Nonredundant Roles of Src-Family Kinases and Syk in the Initiation of B-Cell Antigen Receptor Signaling

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Nonredundant Roles of Src-Family Kinases and Syk in the Initiation of B-Cell Antigen Receptor Signaling

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When a BCR on a mature B cell is engaged by its ligand, the cell becomes activated, and the Ab-mediated immune response can be triggered. The initiation of BCR signaling is orchestrated by kinases of the Src and Syk families. However, the proximal BCR-induced phosphorylation remains incompletely understood. According to a model of sequential activation of kinases, Syk acts downstream of Src family kinases (SFKs). In addition, signaling independent of SFKs and initiated by Syk has been proposed. Both hypotheses lack sufficient evidence from relevant B cell models, mainly because of the redundancy of Src family members and the importance of BCR signaling for B cell development. We addressed this issue by analyzing controlled BCR triggering ex vivo on primary murine B cells and on murine and chicken B cell lines. Chemical and Csk-based genetic inhibitor treatments revealed that SFKs are required for signal initiation and Syk activation. In addition, ligand and anti-BCR Ab–induced signaling differ in their sensitivity to the inhibition of SFKs. The Journal of Immunology, 2013, 190: 000–000.

The earliest events of immunoreceptor signaling include tyrosine phosphorylation catalyzed by kinases of the Src and Syk families. Src family kinases (SFKs) are cytoplasmic proteins that are associated with the plasma membrane via lipid anchors at their N termini (1, 2). At least five family members (Lyn, Blk, Fyn, Fgr, and Yes) are expressed in B cells (3). The main mechanism underlying the regulation of SFK activity depends on the phosphorylation of two key tyrosine residues. Phosphorylation of the C-terminal tyrosine (Y527 for chicken Src) is mediated by Csk and counteracted by phosphatases CD45 and CD148 in lymphocytes. This phosphorylation induces a closed conformation and inhibition of the kinase (4–7). In contrast, trans-autophosphorylation of the activation loop tyrosine (Y416 for chicken Src) is crucial for full kinase activity (8–10).

Syk and ZAP70 are the only members of the Syk kinase family. The recruitment of these kinases to the plasma membrane is mediated by the binding of their tandem SH2 domains to phosphorylated ITAMs in the immunoreceptor-associated chains (11–13). Activation of Syk is achieved via binding to phosphorylated ITAM and/or by phosphorylation of tyrosine residues in the interdomain B between the C-terminal SH2 domain and the kinase domain (e.g., Y346 for murine Syk) (14–16). These tyrosines seem to be phosphorylated mainly by SFKs, although autophosphorylation of these sites has also been observed under various conditions (14, 17, 18). Similar to SFKs, Syk family kinases contain (auto)phosphorylation sites in the activation loop (Y519/Y520 for murine Syk) (17). Their phosphorylation is critical for Syk-mediated signal transduction, although the exact mechanism remains a matter of debate (19–22).

Engagement of the BCR by Ag leads to the phosphorylation of ITAM in the BCR-associated Igα- and Igβ-chains (23). According to the original model of immunoreceptor signaling, this initial ITAM phosphorylation is mediated by SFKs and is a prerequisite for Syk family kinase recruitment and activation (24). In the analogous situation of TCR, this model is well supported by the existing data (25–27). In B cells, the critical role of BCR-associated ITAMs has also been demonstrated (28). However, the presumption of an essential role of SFKs in the catalysis of ITAM phosphorylation has been challenged by unexpected findings in animals deficient in several SFK members. Mice deficient in Lyn, a predominant SFK in B cells, did not display the predicted inhibition of BCR signaling. Rather, Lyn−/− mice exhibited enhanced B cell activation in response to BCR stimulation and developed an autoimmune syndrome, most likely because of hypophosphorylation of inhibitory receptors (29–31). The dispensability of Lyn in BCR signaling was initially explained by compensation by other SFK family members, mainly Fyn and Blk (32). Indeed, Lyn/Fyn/Blk triple-deficient mice displayed profound block in B cell development that was not observed in Lyn−/− mice. However, the triple-deficient mice did not exhibit any defect in anti-Igβ Ab–induced BCR-like proximal signaling at the pro-B stage (33). These data suggest that SFKs are not essential for the initiation of BCR signaling and support the hypothesis of SFK-independent phosphorylation of Igα and Igβ.

In contrast, the ability of Lyn to positively regulate BCR signaling was demonstrated in mice expressing a constitutively active Lyn mutant, which led to the hyperactivation of important signaling molecules, including Syk (34). A positive role of SFKs in proximal BCR signaling was also revealed in CD45/CD148 double-deficient mice (4) and in Lyn-deficient DT40 chicken B cell lymphoma cells (35), which both exhibited a delayed and weakened BCR response. However, neither of these models confirmed the absolute requirement for SFK activity in BCR signaling, as the signaling was far from completely blocked. In addition, the in-
completely inhibited BCR signaling in Lyn−/− DT40 cells, which are believed to express no other SFKs, was another important line of evidence supporting the existence of SFK-independent BCR signaling (32, 35, 36).

The putative SFK-independent BCR signaling is often explained by the ability of Syk to phosphorylate BCR-associated ITAMs (16, 37, 38). Indeed, Syk was reported to phosphorylate both ITAM tyrosines in Igx when coexpressed in the insect cell line S2 (15), and Syk, but not its T cell paralogue ZAP70, was reported to phosphorylate ITAMs in vitro and in COS cells (14, 39). Moreover, ectopic expression of Syk was able to rescue TCR signaling in the J.CaM1.6 T cell line, which is deficient in SFK Lck (40).

