The catalytic activity of Src-family tyrosine kinase is required for B cell antigen receptor signaling

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Abstract The Src family protein-tyrosine kinases (PTKs) are known to be important for B cell antigen receptor (BCR) signaling. To study the mechanism of action of Src-PTK in BCR signaling, kinase deficient- and Src homology 2 (SH2)-mutants of Src-PTK were transfected into Lyn-deficient B cells and analyzed. Kinase activity of Src-PTK was essential for tyrosine phosphorylation of Syk and calcium mobilization upon receptor ligation, whereas these events were not affected by the mutation of SH2 domain. Receptor-mediated tyrosine phosphorylation of Lyn was still observed in Syk-deficient B cells. These results demonstrate that the BCR-induced phosphorylation of Src-PTK is independent of Syk and that the kinase activity of Src-PTK is critical for BCR signaling.

Key words: Kinase activity; BCR signaling; Src-PTK; SH2 domain; Syk

1. Introduction

Induced phosphorylation of several proteins on tyrosine residues is the earliest biochemical event detectable upon B cell antigen receptor (BCR) stimulation [1,2]. The BCR associates with two classes of tyrosine kinases; Src-family protein tyrosine kinase (PTK) and Syk kinase. These PTKs become activated as a result of receptor cross-linking. The activation of these kinases leads to rapid tyrosine phosphorylation of several proteins including the BCR Igα, Igβ chains, phosphatidylinositol (PI)-3 kinase, p21^{ras}-GTPase activating protein (GAP), VAV, and phospholipase C (PLC)-γ2 [3,4].

The importance of Src-PTKs in BCR signaling is emphasized by the finding that mutant B cell clones devoid of Lyn respond to BCR stimuli with a very limited tyrosine phosphorylation of cellular substrates [5]. Activity of Src-PTKs can be regulated by tyrosine phosphorylation. There are two known sites of regulation: a positive regulatory autophosphorylation site, and a C-terminal tyrosine, a site of negative regulation. Phosphorylation at the negative regulatory site is believed to maintain the enzyme in an inactive conformation by an intramolecular interaction with the Src homology 2 (SH2) domain of the kinase [6,7].

In addition to regulation of Src-PTK by phosphorylation, it is proposed that a spatial reorientaion of Src-PTK alters its kinase activity during BCR stimulation. In vitro binding data showed that Fyn or Lyn interacts with resting BCR primarily through the non-phosphorylated Igα chain Immunoreceptor Tyrosine based Activation Motif (ITAM) (low-affinity binding) and that tyrosine-phosphorylated Igα ITAM induces the binding to Fyn SH2 domain (high-affinity binding), leading to the increased kinase activity of Fyn [8]. However, the in vivo role of the Src-PTK SH2 domain in BCR signaling process has not been established. Moreover, it has not been clearly demonstrated that the kinase activity of Src-PTK plays a role in receptor signaling.

To address the in vivo functions of Src-PTK domains in regulating BCR signal transduction, we mutated residues required for SH2 or kinase function of Src-PTK and analyzed the ability of these constructs to restore BCR signaling. In this report, we demonstrate that the catalytic activity of Src-PTK is essential for BCR signaling and suggest that the SH2 domain of Src-PTK is not crutical for early signaling events.

2. Materials and methods

2.1. Cell culture, DNA transfection, and antisera

Chicken wildtype DT40 cells and various mutant cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. Kinase-deficient and SH2-mutant cDNAs of mouse *fyn* (Lys^{296} to Met^{296} and Arg^{176}-Glu^{177}-Ser^{178} to Asp^{176}-Glu^{177}-Cys^{178}, respectively) [9] were created by polymerase chain reaction and was cloned into pApuro vector [5]. Resulting constructs were confirmed by DNA sequencing. These cDNAs were transfected into *lyn*-negative DT40 cells [5] by electroporation using Gene pulser apparatus (Bio-Rad Laboratories) at 550 V, 25 μF, and selected in the presence of 0.5 μg/ml puromycin. Expression of mutated Fyn was assessed by immunoblotting.

