Brief Definitive Report

Syk Activation by the Src-family Tyrosine Kinase in the B Cell Receptor Signaling

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Summary

Signaling through the B cell antigen receptor (BCR) results in rapid increases in tyrosine phosphorylation on a number of proteins. The BCR associates with two classes of tyrosine kinase: Src-family kinase (Src-protein–tyrosine kinase [PTK]; Lyn, Fyn, Blk, or Lck) and Syk kinase. We have investigated the interaction between the Src-PTK and the Syk kinase in the BCR signaling. In contrast to wild-type B cells, BCR-mediated tyrosine phosphorylation of Syk and activation of its in vitro kinase activity were profoundly reduced in *lyn*-negative cells. The requirement of the Src-PTK to induce tyrosine phosphorylation and activation of Syk was also demonstrated by cotransfection of *syk* and *src*-PTK cDNAs into COS cells. These results suggest that the Src-PTK associated with BCR phosphorylates the tyrosine residue(s) of Syk upon receptor stimulation, enhancing the activity of Syk.

The antigen receptor on B lymphocytes (BCR) is a surface immunoglobulin that associates with additional molecules involved in receptor transport and signal transduction (1–5). Stimulation of the BCR initiates a biochemical cascade in which protein-tyrosine kinase (PTK) activity is the earliest known event (6, 7). This PTK activation results in the tyrosine phosphorylation of several proteins, including the BCR Ig-α, Ig-β chains (8), phosphatidylinositol (PI)-3 kinase (9, 10), guanosine triphosphate-activating protein (GAP) (11), protooncogene *vav* (12–14), and phospholipase C-γ2 (15).

Since the BCR complex does not have any intrinsic kinase activity, it is implicated that cytoplasmic PTK(s) is involved in initiating BCR signaling. One of the BCR-associated kinases is Src-PTK including Lyn, Fyn, Lck, and Blk (16–18). Recent evidence indicates that the activity of each of these enzymes is increased after cross-linking of the BCR (16). In addition to Src-PTK, BCR associates with the recently characterized Syk tyrosine kinase (19–21). Unlike the Src-PTK, Syk bears two SH2 domains and no NH_2-terminal myristoylation site (20). Syk has been shown to be tyrosine phosphorylated and activated upon cross-linking of the BCR (21, 22). At present, it is not clear whether Syk activates the Src-PTK and/or vice versa, nor which of these two types of kinase plays a more important role through the BCR signaling. In this study, we focus upon how Src-PTK affects the activity of Syk through BCR signaling.

Materials and Methods

Cell Culture and DNA Transfection. COS-7 cells were cultured in DME containing 10% FCS. Wild-type and *lyn*-negative DT40 cells were cultured in RPMI 1640 supplemented with 10% FCS. Methods to establish *lyn*-negative DT40 cells were described in detail (23). Briefly, using gene targeting constructs including chicken genomic *lyn* and drug selection markers such as Neo, we created the *lyn*-negative cells by homologous recombination. Three alleles of *lyn* locus were sequentially disrupted and after isolating the three alleles targeted clone, we confirmed that this clone has incorporated a single copy of each construct. Transfection of *lyn* cDNA into the *lyn*-negative cells restored the normal BCR-mediated functions such as Ca^{2+} mobilization or overall tyrosine phosphorylation pattern (23). Mouse *fyn* cDNA (from R. Perlmutter, University of Washington, Seattle, WA) (24), human *lyn* cDNA (American Type Culture Collection, Rockville, MD) (25) were cloned into the pcEXV-3 vector. A point mutation in the ATP binding site of porcine *syk* cDNA (20) and *fyn* cDNA was created by polymerase chain reaction. Wild-type and kinase-negative *syk* cDNAs were cloned into the pcDL-SRα296 vector (26). DNA (15 μg each DNA/60-mm dish) was transfected into COS-7 cells using the calcium–phosphate method. For DNA transfection into DT40 cells, *syk* cDNA was cloned into the pApuro vector (23). DNA was linearized, transfected into wild-type and *lyn*-negative DT40 cells (23) by electroporation, and selected in the presence of puromycin (0.5 μg/ml). The expression of Syk in drug-resistant clones was analyzed by Western blotting.