The existence of SFK-independent BCR signaling and the ability of Syk to phosphorylate BCR-associated ITAMs are broadly accepted (11, 16, 37, 38), although several important related issues have been poorly addressed so far, including the relationship between SFK-dependent and -independent signaling. SFK/Syk interplay, and the overall role of SFKs as both positive and negative regulators in the proximal steps of BCR signaling. Another important problem is that most of the evidence supporting the model of Syk-mediated ITAM phosphorylation is based on in vitro analyses and overexpression studies in cells of non–B-lymphoid origin. One of the reasons for the apparent neglect of these questions may be the lack of a suitable B cell model of SFK deficiency arising from the redundancy of multiple Src family members in B cells and their requirement for B cell development.

In this study, we used a number of novel tools including phospho-specific Abs and chemical- and protein-based inhibitors of SFKs and Syk to revisit this issue. Our results show that SFKs are indispensable for BCR signaling triggered by Ag. In contrast, we could not find any evidence for Syk-mediated ITAM phosphorylation in B cell lines and primary B cells. This brought us to the conclusion that for BCR signaling, the model of sequential activation of Src and Syk family kinases is also fully valid.

Materials and Methods

Abs and reagents

Abs to the following Ags were used: phospho-ERK (T202/Y204), amino acid numbers correspond to mouse protein, if not indicated, phospho-Igκ (Y182), phospho-Syk (Y346), phospho-Syk (Y519/520), phospho-Src (Y416), phospho-Lyn (Y507), phospho-Akt (S473), phospho-Src (chicken Y527), non–phospho-Src (chicken Y527), Lyn (all rabbit from Cell Signaling Technology, Danvers, MA), phospho-SLP65 (Y84) (BD Pharmingen, BD Biosciences, San Jose, CA), GAPDH (rabbit; Sigma-Aldrich, St. Louis, MO), c-Src (rabbit, N-16; Santa Cruz Biotechnology, Santa Cruz, CA), CD3-PE (ExBio Praha, Vestec, Czech Republic), CD11b-FITC, CD19-FITC, CD21-FITC, Thy1.1-FITC (both eBioscience, San Diego, CA), Ig κ L chain-FITC (AbD Serotec, Kidlington, U.K.), CD69-allophycocyanin, Ig κ L chain-allophycocyanin (both BioLegend, San Diego, CA), IgM-Pacific Blue (clone B76; conjugated in-house; American Type Culture Collection, Manassas, VA), B220-Pacific Blue (clone RA3-6B2; conjugated in-house; obtained from Dr. R. Coffman, DNAX Research Institute, Palo Alto, CA), B220-Pacific Blue (clone RA3-6B2, conjugated in-house). The following secondary Abs were used: goat anti-rabbit–Dy649 (Jackson Immunoresearch, West Grove, PA), goat anti-mouse–Alexa Fluor 488 (Invitrogen, Carlsbad, CA), goat anti-mouse–HRP IgG specific (Sigma-Aldrich), and goat anti-rabbit–HRP (BioRad, Hercules, CA).

The following reagents were used for cell stimulation: anti-CD3 Ab (hamster IgG1 145–2C11; Exbio Praha), goat anti-Armenian hamster IgG, goat anti-mouse IgG (F(ab)2), goat anti-chicken IgY (F(ab)2) Abs (all from Jackson Immunoresearch, 4-hydroxy-3-nitrophenylacetate (NP) conjugated to BSA at molecular ratio 25:1 (NP25-BSA) and 5:1 (NP5-BSA), 4-hydroxy-3-iodo-5-nitrophenylacetate (NP) conjugated to BSA at molecular ratio 25:1 (NP25-BSA), and NP conjugated to Ficol at molecular ratio 401, 50% (NP50-Ficol) (all from Biosearch Technologies, Novato, CA). The following cell lines were used: P8, Syk inhibitor IV–BAY 61-3606 (both from Calbiochem, Merck, Darmstadt, Germany), and piceatannol (Sigma-Aldrich).

Cell lines and primary cells

All of the cell lines were cultured in the indicated media supplemented with 10% FBS, 2 mM glutamine, 20 μg/ml gentamicin, 50 μg/ml streptomycin, and 10°/U/ml penicillin at 37°C in 5% CO2. The K46-mBSA cells (obtained from Dr. J. Cambier, National Jewish Medical Research Center, Denver, CO) were cultivated in IMDM, the Phoenix Eco (Origene, Rockville, MD) cells were cultivated in DMEM. The DT40 cells (obtained from Dr. A. Weiss, University of California, San Francisco, San Francisco, CA, with kind permission from Dr. T. Kurosaki, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) were cultivated in RPMI supplemented with 1% chicken serum.

Murine spleens were collected from healthy C57BL/6 mice (Institute of Molecular Genetics Animal Facility), B1-8i−/− transgenic mice (41) (The Jackson Laboratory, Bar Harbor, ME), or B1-8i−/− heterozygotes. The B1-8i−/− and B1-8i+/+ were phenotypically identical and used interchangeably. A single-cell splenocyte suspension was prepared, and erythrocyte lysis was performed in ACK buffer (150 mM NH4Cl, 0.1 mM EDTA (disodium salt), 1 mM KHCO3). Splenic B cells were isolated from the whole-spleen suspension by negative selection using anti-CD43 and anti-CD11b magnetic beads on an AutoMACS magnetic cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany), and the purity (>95%) was determined by flow cytometry. Lambda L chain–positive B cells were isolated from the whole-spleen suspension as B220 Igκ+ double-positive cells using a BD Influx (BD Biosciences). The experiments were approved by the Institutional Review Board and Animal Care and Use Committee of the Institute of Molecular Genetics.

DNA transfection and transduction

LAT-Csk-mOrange, LAT-FRB-IRES-Thy1.1, and Csk-FKBP-mOrange in the MSCV vector were described previously (42). Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer’s instructions for the transfection of Phoenix Eco cells to produce viral particles. Retrovirus-containing supernatants were then harvested, supplemented with Polybrene (10 μg/ml; Sigma-Aldrich), and added to the cells. The cells were then centrifuged at 1250 × g for 90 min. Infected K46 cells were sorted by a BD Influx FACs (BD Biosciences). B220 Igκ+ splenocytes from B1-8i+/+ or B1-8i−/− mice were cultivated for 3 d in IMDM supplemented with LPS (1 μg/ml), IL-4 (50 ng/ml), and anti-B CR Ab (anti-BCR; 5 μg/ml) before the infection. After the infection, the cells were cultivated in IMDM supplemented with LPS and IL-4 for 2 d and used in experiments.