The mAb M4, an anti-chicken IgM, was used for stimulation of BCR on DT40 cells [10]. Abs against chicken Lyn and Syk were already described [5]. Abs against phosphotyrosine (4G10) and Fyn were purchased from Upstate Biotechnology and Oncogene Sciences, respectively.

2.2. Immunoprecipitation analysis

In experiments shown in Figs. 1, 4 and 6, cells were solubilized in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA) containing 50 mM NaF, 10 μM molybdate, 0.2 mM Na_3VO_4 supplemented with protease inhibitors as described in [11]. Insoluble material was removed by centrifugation at 12,000 × *g* for 10 min. These clarified lysates were incubated sequentially (1 h, 4 °C for each incubation) with Abs and protein A-Sepharose. The immunoprecipitates were washed four times with lysis buffer. Whole cell lysates shown in Fig. 3, were prepared from non-stimulated or M4-stimulated DT40 cells using SDS sample buffer. Whole cell lysates or immunoprecipitates were fractionated on 8% SDS-PAGE and transferred to nitrocellulose. The blots were blocked with 5% milk in 25 mM Tris (pH 7.9), 150 mM NaCl with 0.05% Tween-20, and incubated with primary Ab for 1 h at room temperature. Filters were developed with a goat anti-mouse or donkey anti-rabbit secondary Ab conjugated to horseradish peroxidase using the Enhanced Chemiluminescence (ECL) detection system (Amersham).

For in vitro kinase assay, the immunoprecipitates were washed with

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Abbreviations: PTK, protein-tyrosine kinase; BCR, B cell antigen receptor; SH, Src homology; Ab, antibody; mAb, monoclonal antibody; TCR, T cell antigen receptor.

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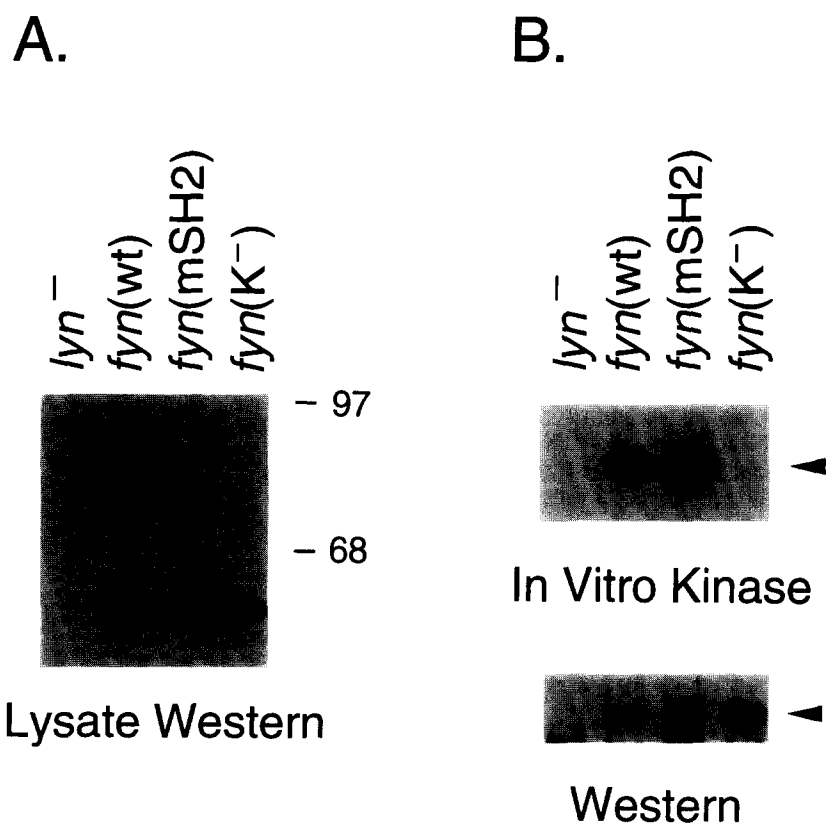

Fig. 1. Expression of mutant Fyn in Lyn-deficient DT40 cells. DT40 cells (0.5×10^6 cells/lane) expressing indicated constructs were lysed with NP-40 buffer and electrophoresed on a 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anti-Fyn Ab (A). NP-40 lysates (2×10^6 cells/lane) were immunoprecipitated by anti-Fyn Ab, divided, and half of them were used for in vitro kinase assay (top in B). The remaining half were used for Western blotting with anti-Fyn Ab (bottom in B).

kinase buffer (20 mM Tris, pH 7.4, 10 mM $MnCl_2$) after washing with lysis buffer. The immunoprecipitates were then suspended in 30 μl of kinase buffer with 1 μM ATP and 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol), and the kinase reaction was carried out at 30°C for 10 min.

