cells. We could confirm that the μ chain-containing receptor complexes on the pre-B cell lines were lower than on the B cell lines tested. Nevertheless, the inducible tyrosine phosphorylation upon anti-μ cross-linking was similar for pre-B and B cell lines. In addition, anti-μ stimulation of the pre-B cells clearly induced tyrosine phosphorylation of PI3-kinase and the activation of PI3-kinase activity to a similar extent as that observed in B cells.

Activation of protein tyrosine kinases, such as Lyn and Fyn src-family members associated with BCR complex, is one of the earliest events initiated by stimulation via BCR complex on B cells (14). Takata and co-workers showed that Syk is involved in inositol 1,4,5-trisphosphate (IP$_3$) generation mediated by PLC-γ, whereas Lyn is involved in the regulation of Ca$^{2+}$ mobilization independent of IP$_3$ generation (29). However, constitutive activation of both Lyn and Syk is not sufficient to initiate other biochemical events, suggesting that additional signals induced by BCR cross-linkage are required for full transduction of BCR signaling (50). Although both Syk and Lyn exist in similar levels in the pre-B and B cell lines employed in our studies, cross-linkage of the pre-BCR did not result in an increase in tyrosine phosphorylation of Syk or kinase activity. Bossy and co-workers, who previously examined the structure, biosynthesis and transduction properties of the pre-BCR complex of Nalm-6 (35), observed a low level Ca$^{2+}$ mobilization response in comparison with the BCR-triggered response of mature B cell lines. However, in our experiments cross-linkage of the pre-BCR on Nalm-6 and 697 pre-B cells did not induce a detectable Ca$^{2+}$ mobilization response. Our results accord well with the observation that stimulation via the BCR in a Syk-deficient B cell line did not result in a Ca$^{2+}$ mobilization response (29). Sidorenko and co-workers recently analyzed the components associated with Syk in human B cell lines, representative of various stages in B cell differentiation (48). In mature B cell lines including Daudi, p53/S6 Lyn and an unidentified pp120 protein are co-precipitated with p72 Syk. In Daudi B cells, Lyn and Syk were associated, but in Nalm-6 pre-B cells Lyn was not associated with Syk. Lyn molecules from the Daudi cells could not re-associate with Syk from the Nalm-6 cells suggesting to these investigators that a specific Lyn–Syk association is necessary for the activation of Syk (48).

Activation of Syk leads to the tyrosine phosphorylation of PLC-γ, resulting in the further activation of PKC and Ca$^{2+}$ mobilization response by producing diacylglycerol and IP$_3$ (29). Using specific antibodies against the PKC-α and PKC-β isozymes, Western blot analysis revealed the differential expression of PKC-α and PKC-β. Daudi expresses both PKC-α and PKC-β, but Nalm-6 expresses PKC-α only and BALL1 expresses PKC-β only. Cross-linking of pre-BCR did not induce the calpain-dependent activation of PKC-α or the translocation of PKC-α toward the membrane fraction of Nalm-6 (Figs 6 and 7).

PI3-kinase activities originally appeared with the transforming protein tyrosine kinases v-ros and v-src as the associated enzymes. PI3-kinase contains two major proteins of p85 and p110. It was not clear what kind of tyrosine kinase(s) is
involved in the tyrosine phosphorylation of each subunit of PI3-kinase in B cells. Our results may suggest that activation of Syk is not essential in the tyrosine phosphorylation of p85 and p110 subunits.

It is not clear what kind of function(s) pre-BCR possesses? We tested the change of growth after anti-μ cross-linking. Anti-μ cross-linking did not cause any significant change of cell growth in 4 days culture in vitro (data not shown). Our results demonstrated clearly that cross-linking of BCR-related structure on Nalm-6 induces tyrosine phosphorylation of p85 and p110 subunits of PI3-kinase, which is probably the first evidence demonstrating a clear biochemical change related to PI3-kinase induced by signal(s) through pre-BCR.