Cell activation

For Western blotting, the cells were washed and resuspended in serum-free IMDM at a concentration of 25–50 × 106 cells/ml. In some experiments, the indicated inhibitors were added 5–15 min before activation. Subsequently, the cells were stimulated as indicated at 37°C. The activation of cells was stopped by addition of an equal volume of 0.1% SDS-PAGE sample buffer, followed by heating of the samples (94°C for 3 min) and sonication. The samples were analyzed by SDS-PAGE followed by Western blotting. For pervanadate-induced activation, the cells (5 × 105 cells/ml) were incubated with 1 mM perva- nadate for 20 min.

Cell lines were harvested and resuspended in IMDM/10% PBS and prewarmed for 10 min at 37°C with the indicated inhibitor, if applicable. Cells were stimulated as indicated, and the activation was stopped by fixation in 2% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA).

If not indicated otherwise, we used the following doses of activators: 10 μg/ml anti-BCR Ab, 1 μg/ml NP-BSA, 1 μg/ml NP-Ficol.

Flow cytometry

For calcium measurement, the cells were loaded with 5 μM Fura Red dye (Invitrogen) in serum-free IMDM at 37°C for 30 min. Subsequently, the cells were labeled with the indicated Abs in PBS with 1% BSA and 10% FBS, and cells were placed on ice for 5 min at 37°C and incubated with the indicated inhibitors, if applicable, before the measurement. The relative intracellular calcium concentration was measured as a ratio of the Fura Red (43) fluorescence intensities elicited by excitation wavelengths of 405 nm (detection at 635–720 nm) and 488 nm (detection at 655–695 nm). The cells were stimulated as indicated 30 s after the measurement began. Before specific marker-dependent gating, spleen lymphocytes were gated based on their forward scatter versus side scatter properties. The median relative calcium influx is shown.

Intracellular staining was performed on cells fixed in 2% paraformaldehyde at room temperature and permeabilized in 90% methanol on ice.
The cells were stained with the indicated Abs in PBS/15% goat serum at room temperature for 45 min. Data were collected using a BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, San Carlos, CA). For the calcium response analysis, FlowJo’s Kinetic platform was used. The resulting curves were smoothed using the Moving Average function of the software (displayed value for each time point is the result of averaging three measured values, including this time point and its neighbors).

**Quantitative RT-PCR**

RNA was isolated using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). Reverse transcription was performed with RevertAid reverse transcriptase (Fermentas; Thermo Fisher Scientific) using anchored oligo (dT)20 primers. Quantitative PCR was performed using a LightCycler 480 SYBR Green I Master chemistry (Roche Applied Science, Indianapolis, IN) in triplicates with following primers (5′-3′): FYN-forward, TCCTCTCACAGGGACGCT, FYN-reverse, CCAGTCTCTCCGGAGCCTG; LYN-forward, AAATGAAAGTTATGGAGCCTG; LYN-reverse, GAGCAGGGATTGGCCCTTCA, YES-forward, AGCCAGATTGTATGCGTGTG, YES-reverse, TTCCTGACTACATTCTCGG; YRK-forward, GTGCGGTACCCAGGGATGAA, YRK-reverse, CGACGTC- GCACATCACATC; SRC-forward, CACCGTGACGTCGCCTCAG, SRC-reverse, CACGAGAAGGGGCTAAA, LCK-reverse, TCCGGCCGTAGGTAAC; HCK-reverse, TCTGTGTTCATCATGTTGCTTGC; LCK-forward, CAGGCACCATCACATC; HCK-forward, AGAGAAGATGCAGTTAGGCCG, GATATGGTCGGT; YES-reverse, TTCCCGATTCACCATTCCTGG; YRK-reverse, GAGCCAGGAGTTGCCTTTCA, YES-forward, AGCAGCATTAGCTGT; LYN-forward, AAATTGAAAGTTATTGAGGAGCTGG, LYN-reverse, GATCTCTCATACAGGGACGCT, FYN-reverse, CCAGTCTCCTTCCGGAGCCTG; LYN-forward, AAATTGAAAGTTATTGAGGAGCTGG, LYN-reverse, GATCTCTCATACAGGGACGCT, FYN-reverse, CCAGTCTCCTTCCGGAGCCTG.

**Statistics**

For the calculation of statistical significance, a two-tailed Student t test (unequal variance) was used. All of the presented data are representative of at least three independent experiments.

**Results**

**Inhibition of SFKs delays but does not abolish the BCR signaling**

The primary focus of this study was the role of SFKs and Syk in the initiation of BCR signaling. Because several reports supported the concept of SFK-independent BCR signaling and/or the ability of Syk to initiate the BCR transduction cascade by phosphorylating ITAMs in Igα/Igβ, we evaluated the effects of the SFK inhibitor PP2 (44) on BCR signaling in splenic B cells and on TCR signaling in splenic T cells. We cross-linked the BCR or TCR complexes using an anti-IgM F(ab)2 polyclonal Ab (anti-BCR Ab, anti-BCR) or hamster anti-CD3 Ab in combination with an anti-hamster Ab (anti-TCR), respectively. Whereas 5 μM PP2 was able to completely inhibit calcium influx after TCR stimulation, the BCR-induced calcium response was delayed but not diminished (Fig. 1A, gating strategy in Supplemental Fig. 1A). Interestingly, after 5 μM PP2 treatment, phosphorylation of the SFK activation loop tyrosine in T cells was higher than that in B cells, suggesting higher residual SFK activity in T lymphocytes (Supplemental Fig. 1B). An increase in PP2 concentration to 20 μM further delayed the calcium influx but again did not diminish the amplitude at later time points (Fig. 1B). In contrast, the inhibition of Syk using Syk inhibitor IV (45) did not delay the initiation of calcium response; rather, it reduced the amplitude during the entire monitored period in a concentration-dependent manner (Fig. 1C). Similar effects were observed after the inhibition of Syk by piceatannol (Supplemental Fig. 1C). BCR-induced phosphorylation of Igα and Syk, as well as the downstream signaling protein Erk, was delayed in the presence of PP2 in a manner similar to that of the delay in the calcium response (Fig. 1D).