2.3. Calcium mobilization and fluorescence-activated cell sorter (FACS) analysis

Measurements of intracellular free calcium were performed using fura-2/AM. Cells (5×10^6/ml) were washed once and loaded with 3 μM fura-2/AM in PBS containing 20 mM HEPES (pH 7.2), 5 mM glucose, 0.025% BSA and 1 mM $CaCl_2$. After incubation for 45 min at 37 °C, cells were washed twice and diluted to 10^6 cells/ml. Fluorescence of cell suspension was continuously monitored with a fluorescence spectrophotometer Hitachi F-2000 at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calibrated and computed as described in [12].

For FACS analysis, DT40 cells were washed, subsequently incubated with FITC-conjugated anti-chicken IgM (Bethyl Laboratories), and analyzed using a FACSort (Becton Dickinson).

3. Results

We previously showed that the function of Lyn is compensated by other Src-PTKs, Fyn and Lck [5]. To study the functional importance of the SH2 domain and catalytic activity of Src-PTK in BCR signaling, we prepared mutants of Fyn that were defective in phosphotyrosine-binding site of the SH2 domain (Arg^{176}-Glu^{177}-Ser^{178} to Asp^{176}-Glu^{177}-Cys^{178}; mSH2) and phosphotransferase activity (Lys^{296} to Met^{296}; K^-), and expressed these in Lyn-deficient DT40 B cells. The expression of mutated Fyn was monitored by immunoblotting and in vitro kinase assay (Fig. 1A and B). Even though the amount of immunoprecipitated Fyn(mSH2) was almost same as that of

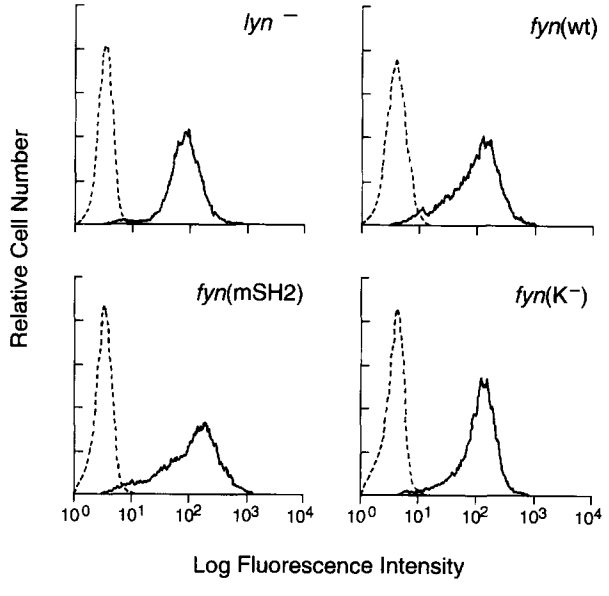

Fig. 2. Cell surface expression of BCR on DT40 cells. Transfected DT40 cells were stained with FITC-labeled anti-chicken IgM. Unstained cells are the negative control in each case.