Immunoprecipitation Analysis. DT40 cells were stimulated by a mAb, M4 (27), that recognizes chicken IgM (from C. Chen, Univer-

sity of Alabama, Birmingham, AL). 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 molibrate, and 0.2 mM sodium vanadate (28) supplemented with protease inhibitors as described in (20). Cell lysates were sequentially incubated (1 h at 4°C for each incubation) with antibodies and protein A–Sepharose. Polyclonal rabbit antisera to Fyn and Lyn were purchased from Oncogene Science Inc. (Manhasset, NY) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively. Antibody against porcine Syk was already described (20). For immunoblotting, samples were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Corp., Arlington Heights, IL). Filters were incubated with a monoclonal antibody 4G10 (antiphosphotyrosine antibody; Upstate Biotechnology Inc., Lake Placid, NY), anti-Syk, or anti-Lyn antibody. After washing, filters were developed using a sheep anti–mouse or a donkey anti–rabbit IgG antibody conjugated to horseradish peroxidase and enhanced chemiluminescence (ECL) (Amersham Corp.).

In Vitro Kinase Assay. Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA) supplemented with phosphatase inhibitors and protease inhibitors described above. Cell lysates were immunoprecipitated with anti-Syk antibody and protein A–Sepharose. The immunoprecipitates were washed four times with lysis buffer followed by a final wash with 20 mM Hepes, pH 8, and 150 mM NaCl. Added to each sample was 50 μl of kinase buffer (20 mM Hepes, pH 8, 150 mM NaCl, 10 mM magnesium acetate) containing 10 μCi of γ-[^{32}P]ATP (>3000 Ci/mmol). The reactions were allowed to incubate at 30°C for 10 min and terminated by the addition of sample loading buffer.

Results and Discussion

Since Syk is drastically tyrosine phosphorylated upon cross-linking of the BCR (21, 22), we examined the possibility that Src-PTK phosphorylates Syk through the BCR signal transduction. To address this issue, wild-type and *lyn*-negative DT40 chicken B cell lines were used. RNA blot analysis of DT40 cells revealed that *lyn* and *syk* are expressed in this cell line. Transcripts of the *src*, *lck*, *fyn*, *blk*, *yes*, *hck*, or *zap-70* could not be detected, showing that *lyn* and *syk* are expressed dominantly. Stimulation of BCR evoked a drastic change in tyrosine phosphorylation on a number of proteins, whereas the induction of phosphorylation on many of these substrates was abolished in *lyn*-negative cells (23). Although endogenous Syk is expressed in DT40 cells, we transfected porcine *syk* cDNA into wild-type and *lyn*-negative cells in order to easily detect the expression of Syk. Wild-type and *lyn*-negative DT40 cells transfected with *syk* cDNA were stimulated with anti-BCR mAb M4, and immunoprecipitated with anti-Syk antibody which recognizes only porcine Syk. These immunoprecipitates were analyzed by antiphosphotyrosine mAb 4G10 and anti-Syk Ab (Fig. 1 *A*). Syk was tyrosine phosphorylated upon BCR stimulation in wild-type DT40 cells, with very rapid kinetics. In contrast, the induction of tyrosine phosphorylation on Syk in *lyn*-negative cells was barely detected; overexposure of this blot showed the weak 4G10 reactive species of Syk by 3 min stimulation of BCR. These results showed that Lyn is primarily involved in the induction of tyrosine phosphorylation of Syk through BCR signaling in DT40 cells. The antiphosphotyrosine mAb reactive species of Syk migrated a little more slowly than anti-Syk Ab reactive one. This difference probably reflects the tyrosine phosphorylation of Syk.

Syk has been already shown to be activated by cross-linking of the BCR (21, 22). To examine whether this activation is dependent on Src-PTK, we carried out in vitro kinase assay of Syk in wild-type and *lyn*-negative DT40 cells after BCR stimulation. As shown in Fig. 1 *B*, cross-linking of BCR stimulated autophosphorylation activity of Syk in wild type cells, consistent with previous reports (21, 22). This rapid activation of Syk was not observed in *lyn*-negative cells. However, a 5-min stimulation of BCR on *lyn*-negative cells resulted in about a twofold increase of autophosphorylation activity of Syk. In wild-type DT40 cells, the dominant autophosphorylated Syk after BCR stimulation migrated a little more slowly than the major Syk molecule, suggesting that the antiphosphotyrosine mAb reactive Syk corresponds to this activated autophosphorylated molecule. These results suggest that Lyn-dependent phosphorylation increases the autophosphorylation activity of Syk through BCR stimulation in DT40 cells.