The relative resistance of BCR signaling to the inhibition of SFKs and the differential effects of SFK and Syk inhibitors on BCR signaling could be explained by two hypotheses. Either the SFK inhibition was incomplete and the residual SFK activity was sufficient to initiate BCR signaling, or there is an alternative mechanism for BCR activation that is independent of SFKs. To analyze the SFK activity in PP2-treated cells, we reprobed the membranes from Fig. 1D with an Ab targeting the activation loop phosphorytase in the kinase domain. Although this tyrosine was substantially hypo-phosphorylated in the presence of PP2 in resting cells, after BCR triggering, it became markedly phosphorylated with a delay similar to that of the other examined proteins (Fig. 1E). These results suggest that the residual SFK activity may be responsible for the initiation of the observed signaling events in the presence of PP2.

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Inhibition of SFKs delays BCR signaling. (A) Mouse splenocytes were stained for CD19 and CD11b or for CD3 and CD11b, and treated or not with 5 μM PP2. T cells were stained with saturating anti-CD3 hamster Ab on ice before activation by the anti-hamster secondary Ab (10 or 50 μg/ml) and gated as CD19+/CD11b+ cells. B cells were activated by anti-BCR (10 or 50 μg/ml) and gated as CD3+/CD11b+ cells. The calcium influx was monitored. See Supplemental Fig. 1A for analysis of the gating strategy. (B and C) Mouse splenocytes were stained for CD3 and CD11b, activated by anti-BCR in the absence of the indicated concentration of PP2 or Syk inhibitor IV, and the calcium influx was monitored. B cells were gated as CD3+/CD11b+. (D) Western blot analysis of lysates from splenic B cells activated by anti-BCR in the indicated concentration of PP2. Samples were collected at the indicated time points after activation, and phosphorylation of Syk, Erk, and Igα was determined by Western blotting. (E) The membranes from (D) were reprobed with an Ab to SFK pY416.

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Inhibition of SFKs blocks BCR signaling induced by a BCR ligand

Although BCR cross-linking via a specific Ab is widely used to mimic the BCR engagement by its ligand, such cross-linking could potentially induce effects that do not occur upon physiological BCR triggering. To analyze the effects of SFK inhibition on ligand-induced BCR signaling, we used the B cell lymphoma cell line K46-wt (here referred to as K46 wild type [WT]), which expresses transgenic BCR (B1-8) specific for NP (46). NP-conjugated BSA (NP25-BSA) induced a rapid calcium influx, but the increase was more transient than the anti-BCR–triggered response (Fig. 2A). In agreement with our data from primary B cells, anti-BCR–induced signaling was only delayed by the inhibition of SFKs in K46 cells. Surprisingly, the NP25-BSA–mediated response was substantially reduced by 5 μM PP2, and 20 μM PP2 was able to inhibit the calcium response completely (Fig. 2A), which suggests a qualitative difference between Ab- and ligand-induced BCR signal transduction. The higher sensitivity of the ligand-dependent signaling to PP2 was not the result of suboptimal concentration of NP25-BSA (1 μg/ml), as even 10-fold lower concentration was sufficient to induce maximum response (Supplemental Fig. 2A).

In addition to PP2, we also used a genetically encoded protein inhibitor based on Csk, which is a kinase that very specifically phosphorylates the inhibitory tyrosine of SFKs. The Csk inhibitor consisted of an extracellular and transmembrane domain of the lipid raft protein LAT fused to constitutively active Csk (with mutations in the SH2 and SH3 domains to prevent binding to cellular proteins). This construct was C-terminally tagged with mOrange fluorescent protein (Fig. 2B) to permit the tracking of construct-expressing cells. The LAT-Csk construct was previously shown to be a potent inhibitor of TCR signaling (42). We also used a tunable variant of this inhibitor comprising the LAT transmembrane domain fused to an FRB domain and Csk-mOrange fused to an FKBP domain (Fig. 2B). The recruitment of constitutively active Csk to the plasma membrane could be induced by adding a rapamycin derivative AP21967 that dimersizes the FRB and FKBP domains. Such recruitment has been shown to regulate the inhibitory effect on SFKs (42).

The expression of LAT-Csk, but not kinase-dead LAT-Csk, resulted in dramatic hyperphosphorylation of Lyn at the inhibitory tyrosine (Y507) and hypophosphorylation of SFKs at the activation tyrosine (Y416), which indicates that LAT-Csk is a potent SFK inhibitor in B cells (Fig. 2C). The expression of dimLAT-Csk induced similar effects, although to a lesser extent. The inactivation of SFKs, measured by phosphorylation of the key tyrosine residues, was enhanced by AP21967 (Fig. 2C).