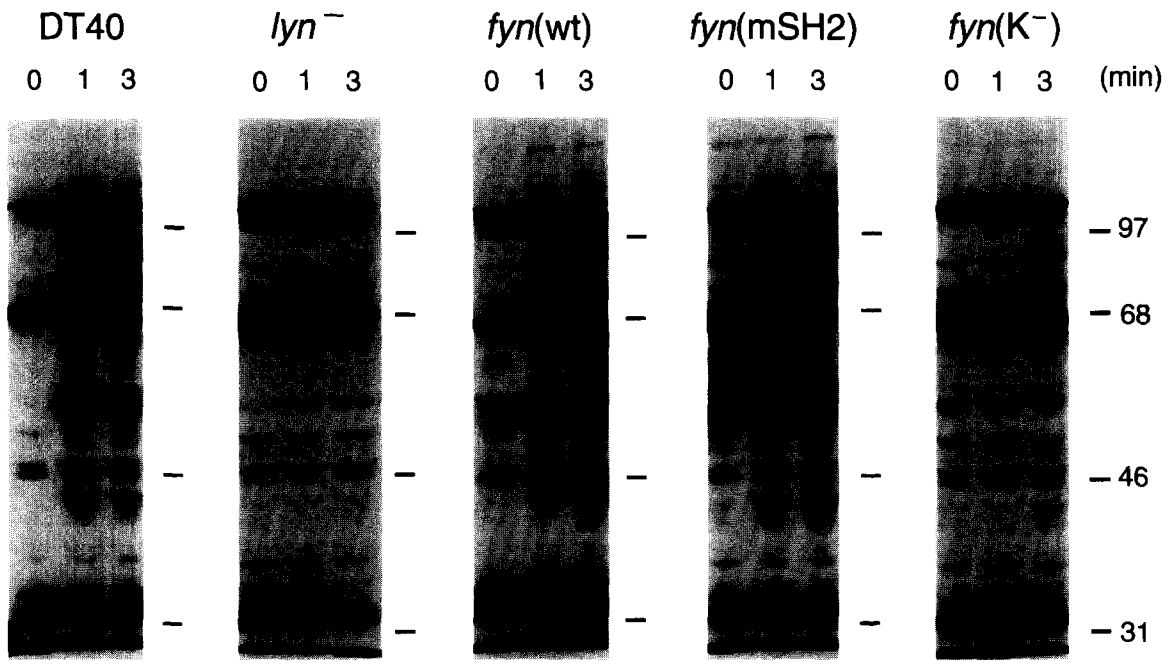

Fig. 3. Tyrosine phosphorylation of whole cell proteins following BCR stimulation. At the indicated times following the addition of M4 (4 μg/ml), whole cell lysates were prepared from 2.5×10^6 cells and loaded on a 8% SDS-PAGE. After transfer to nitrocellulose, the phosphotyrosine containing proteins were detected by immunoblotting with anti-phosphotyrosine mAb 4G10.

wildtype Fyn, autophosphorylation magnitude of Fyn(mSH2) was about 2-fold lower than that of wildtype Fyn. Phosphorylation activity for an exogenus substrate enolase was also decreased by this SH2 mutation (data not shown). Cell surface expression of BCR on these transfectants was assayed by FACS, and demonstrated essentially the same level as wildtype DT40 and Lyn-deficient cells (Fig. 2).

Tyrosine phosphorylation of cellular proteins was analyzed by anti-phosphotyrosine immunoblotting of extracts from Lyn-deficient DT40 cells expressing Fyn(wt), Fyn(mSH2), and Fyn(K^-), following anti-BCR mAb cross-linking (Fig. 3). While Fyn(wt) showed the similar phosphorylation pattern to wildtype DT40 cells, Fyn(K^-) exhibited almost same pattern as parental Lyn-deficient DT40 cells, indicating the requirement of the kinase activity of Src-PTK for BCR-induced global tyrosine phosphorylation. Although Fyn(mSH2) showed the decreased in vitro kinase activity (Fig. 1B), tyrosine phosphorylation in DT40 cells expressing Fyn(mSH2) at the resting state was somewhat increased, compared with that of Fyn(wt). Nevertheless, tyrosine phosphorylation of 40-, 78-, 90-kDa proteins was clearly induced upon receptor crosslinking in DT40 cells expressing Fyn(mSH2).