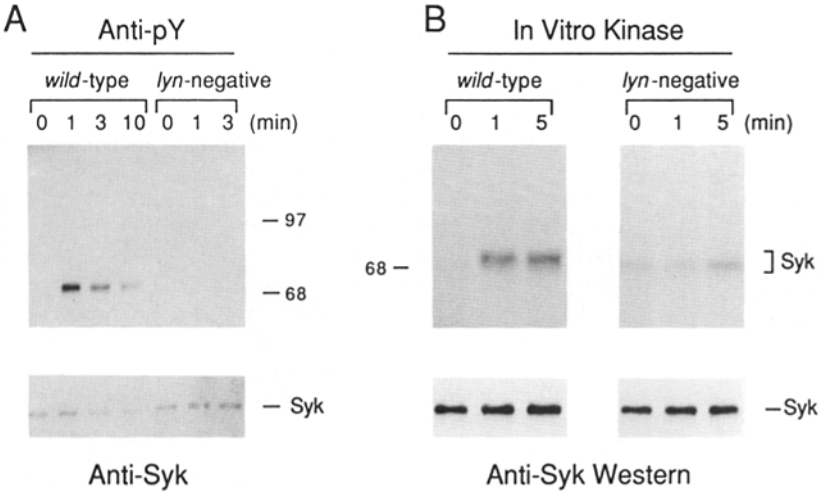

Figure 1. Stimulation of tyrosine-phosphorylation on Syk (*A*) and autophosphorylation activity of Syk (*B*) in wild-type and *lyn*-negative DT40 cells. (*A*) Cells (2×10^6/ml) were stimulated by mAb M4 (4 μg/ml) for indicated time, lysed by NP-40 buffer, and immunoprecipitated with anti-Syk Ab. Immunoprecipitates were electrophoresed on an 8% SDS-PAGE gel, transferred, and incubated with antiphosphotyrosine mAb 4G10 (*top*). After the filter was stripped, the same blot was reprobed with anti-Syk Ab (*bottom*). (*B*) Cells (2×10^6/ml) were stimulated by mAb M4 for indicated time, lysed by modified RIPA buffer, and immunoprecipitated with anti-Syk Ab. Immunoprecipitates were divided, and half of them were used for in vitro kinase assay (*top*). The remaining half were used for Western blotting with anti-Syk Ab (*bottom*). Samples were electrophoresed on 8% SDS-PAGE gels.

To provide more insights into the interaction between Src-PTK and Syk, we cotransfected the *src*-PTK and *syk* cDNAs into COS cells (Fig. 2). Tyrosine phosphorylation of Syk was only observed in the presence of Lyn, as judged by antiphosphotyrosine mAb reactivity. In contrast, the tyrosine phosphorylation of Lyn was not drastically changed by the presence of Syk; the increased tyrosine phosphorylation of Lyn in the presence of Syk is accounted for by the expression extent of Lyn (data not shown). Comparison of anti-Syk and antiphosphotyrosine blotting showed that the antiphosphotyrosine mAb reactive molecule is corresponding to the upper band of the anti-Syk Ab reactive species. To examine the specificity of each Src-PTK member in its capability of phosphorylation on Syk, cotransfection of *fyn* or *lck* with *syk* cDNAs was carried out. Similar to cotransfection with *lyn* and *syk* cDNAs, Syk was tyrosine phosphorylated in the presence of Fyn (Fig. 2) or Lck (data not shown), indicating no strict specificity for Src-PTK members.

To exclude the possibility that the observed tyrosine phosphorylation of Syk is due to the enhanced autophosphorylation activity induced by Src-PTK, cotransfection of kinase-negative *syk* and *lyn* cDNAs was carried out. The Syk kinase-negative mutant was also tyrosine phosphorylated in the presence of Lyn, suggesting that Lyn phosphorylates Syk directly, rather than affects the autophosphorylation activity of Syk. However, in the presence of Lyn, the extent of tyrosine phosphorylation of the wild-type Syk was about threefold higher than that of the kinase-negative one (Fig. 3, *left*). Thus, this observation suggests that tyrosine phosphorylation of Syk by Lyn may enhance Syk autophosphorylation or that Lyn may not only phosphorylate Syk, but also enhance Syk autophosphorylation activity through a phosphorylation-independent mechanism. Cotransfection experiments with kinase-negative *fyn* and *syk* cDNAs showed that kinase activity of Fyn is essential for phosphorylation of Syk (Fig. 3, *right*).