We then tested the effects of LAT-Csk on the anti-BCR– and ligand-induced calcium response in K46 cells. In agreement with the PP2-based experiments, LAT-Csk completely inhibited the

FIGURE 2. Inhibition of SFKs has different effects on Ab- and ligand-induced BCR signaling. (A) K46 WT cells were activated by anti-BCR or NP25-BSA in the presence of the indicated concentrations of PP2, and the calcium influx was monitored. (B) Schematic representation of genetic Csk-based inhibitors of SFKs. (C) K46 cells treated or not with 5 μM PP2 for 5 min, K46-LAT-Csk and K46-LAT-Csk-KD cells, and K46:dimLAT-Csk cells treated or not with 1 μM AP21697 for 5 min were lysed and examined for phosphorylation of the activation tyrosine of SFKs and the inhibitory tyrosine of Lyn. Staining for total Lyn served as a loading control. (D) K46-LAT-Csk or K46-LAT-Csk-KD were activated by anti-BCR or NP25-BSA, and the calcium influx was monitored. Only mOrange+ cells were gated. Density plots for cells expressing active LAT-Csk are shown (E). (F and G) K46:dimLAT-Csk cells were activated by anti-BCR or NP25-BSA after incubation for 5 min with or without 1 μM AP21697. The calcium influx was monitored. Only mOrange+ cells were gated. Density plots for cells treated with the AP21697 are shown (G).
NP25-BSA–induced calcium influx, but the cells were still able to respond to anti-BCR, although with a substantial delay (Fig. 2D, 2E). The difference in the sensitivity to SFK inhibition between the ligand-triggered and anti-BCR–triggered BCR calcium responses was reproduced in K46:dimLAT-Csk cells after the addition of the AP21967 dimerizer. Again, the anti-BCR–mediated calcium response was delayed, whereas the ligand-triggered signaling was almost completely abolished (Fig. 2F, 2G). The dimerizer did not affect the calcium signaling in K46 WT cells (data not shown). LAT-Csk also inhibited the transcriptional BCR response, as revealed by impaired CD69 upregulation after stimulation with both anti-BCR and NP25-BSA (Supplemental Fig. 2B).

The experiments with NP25-BSA indicated that ligand-induced BCR signaling is blocked, but anti-BCR–triggered signaling is only delayed upon the inhibition of SFKs. It was possible that NP25-BSA was a weaker agonist than anti-BCR (i.e., because of low affinity of its interaction with BCR, low receptor occupancy, or limited flexibility of the protein carrier). To address this issue, we tested B1-8 agonists with lower NP-BSA ratio (NP25-BSA), higher affinity to the BCR (NIP-BSA) (47), or attached to a more flexible carrier (NP40-Ficoll). Neither the decrease in NP-BSA ratio nor the higher affinity ligand had any effect on the response in nontreated or PP2-treated cells (Supplemental Fig. 2C).

In contrast, a potent T cell–independent ligand NP40-Ficoll induced stronger response than NP-BSA and anti-BCR in K46 cells (Fig. 3A). However, NP40-Ficoll–induced calcium signaling was totally inhibited in K46:LAT-Csk cells (Fig. 3B). PP2 did not block NP40-Ficoll–triggered calcium flux completely, but again, it had a significantly stronger impact on NP40-Ficoll than on anti-BCR–mediated signaling (Fig. 3C).

The BCR-induced phosphorylation of SFKs, Syk, Igα, Erk, and Akt was comparable in anti-BCR, NP25-BSA, and NP40-Ficoll–treated cells at 1 min after activation in K46:LAT-Csk-KD cells (Fig. 3D). The NP25-BSA–induced phosphorylation appeared to be more transient for Erk, Igα, and Syk, but not Akt and SFKs upon stimulation for 5 min. LAT-Csk delayed the phosphorylation of Syk, Igα, Erk, and Akt in anti-BCR–activated cells, but almost completely abolished the phosphorylation of these proteins in NP25-BSA– and NP40-Ficoll–treated cells (Fig. 3D). These results support the hypothesis that anti-BCR–mediated signaling is partially resistant to the inhibition of SFKs, whereas higher SFK activity is required for ligand-induced signaling. The anti-BCR induced higher level of the activation loop phosphorylation than the ligands in K46:LAT-Csk-KD cells even at 1 min after activation, when the phosphorylation of other examined proteins was comparable (Fig. 3D). The stronger activation of SFKs by the anti-BCR could explain the partial resistance to SFK inhibition.

Interestingly, in contrast with PP2-treated primary B cells (Fig. 1E), we did not observe anti-BCR–induced phosphorylation of the activation site of SFKs in K46:LAT-Csk cells (Fig. 3D), which could be explained by the likely induction of the closed conformation of SFKs upon the inhibition by LAT-Csk. This conformation stabilizes the activation loop in the inactive position where the activation loop tyrosine might be relatively difficult to access (48). PP2-inhibited Lyn is characterized by a very low level of Y507 phosphorylation (Fig. 2C), thus, the majority of this kinase is likely in the open active-like conformation even when interacting with PP2 (43, 47). The activation loop phosphorylation site is thus more accessible and is possibly phosphorylated (in trans), even in the PP2-bound kinase domain.

**Differences between anti-BCR– and ligand-induced signaling in primary cells**

To verify our results from the K46 cells, we used primary splenocytes from the B1-8I knock-in mouse strain, which expresses the B1-8 transgenic IgH (41). When paired with an Igα L chain, the B1-8 H chain produces an NP-specific BCR (51). Because few B cells express the α L chain, we had to sort or gate them (Supplemental Fig. 3A, 3B).

We treated the splenocytes with 5 or 20 μM PP2 and monitored the calcium influx in the Igα–B cells after activation by anti-BCR, NP25-BSA, or NP40-Ficoll. In the nontreated samples, the highest response was observed after activation by NP40-Ficoll (Fig. 4A). The 5 μM PP2 substantially inhibited NP25-BSA–mediated calcium signaling but had only a minor effect on the NP40-Ficoll– and anti-BCR–mediated response. In contrast, the treatment with 20 μM PP2 resulted in significantly stronger inhibition of the response mediated by NP40-Ficoll relative to the response mediated by anti-BCR (Fig. 4A).