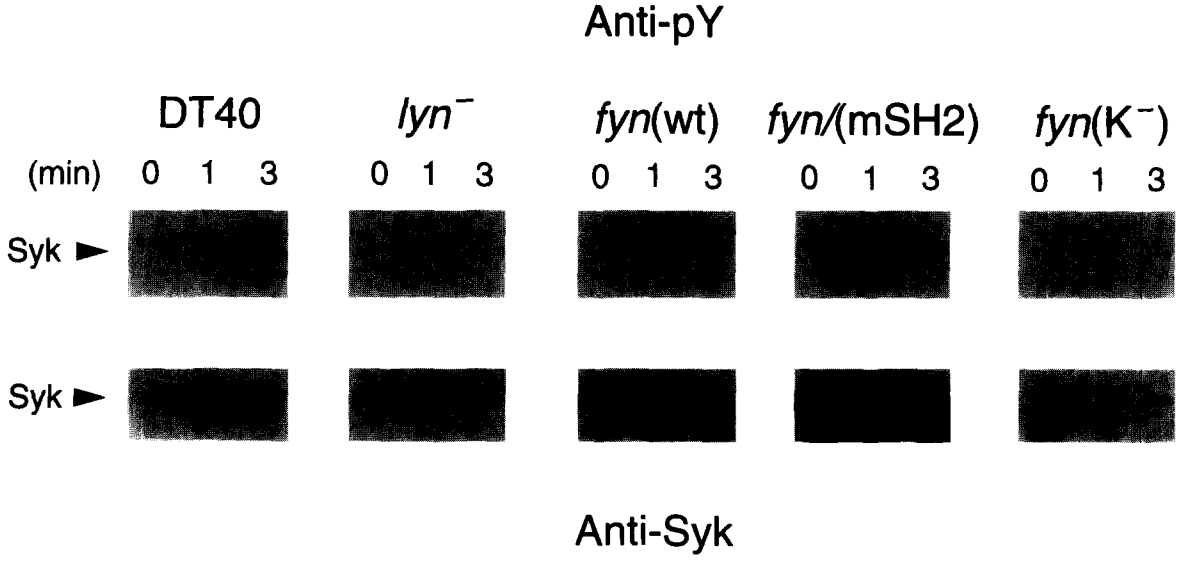

Fig. 4. Tyrosine phosphorylation of Syk after BCR stimulation. Cells were stimulated as described in Fig. 3 legend, lysed by NP-40 buffer, immunoprecipitated with anti-Syk Ab. Samples were loaded on a 8% SDS-PAGE, transferred to nitrocellulose, incubated with anti-phosphotyrosine mAb 4G10 (upper). After filters were stripped, the same blots were reprobed with anti-Syk Ab (bottom).

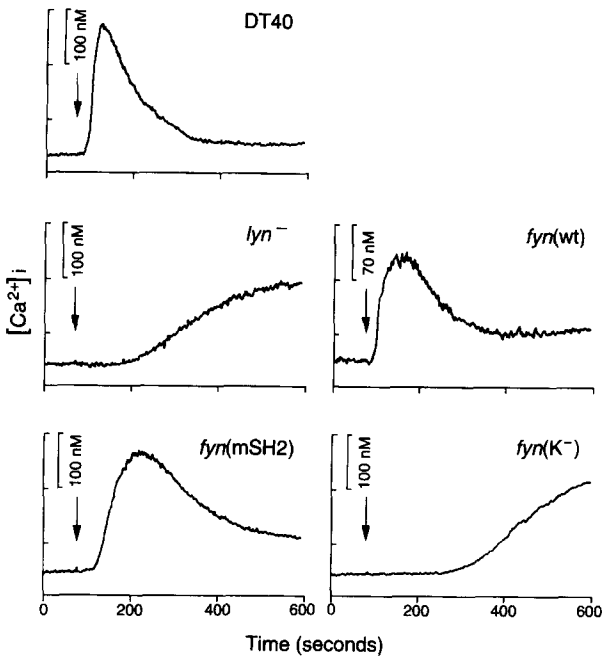

Fig. 5. Calcium mobilization upon BCR stimulation of DT40 cells. Cells were loaded with fura-2/AM, and the samples (1×10^6 cells/ml) were stimulated with M4 (1 μg/ml). As a control, cells were stimulated with thapsigargin (10 μg/ml), yielding similar amount of calcium mobilization in all clones.