There is suggestive evidence that the expression of pre-BCR is essential for the maturation of early progenitor cells to early and late stage pre-B cells and into mature slg^+ B cells. In λ5 knock-out mice, the rate of B cell production is decreased to 5-10% of normal mice although the development of pro-B cells and pre-B cells appears normal. Pre-BCR complex is supposed to be involved in the regulation of Ig gene rearrangement (51). Ehlrich and co-workers, however, showed that Ig heavy and light chain genes rearrange independently at an early stage of B cell development and do not require pre-BCR complex (52). Surface expression of surrogate light chain is confined to the early stage of murine pre-B cell development and becomes undetectable once μ heavy chain is produced, which might suggest that pre-BCR initiates the transition from the pro-B to early pre-B cell to the pre-B cell stage (53). In human, the expression of pre-BCR is confined to a relatively late stage in normal pre-B cell differentiation, during which receptor-cross-linking does not impede cell growth of B cell differentiation (36). To understand clearly the physiological function(s) of pre-BCR in the early B cell differentiation, it would be helpful to demonstrate clear molecular evidence of the signal transduction through pre-BCR. It was difficult to determine the molecular changes of the signal transduction through pre-BCR because of the absence of cell lines which can represent clear evidence for active signal transduction in pre-B cells. Pre-BCR can mediate only partial but clearly mediate a certain signal(s) into cells. It still remained whether the activation of partial signal transduction pathways might be caused because of in vitro tumor cell lines. In fact, a variant cell line of murine pre-B cells established by Takemori et al. shows the intracellular Ca^2+ response upon stimulation to pre-BCR (54) and the pre-B cell line often do not express one or some molecules that are commonly seen in mature B cell lines (55). To try to generalize the present results of pre-BCR signal transduction, we included results of another human pre-B cell line 697 (Figs 1A, 2, 4A, 5A, 6A and 9A) and a mouse pre-B cell line NFS-5 (Figs 1B, 4B, 5B, 8B and 9B) that are expressing the μ heavy chain NalM-pre-B or Ig heavy chain in a relatively late stage in normal pre-B cell differentiation (38,39). These two cells showed similar molecular changes after cross-linking of pre-BCR. Furthermore, we considered the possibility that the point mutations might have occurred in the syk gene of Nalm-6, which cannot play the kinase function in BCR-mediated signal transduction. We isolated a syk cDNA clone from Nalm-6 by PCR and determined the nucleotide sequence of the entire coding regions (data not shown). The syk cDNA clone has the identical sequence to that reported previously (40).

Lyn-deficient mice have normal development in the phenotype of B cells, although the number of B cells is reduced (56,57). Syk-deficient mice, however, showed a severe block at the pro-B to pre-B cell transition, suggesting that Syk might have a crucial role in pre-BCR signaling (58,59). Our results did not demonstrate the involvement of Syk when stimulated with anti-μ antibody on pre-B cells, but the results do not necessarily neglect the involvement of Syk kinase in the activation or differentiation of pre-B cells. As proposed by Pillai and co-workers (60,61), the constitutive ligand-independent signaling by the pre-BCR might play a crucial role in pre-B cells for the development of pre-B cells, while the cell surface pre-BCR could mediate a different kind of signal causing the activation of PI3-kinase without the activation of Syk kinase.

An interesting question is whether there is any difference in pre-BCR signaling between by anti-μ cross-linking and by the natural ligand. It is also necessary to further study the signals which can induce full activation of BCR-related signal transduction pathways in Nalm-6. Normal pre-BCR might mediate a partial signal transduction, which is further augmented by other co-stimulatory signals given to molecules expressed on the surface of pre-B cells in vivo. These may include cognate cell interaction with pre-B cells, T cells or stromal cells in the bone marrow microenvironment. Soluble cytokines or ligands to several differentiation markers known as CD molecules might be a candidate molecule for the secondary activation of pre-B cells. The antigen molecule(s) or ligand of pre-BCR is still not clear, although it is most likely expressed on the surface of stromal cells. Normal pre-B cells might require two kinds of signals given to pre-BCR and co-stimulatory molecules on pre-B cells. To address these issues, it might be useful to know the signal(s) responsible for the tyrosine phosphorylation of Syk and the induction of Syk activity in addition to the signal transduction leading to the PI3-kinase pathway in pre-B cells.

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Abbreviations

- BCR: B cell antigen receptor
- IP₃: inositol 1,4,5-trisphosphate
- PI3-kinase: phosphatidylinositol 3-kinase
- PLC: phospholipase C
- PKC: protein kinase C

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