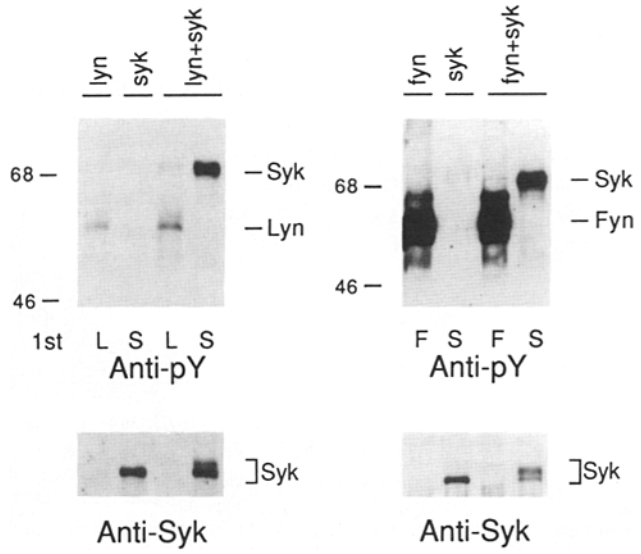

Figure 2. Tyrosine phosphorylation of Syk induced by Src-PTK in COS cells. COS cells were transfected with indicated combinations of cDNAs. Cells were lysed by NP-40 buffer, and immunoprecipitated with anti-Lyn (*L*), anti-Syk (*S*), or anti-Fyn (*F*) Ab. Immunoprecipitates were electrophoresed on 8% SDS-PAGE gels, transferred, and incubated with antiphosphotyrosine mAb 4G10 (*top*). After filters were stripped, the same blots were reprobed with anti-Syk Ab (*bottom*).

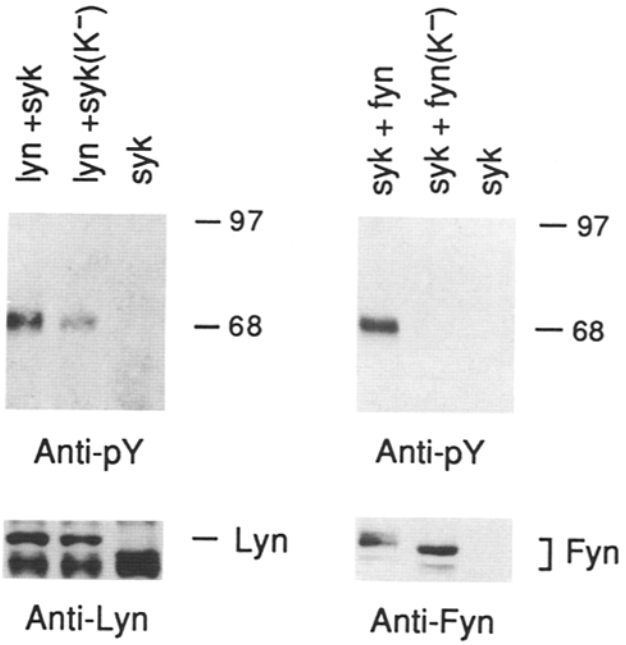

Figure 3. Effect of kinase activity (Syk or Src-PTK) on tyrosine phosphorylation of Syk in COS cells. COS cells were transfected with indicated combinations of cDNAs. Kinase-negative *syk* or *fyn* cDNA is shown by syk(K^-) or fyn(K^-), respectively. Transfected COS cells were lysed by NP-40 buffer, and immunoprecipitated with anti-Syk Ab. The immunoprecipitates were electrophoresed on 8% SDS-PAGE gels, transferred, and incubated with antiphosphotyrosine mAb 4G10 (*top*). Lysates were blotted, and incubated with anti-Lyn or anti-Fyn Ab (*bottom*).

To determine the change in Syk activity induced by Src-PTK in COS cells, we compared the autophosphorylation activity of Syk in the absence or presence of Src-PTKs. Fig. 4 shows that the autophosphorylation activity of Syk is increased by Lyn, or Fyn, and that most of this enhanced activity corresponds to the antiphosphotyrosine mAb reactive species of Syk (Fig. 4, upper band in anti-Syk blotting; *bottom left*). Under these conditions, the association of Lyn or Fyn with Syk could not be detected, as judged by the appearance of phosphate-labeled Lyn or Fyn. Autophosphorylation activity of Lyn or Fyn was not changed by the presence of Syk (data not shown). The overall tyrosine phospho proteins of the COS cell extract transfected with *syk* alone or cotransfected with *syk* and *lyn* cDNAs, was also analyzed with an antiphosphotyrosine mAb. The tyrosine phospho proteins of the COS cell extract cotransfected with *syk* and *lyn* cDNAs was significantly increased, compared with either alone. This induction of tyrosine phosphorylation of cellular proteins is attributed to the enhanced kinase activity of Syk induced by Lyn, since the tyrosine phospho proteins of the COS cell extract cotransfected with *lyn* and kinase-negative *syk* cDNAs