Similar effects of PP2 were observed at the level of Igα and Erk phosphorylation. We measured the percentage of cells activating Erk upon treatment with the BCR agonists. The Erk response was...
strongly inhibited by 5 μM PP2 after NP40-Ficoll and NP25-BSA stimulation but much less affected upon activation by anti-BCR, whereas 20 μM PP2 almost completely abolished the ligand-induced Erk phosphorylation and partially inhibited the phosphorylation of Erk mediated by anti-BCR (Fig. 4B). Although NP40-Ficoll was the best inducer of Igα phosphorylation in non-treated cells, the BCR-induced phosphorylation of Igα was more efficiently inhibited after NP40-Ficoll and NP25-BSA stimulation than after anti-BCR stimulation (Fig. 4C).

We also transduced B cell blasts derived from Igλ+ splenocytes isolated from B1-8i transgenic mice with a retroviral vector encoding active or kinase-dead variants of LAT-Csk. The active LAT-Csk severely inhibited the NP40-Ficoll- and NP25-BSA-induced calcium influx, but only delayed and partially inhibited the anti-BCR–induced calcium response (Fig. 4D). The inhibitory effects of LAT-Csk on BCR-induced calcium signaling were concentration dependent, but unable reach the plateau at the expression levels of LAT-Csk we could achieve, indicating that these expression levels

FIGURE 4. Requirement of SFKs for ligand-induced BCR signaling in primary B cells. (A) Splenocytes isolated from B1-8i transgenic mouse were activated by anti-BCR, NP25-BSA, or NP40-Ficoll in the presence of the indicated concentrations of PP2. The calcium influx in Igκ/B220+ cells was monitored. The maximum response was calculated as maximum median relative calcium concentration in the particular sample. Mean ± SEM, n = 4, *p < 0.05, **p < 0.01, n.s., not significant (between the indicated ligand treatment and the respective anti-BCR treatment). (B) Splenocytes isolated from B1-8i transgenic mouse were activated by anti-BCR, NP25-BSA, or NP40-Ficoll with the indicated concentrations of PP2. The phosphorylation of Erk was analyzed in Igκ/IgM+ cells. The relative pErk response was calculated as a percentage of pErk+ cells for each sample. Mean ± SEM, n = 4, *p < 0.05, **p < 0.01 (between the indicated ligand treatment and the respective anti-BCR treatment). (C) Splenocytes from B1-8i transgenic mouse were activated as in (B) and stained with anti-pIgα. (D) B220+/Igκ+ splenocytes were transduced with LAT-Csk or LAT-Csk kinase-dead. The cells were activated as indicated, and the calcium influx was monitored. The ratio of the maximal median relative calcium concentration between the LAT-Csk and LAT-Csk kinase-dead samples was calculated. Mean ± SEM, n = 3.
were not sufficient to fully inhibit SFKs in LAT-Csk+ cells (Supplemental Fig. 3C).

**Syk does not phosphorylate Igα**

Inhibition of SFKs by chemical and genetic inhibitors efficiently blocked BCR signaling induced by BCR ligands. In contrast, anti-BCR-induced signaling exhibited a partial resistance to the inhibition of SFKs. There are two possible explanations for these effects: 1) anti-BCR triggers an SFK-independent signaling pathway, or 2) the residual SFK activity is sufficient and required to initiate the downstream signaling pathways.

Because SFK-independent BCR signaling has been linked to the ability of Syk to compensate for SFK activity (37, 38), we analyzed the ability of Syk to phosphorylate ITAMs in B cells. The inhibition of Syk in K46:LAT-Csk or K46:LAT-Csk-KD cells decreased anti-BCR-induced phosphorylation of the catalytic loop tyrosines of Syk (Y519/520) and proteins downstream of Syk (SLP65, Erk, and Akt; Fig. 5A). In contrast, the phosphorylation of Igα and the interdomain B tyrosine of Syk (Y346) was not affected by the inhibition of Syk in both cell lines (Fig. 5A). Treatment of K46:LAT-Csk cells with PP2 further delayed the phosphorylation of Igα, Syk, and Erk (Fig. 5B), which shows that LAT-Csk was unable to inhibit the SFKs completely. The combination of genetic and chemical inhibitors did not block the anti-BCR–induced response entirely, probably because of the partial inhibition of Csk kinase activity in LAT-Csk chimera by PP2 (52) (Supplemental Fig. 4). We performed similar experiments in primary splenic B cells stimulated with anti-BCR after being treated with PP2, Syk inhibitor IV, PP2+Syk inhibitor IV, or left untreated. Inhibition of Syk diminished the anti-BCR–induced phosphorylation of Syk at tyrosines Y519/520 and the phosphorylation of proteins downstream of Syk, but did not affect the phosphorylation of Igα and Syk at tyrosine Y346, regardless of the inhibition of SFKs (Fig. 5C). Treatment with Syk inhibitor IV did not influence the timing of the anti-BCR–induced phosphorylation of Igα and Syk at Y346 (Fig. 5D).

Collectively, these results show that Syk activity does not play a role in the anti-BCR–induced phosphorylation of Igα (at least at the ITAM tyrosine Y182) in SFK-deficient and -sufficient B cells. Moreover, the data indicate that whereas the catalytic loop tyrosine of Syk is, at least partially, an autocatalytic site, the interdomain B tyrosine 346 is a substrate for SFKs. We conclude that

![FIGURE 5. BCR-induced phosphorylation of Igα is independent of Syk activity. (A) K46:LAT-Csk and K46:LAT-Csk-KD cells were activated by anti-BCR in the presence or absence of 2.5 μM Syk inhibitor IV. Phosphorylation of the indicated proteins 1 and 5 min after the activation was analyzed by Western blotting. Staining of GAPDH served as a loading control. (B) K46:LAT-Csk cells were treated or not with 20 μM PP2 and activated by anti-BCR. Phosphorylation of Erk, Igα, and Syk at the indicated time points was analyzed by Western blotting. (C and D) Splenic B cells were treated or not with 1 μM Syk inhibitor IV and/or 5 μM PP2, and activated by anti-BCR. Phosphorylation of the indicated proteins was analyzed by Western blotting.](http://www.jimmunol.org/)
the initiation of anti-BCR–induced BCR signaling in our models with significant SFK inhibition still depends on the residual activity of SFKs.