One potential substrate of Src-PTK in the context of BCR signaling is Syk [13]. Thus, the effect of kinase activity and SH2 domain of Fyn on BCR-induced phosphorylation of Syk was examined by immunoprecipitation of Syk from cells expressing Fyn(wt), Fyn(K⁻), and Fyn(mSH2), followed by immunoblotting with anti-phosphotyrosine mAb (Fig. 4). Fyn(K⁻) was incapable of phosphorylating Syk, consistent with the overall tyrosine phosphorylation pattern in DT40 cells expressing this mutant (Fig. 3). In cells expressing Fyn(wt) and Fyn(mSH2), phosphorylation of Syk was induced upon receptor cross-linking, indicating that the SH2 domain of Src-PTK is not essential for BCR-induced phosphorylation of Syk.

The conclusion that the kinase activity of Src-PTK, not SH2 domain, is required for BCR-induced early signaling events was further confirmed by receptor-induced calcium mobilization data (Fig. 5). Fyn(mSH2) evoked the normal calcium mobilization upon BCR stimulation, whereas Fyn(K⁻) exhibited the similar calcium mobilization pattern to that of Lyn-deficient cells.

To test the proposed idea that Syk acts as a kinase upstream of Src-PTKs in antigen receptor signaling [14], we examined the BCR-mediated tyrosine phosphorylation of Lyn in wildtype and Syk-deficient DT40 cells. As shown in Fig. 6, tyrosine phosphorylation of Lyn in wildtype DT40 cells was sligtly induced after 1 min stimulation of M4. This BCR-induced phosphorylation was not significantly affected by the absence of Syk. These results suggest that the BCR-induced phosphorylation of Lyn is independent of Syk.

4. Discussion

Numerous studies have defined the importance of Src-PTK expression in regulating an essential component(s) in the signal transduction pathway utilized by antigen receptors [3,4]. In Lyn-deficient B cells [5] and Lck-deficient T cells [15], signals emanating from the stimulated BCR and T cell antigen receptor (TCR) appear to be blocked at an early stage in the signaling pathway. This BCR-mediated responses can be restored by introducing another Src-PTK, Fyn or Lck [5], indicating some functional redundancy among Src-PTK family, at least in early signaling events.

Mutation of SH2 domain of Fyn caused a decrease in tyrosine kinase activity in vitro (Fig. 1B). However, this SH2 mutation increased phosphotyrosine-containing proteins at the resting state of DT40 cells (Fig. 3). These results are in good agreement with the previous report that deletion of the SH2 domain of Lyn causes an increase in tyrosine phosphorylation in NIH 3T3 cells despite its lower in vitro kinase activity [16]. In contrast to the lower kinase activity of the SH2 mutants of Fyn and Lyn, mutations in Src SH2 domain lead to the dramatic increase in kinase activity [17,18]. These observations suggest that the enzymatic regulation of Fyn and Lyn is different from that of Src, and that the SH2 domain of Fyn and Lyn may play a role in repressing the ability of Fyn and Lyn to phosphorylate substrates through a mechanism independent of regulating intrinsic kinase activity of Fyn and Lyn.