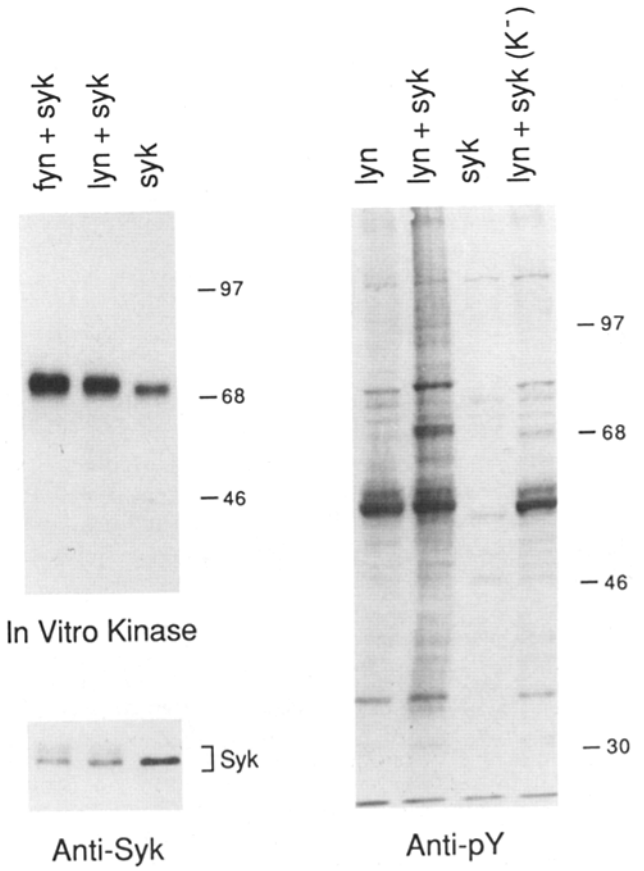

Figure 4. Stimulation of phosphorylation activity of Syk by Src-PTK in COS cells. COS cells were transfected with indicated combinations of cDNAs. Transfected COS cells were lysed by modified RIPA buffer, and immunoprecipitated by anti-Syk Ab. Immunoprecipitates were divided, and half of them were used for in vitro kinase assay (*top left*). The remaining half were used for Western blotting with anti-Syk Ab (*bottom left*). Transfected cells were dissolved in NP-40 buffer, electrophoresed on an 8% SDS-PAGE gel (same amount of protein content per lane), blotted, and incubated with antiphosphotyrosine mAb 4G10 (*right*).

was similar to that transfected with *lyn* cDNA alone (Fig. 4, *right*).

Analysis of wild-type and *lyn*-negative DT40 B cells revealed that the tyrosine phosphorylation of Syk through BCR stimulation requires Lyn in DT40 cells. However, these data do not distinguish the possibility that Syk is phosphorylated directly or indirectly by Lyn through BCR signaling. Kinase-negative Syk was tyrosine phosphorylated only in the presence of Lyn and kinase-negative Fyn was unable to phosphorylate Syk in COS cells, suggesting that Syk is directly phosphorylated by Src-PTK.

Even in the *lyn*-negative DT40 cells, autophosphorylation activity of Syk is about twofold stimulated by cross-linking of BCR, indicating that Syk is activated by receptor aggregation in the absence of Lyn to some extent. This result is consistent with the previous report that stimulation of the Syk chimera bearing a CD16 extracellular domain and a Syk kinase intracellular domain, induced protein tyrosine phosphorylation (29). However, in the wild-type DT40 cells, BCR-mediated autophosphorylation activity of Syk was more rapid and drastic. These results demonstrate that Lyn enhances the phosphorylation activity of Syk through BCR signaling in DT40 cells. The tyrosine phosphorylated species of Syk seems to have an activated autophosphorylation activity, judged by the gel mobility shift, implicating the correlation between phosphorylation of Syk and its activity. This is also supported by the observation that in COS cells, the tyrosine phosphorylated species of Syk induced by Lyn or Fyn has an increased autophosphorylation activity. In TCR signaling, it was already proposed that ZAP-70, which is a homologue of Syk, requires Lck or Fyn for synergistic induction of PTK activity (30). Our data using COS cells are consistent with the previous report that coexpression of Src-PTK with ZAP-70 leads to a remarkable increase in tyrosine phosphorylation (31, 32).

Although our results suggest that the phosphorylation of Syk by Src-PTK activates its kinase activity, it is still possible that Syk may be activated through another mechanism by Src-PTK. For example, the association of Src-PTK with Syk may enhance Syk activity. Thus, our observations provide the possibility that the activation of Syk through phosphorylation by Src-PTK is one of the mechanisms accounting for vast tyrosine phosphorylation through BCR stimulation.

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