**SFK activity is important for sustained BCR signaling**

Although the inhibition of Syk did not reveal any role in Igα phosphorylation, we tested the possibility that Syk activity is sufficient to sustain ongoing signaling upon the acute inhibition of SFKs. The inhibition of SFKs by 5 μM PP2 after BCR triggering resulted in rapid inhibition of the calcium signaling and dephosphorylation of the activation tyrosine of the SFKs, interdomain B tyrosine of Syk, and Igα (Fig. 6A, 6B). After the immediate decrease, the signaling partially recovered, especially with regard to calcium flux and Syk interdomain B tyrosine phosphorylation, probably as a result of the incomplete inhibition of SFKs as revealed by the phosphorylation of the activation loop tyrosine (Fig. 6A, 6B). The acute inhibition of anti-BCR–induced calcium signaling was also achieved by adding a dimerizer to K46:dimLAT-Csk cells (Fig. 6C). These experiments showed that SFKs are involved in not only the initiation but also the maintenance of BCR signaling.

**BCR signaling in DT40 cells is dependent on SFK activity**

Lyn was the only SFK detected in the chicken B cell lymphoma cell line DT40 at the level of mRNA (35). Lyn−/− DT40 cells were characterized by delayed, but not completely inhibited, anti-BCR–induced signaling, which suggests the existence of an SFK-independent BCR signaling pathway (35). Because our data indicate the requirement for SFKs in the initiation of BCR signaling, we tested whether BCR signaling in Lyn−/− DT40 cells is truly SFK independent. Treatment of DT40 WT cells with PP2 delayed calcium signaling, as expected (Fig. 7A). Importantly, PP2 also delayed calcium signaling in Lyn−/− DT40 cells in a concentration-dependent manner (Fig. 7A). Inhibition of the SFKs also delayed Akt and Erk phosphorylation in WT and Lyn−/− DT40 cells (Fig. 7B). Staining for the phosphorylated activation loop tyrosine in SFKs revealed that DT40 Lyn−/− cells express at least one SFK that is activated after BCR engagement (Fig. 7B).

To investigate whether DT40 Lyn−/− cells express another Src family member, we used a rabbit mAb that recognizes the activation loop phosphorytrosine of multiple SFKs (pSFK Y416). We used this Ab to immunoprecipitate SFKs from nontreated and pervanadate-treated WT and Lyn−/− DT40 cells. Cell lysates and immunoprecipitated material were stained with mAbs and polyclonal Abs to the SFK activation loop phosphorytosine, an Ab to the phosphorylated C-terminal tyrosine of Src, and an Ab to the nonphosphorylated C-terminal tyrosine of Src, and an Ab to Lyn (Fig. 7C). Although the phospho-specific Abs exhibited some level of off-target reactivity, especially after the pervanadate treatment, the additional bands not corresponding to the m.w. of the SFKs were substantially reduced after the immunoprecipitation. In samples from WT and Lyn−/− DT40 cells, two different Abs to pSFK Y416 stained a protein corresponding to an SFK as confirmed by its m.w. The signal was much stronger in DT40 WT cells, apparently because of the expression of Lyn. In addition, Abs to the C-terminal pY527 or nonphosphorylated Y527 (non-pY527) of Src stained a band with the same m.w. in lysates and pSFK Y416 immunoprecipitates from both cell lines. Whereas the pervanadate treatment enhanced the signal detected by the pY527-specific Ab, it reduced the signal from the non-pY527 Ab in the DT40 WT and Lyn−/− lysates.

To identify the non-Lyn SFK(s) in DT40 cells, we performed quantitative RT-PCR analysis using RNA isolated from DT40 WT and Lyn−/− cells and primers specific for LYN, LCK, FYN, BLK, SRC, HCK, YES, and YRK genes. Although the Lyn encoding transcript was the most abundant, we could detect mRNAs of other family members, among which Fyn mRNA was the most prominent (~20% of Lyn mRNA levels; Fig. 7D). Together, these results imply that DT40 Lyn−/− cells express SFK Fyn that is activated after BCR cross-linking and promotes BCR signaling in these cells.

**Discussion**

A widely accepted model of BCR signaling proposes that BCR engagement leads to the activation of SFKs, mainly Lyn, that
Influx was monitored. (Y416) were analyzed by Western blotting. (S473), Erk (T193/Y195), and the activation loop tyrosine of the SFKs were stained with Abs to anti-SFK pY416 (monoclonal and polyclonal), with the anti-pSFK (Y416) mAb. Lysates and immunoprecipitated material were treated with pervanadate or not and subjected to immunoprecipitation with the anti-BCR in the presence of 0 or 5 μM PP2. The phosphorylation of Akt, pSFK, and Src were quantified using Western blotting. Yes, Src, Lck, and Blk were below the detection limit. Mean ± SEM, n = 3. n.d., Not detected.

The phosphorylation of the ITAM in Igα/Igβ cells were stimulated with the ligand-induced BCR signaling requires SFK activity in K46 B cell lymphoma cells and in primary B cells. Surprisingly, the inhibition of SFKs substantially delayed the initiation of signaling but only partially reduced the intensity of the anti-BCR−induced response. A similar delay in BCR signaling was caused by the absence of Lyn in the DT40 cell line and, to a lesser extent, in primary B cells (30, 35). We analyzed in detail whether residual SFK activity or SFK−independent Syk activation was responsible for the initiation of BCR signaling under SFK deficiency. Combined treatment of the cells with LAT-Csk and PP2, as well as simultaneous inhibition of BCR signaling of Lyn−deficient DT40 cells is dependent on SFK activation feedback mechanism (absent in TCR signaling) (62) could explain the higher sensitivity of TCR signaling to the activity of SFKs and CD19 (60, 61). The existence of such a B cell−specific positive feedback loop at the level of SFKs, possibly involving the cooperation between SFKs and Igα/Igβ (59) and/or SFKs and CD19 (60, 61). The existence of a positive feedback loop at the level of SFKs, possibly involving the cooperation between SFKs and Igα/Igβ (59) and/or SFKs and CD19 (60, 61). The existence of such a B cell−specific SFK activation feedback mechanism (absent in TCR signaling) (62) could explain the higher sensitivity of TCR signaling to the inhibition of SFKs.