It was proposed that tyrosine-phosphorylated Igα ITAM

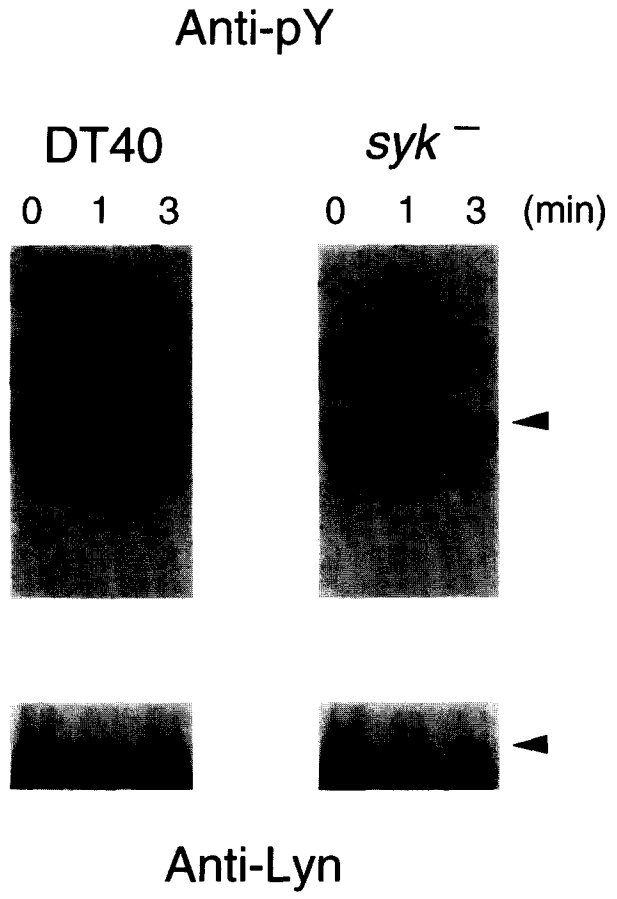

Fig. 6. BCR-induced tyrosine phosporylation of Lyn. Cells were stimulated as Fig. 3 legend, lysed by NP-40 buffer, immunoprecipitated with anti-Lyn Ab. Samples were divided and half were used for anti-phosphotyrosine immunoblotting using 4G10 (upper). Remaining immunoprecipitates were used for immunoblotting of Lyn (bottom).

induces the binding to Fyn SH2 domain (high-affinity binding), leading to the increased kinase activity of Fyn in BCR signaling process [8]. However, BCR-induced calcium mobilization and phosphorylation of Syk were not affected by the SH2 mutation of Src-PTK. Thus our results suggest that initial low-affinity binding of Src-PTK with non-phosphorylated Igα is sufficient for activating Src-PTK upon receptor cross-linking. The association of Lyn with BCR complex after receptor stimulation is readily disrupted by Nonidet P-40 [19], further supporting that Src-PTK is attached to the BCR complex through a mechanism independent of phosphotyrosine-SH2 domain interaction.

In the current study, we demonstrate that Src-PTK must have intact kinase activity for signal transduction through BCR. Induction of cellular tyrosine phosphoproteins in cells expressing Fyn(K^-) was almost the same as that in Lyn-deficient cells (Fig. 3). Moreover, in these cells, Syk was not phosphorylated by receptor stimulation (Fig. 4), indicating the requirement of catalytic activity of Src-PTK for BCR-induced phosphorylation of Syk in B cells. Since Syk associates with Src-PTK, particularly Lyn, in B cells and in vitro [20], phosphorylation of Syk by Src-PTK appears to be direct. The Src-PTK-mediated phosphorylation of Syk is about 5-fold reduced by the mutation of the putative autophosphorylation sites of Syk (Y518/519 to F518/519), indicating that these sites are one of the phosphorylation sites of Syk by Src-PTK in the context of BCR signaling [21]. Phosphorylation of the homologous tyrosine of ZAP-70 (a PTK related to Syk which is expressed in T cells and NK cells) is shown to increase its kinase activity using purified ZAP-70 [22]. Taken together, these observations strengthen the proposal that the Src-PTK associated with BCR phosphorylates Syk, enhancing the activity of Syk in BCR signaling process.

Two models have been proposed to describe the mechanistic activation of BCR or TCR. In the first, the Src-PTKs phosphorylate the tyrosine residues in the ITAM of BCR or TCR subunits which in turn recruit Syk/ZAP-70 in a SH2-phosphotyrosine dependent manner. Association of Syk/ZAP-70 to the antigen receptor results in their phosphorylation by Src-PTKs, permitting phosphorylation of additional downstream substrates [23]. A second model has been proposed in which Syk is constitutively associated with BCR or TCR and functions to activate the Src-PTKs [24]. Based on the latter model, Syk is autophosphorylated upon receptor ligation, permitting the recruitment of Src-PTKs via a SH2-phosphotyrosine dependent manner. Indeed it was demonstrated that SH2 domain of Src-PTK binds to the phosphorylated Syk/ZAP-70 in vitro [14,25]. Data presented in this paper are more consistent with the first model. Although Src-PTK-dependent phosphorylation of Syk is dramatic in BCR signaling [13], Syk did not affect phosphorylation state of Lyn (Fig. 6). Furthermore, in contrast to the functional importance of both SH2 domains of Syk in receptor signaling [21], Src-PTK SH2 domain did not affect early signaling events. Recent studies demonstrating that activation of Src-PTKs temporally proceed the activation of Syk/ZAP-70 provide additional support for the first model [26].

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