Our results failed to show SFK−independent Syk activation and phosphorylation of the ITAM in Igα by Syk. Syk activity did not...
contribute to the phosphorylation of Igα under SFK-sufficient and -deficient conditions, and already activated Syk was unable to sustain ongoing signaling after an acute inhibition of SFKs. These observations challenge the proposed existence of the Syk-mediated, SFK-independent pathway and Syk/ITAM autoactivation loop in BCR signaling (14–16, 38–40, 63). Most previous experiments indicating the ability of Syk to phosphorylate ITAMs and autoactivate itself were performed using cell-free or non-B cell models. However, this activity of Syk may be relatively weak and insufficient to counterbalance the inhibitory activity of tyrosine phosphatases in B cells. Another point of consideration is that we measured the phosphorylation of only the first ITAM tyrosine in Igα (Y182). This tyrosine is critical for the initiation of BCR signaling, and multiple lines of evidence indicate that SFKs have high specificity for this tyrosine (15, 64–66). These observations combined with our data thus support the conclusion that the phosphorylation of the proximal ITAM tyrosine by SFK is a critical step in the initiation of BCR signal transduction that is completely independent of Syk. The identity of the kinase responsible for the phosphorylation of the distal ITAM tyrosine is less clear; thus, there is still a possibility that Syk is more substantially involved.

Our experiments also clarified a somewhat controversial issue concerning the identity of the kinases phosphorylating Syk (17, 18, 37, 63). Although the activation loop tyrosines of Syk (Y519/520) behaved as mainly autokacytolytic sites, the interdomain B tyrosine (Y346) was predominantly phosphorylated by SFKs. Thus, both mechanisms of Syk activation, that is, binding to phosphorylated ITAM and phosphorylation of the interdomain B tyrosines (20), seem to be strictly dependent on the activity of SFKs in B cells. The observed independence of Syk phosphorylation at Y346 from the catalytic activity of Syk also suggests that Syk recruitment to the proximity of the SFKs at the BCR is not regulated by Syk catalytic activity. The finding that Syk phosphorylated ITAMs more efficiently than ZAP70 in some assays (14, 39) can be explained by the higher intrinsic catalytic activity of Syk (67) and/or higher affinity of its SH2 domains for phosphorylated ITAMs (68), not necessarily by the specificity of Syk for the ITAMs.

To our knowledge, the only direct evidence documenting SFK-independent BCR signaling in a B cell lineage was obtained using the DT40 chicken B cell lymphoma cell line. DT40 cells were believed to express only a single SFK, Lyn (32, 35, 36, 53). Thus, the delayed, but not completely inhibited, signaling in Lyn+/− DT40 cells could have been interpreted as SFK-independent BCR signaling (35, 38). We showed that Lyn+/− DT40 cells express another Src family member, Fyn, at the level only 5- to 10-fold lower than Lyn in DT40 WT cells. Low amounts of transcripts of two additional Src family members, Hck and York, were also detected in DT40 cells. The non-Lyn SFKs are activated after BCR engagement and positively regulate Ab-induced BCR signaling in DT40 Lyn−/− cells. Anti-BCR–induced signaling is a widely used model for BCR triggering. To our knowledge, no substantial differences between anti-BCR– and ligand-induced BCR signaling have been reported thus far. However, our data suggest that the anti-BCR cross-links BCRs in a specific way to cause enhanced SFK activation that is pronounced upon the inhibition of SFKs but apparent even in SFK–sufficient cells. However, we cannot exclude the possibility that anti-BCR mimics a specific class of ligands. For instance, B1-8 BCR has a relatively moderate affinity for NP (Kd = 2 μM) (47). Although NIP, which has 20-fold higher affinity to B1-8 than NP (47), behaved similarly in our assays, we cannot exclude the possibility that a BCR-ligand pair with even higher affinity could induce a qualitatively different response. One of the analyzed downstream signaling events was Erk phosphorylation. Triggering of the BCR resulted in bimodal Erk response where inhibition of SFKs by PP2 reduced the percentage of BCR-induced pErk2 (high) cells rather than the pErk signal intensity. This type of response, where the percentage of pErk2 cells corresponded to the strength of the signal, has been observed previously and has been variably explained either by switchlike behavior of the proteins involved in Erk activation or as a result of cell-to-cell heterogeneity in the expression of Erk pathway components combined with negative feedback incorporated into the pathway (see Refs. 69, 70 for more detailed discussion). Interestingly, BCR response induced by a flexible polysaccharide Ag carrier (Ficoll) was not only stronger and more sustained than NP-BSA-induced signaling, but it was also partially resistant to a low concentration of SFK inhibitor. These data might suggest that flexibility in the aggregation of BCRs by an agonist could be an important determinant of the quality of the induced signaling. In the future, it would be interesting to address this issue using a set of additional BCR-ligand pairs. Of note, different requirements for SFK activity were reported for different methods of FceRI triggering in mast cells (71, 72). The high-intensity stimulation of FceRI required much less SFK activity than low-intensity stimulation, but both mechanisms of mast cell activation were SFK dependent, as shown by the PP2 treatment of Lyn-deficient mast cells (71).

We believe that our results contribute to the understanding of the molecular mechanisms underlying BCR signaling, as well as the B cell–specific effects of pharmacological Src and Syk inhibitors used, tested, or considered for the treatment of various malignant and immune diseases (73–75).

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Disclosures

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