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The Syk tyrosine kinase is a key molecule in the development of the B cell lineage and the activation of B lymphocytes after Ag recognition by the B cell Ag receptor (BCR). Several genetic studies with chicken B cells have reported that the recruitment of Syk by BCR is essential for activation of a cascade of signaling molecules including phosphatidylinositol 3-kinase, mitogen-activated protein kinases, Ras signaling pathways, phospholipase C-\(\gamma_2\) activation, and calcium mobilization. The identification of a Syk-deficient mouse IIA1.6/A20 B cell line provided us the opportunity to investigate Syk-mediated signaling in mouse. Surprisingly, phosphatidylinositol 3-kinase, Ras, and mitogen-activated protein kinases were activated upon BCR cross-linking in these Syk-deficient mouse B cells, whereas, as expected from results obtained in chicken B cells, phospholipase C-\(\gamma_2\) activation and calcium mobilization were impaired as well as the NF-\(\kappa\)B pathway. These results indicate that BCR signaling is not strictly dependent on Syk expression in mouse IIA1.6/A20 B cells. Thus, B lymphocyte activation may be initiated by Syk-dependent and Syk-independent signaling cascades. The Journal of Immunology, 2003, 171: 1328–1335.

B lymphocytes recognize Ags through their clonally distributed B cell receptors (BCRs), which play an important role on immune responses of host animals. Cross-linking of BCR by multivalent Ag first results in activation of intracellular signal transduction pathways. Subsequently, the cells induce B cell functions, such as gene expressions for cytokine production and B cell differentiation, and a rapid targeting of the BCR-Ag complex to specialized Ag-processing compartments for Ag presentation to CD4\(^+\) T cells (1–6). The B cell functions seem to be closely related to BCR signalings; however, the molecular mechanisms underlying these functions remain to be solved.

Biochemical analysis has led to description of the various steps in BCR signaling after Ag recognition (4–6). The engagement of BCR leads to the tyrosine phosphorylation by Src family tyrosine kinases (SFKs) of immunoreceptor tyrosine-based activation motifs (ITAMs) in both the Ig-\(\alpha\) and Ig-\(\beta\) subunits of BCR. Syk is then recruited to phosphorylated ITAM via its Src homology 2 domains and is activated, leading to the turning on of various signaling pathways. Syk activates four major signaling pathways: phospholipase (PLC)-\(\gamma_2\), phosphatidylinositol 3-kinase (PI3K), Ras, and Rac. The activation of these pathways leads, in turn, to further signal transduction, such as intracellular calcium mobilization, the activation of Akt, and mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-Jun NH\(_2\)-terminal kinase (JNK), and p38 MAPK (7–14). Most of these Syk targets in BCR signaling were identified in a Syk-deficient chicken DT40 B cell line, and Syk-dependent BCR signaling in mouse B cells is poorly understood because B cell development in the Syk\(^{−/−}\) mouse is blocked at an early stage of B cell differentiation and these mice lack both immature and mature B cells (15–18). Although BCR signaling in the DT40 cell line may differ from that in mouse B cells, these lines of evidence suggest that Syk is required for the initiation of almost all types of BCR signaling.

We recently identified a variant of the well-characterized A20/IIA1.6 B cells that lacked Syk tyrosine kinase. This provided us with an opportunity to investigate the function of Syk in mouse B cell signaling. As described in DT40 cells, BCR engagement did not result in PLC-\(\gamma_2\) activation for the induction of calcium mobilization in these mouse Syk-deficient B cells. Cell complementation with wild-type and kinase-dead Syk clearly demonstrated that PLC-\(\gamma_2\) activation and calcium mobilization were strictly dependent on the kinase activity of Syk in BCR signaling. In contrast, PI3K and Ras were activated by BCR cross-linking in these cells. The lack of Syk resulted in slightly lower levels of BCR-induced phosphorylation of the three MAPKs, ERK2, JNK1, and p38. Full activation of MAPKs was strictly dependent on Syk kinase activity. As a consequence of this partial inactivation of the MAPK pathway, BCR engagement in the Syk-deficient cells induced gene expression, whereas activation of the NF-\(\kappa\)B pathway was impaired. These results clearly demonstrate that fully active BCR signaling is initiated by Syk-dependent and Syk-independent pathways in mouse A20/IIA1.6 cells, and that Syk-independent pathways are responsible for the activation of Ras and PI3K.

Materials and Methods

Antibodies

The anti-\(\kappa\)B\(_\text{Ra}\) antibody 52008 Ab was generously provided by R. Weil (Institut Pasteur, Paris, France) (19). Anti-Syk (N-19), anti-PLC-\(\gamma_2\) (Q-20), anti-ERK2 (C-14-G), anti-JNK1 (C-17), anti-p38 (C-20), anti-Lyn (20), anti-
Fyn (FYN3), anti-Btk (K-23), anti-c-Yes (3), anti-Lck (3A5), and anti-B cell linker protein (BLNK) (H-80) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p44/42 MAPK (Thr202/ Tyr204), anti-phospho-stress-activated protein kinase/JNK (Thr183/Tyr185), anti-phospho-p38 MAPK (Thr180/Tyr182), and anti-phospho-Akt (Ser473) Abs were obtained from Cell Signaling Technology (Saint Quentin, France). Anti-Bruton’s tyrosine kinase (Btk) (65251A), anti-CD19 (1D3), and anti-phosphotyrosine (PY-20) Abs were obtained from BD PharMingen (San Diego, CA). Another anti-phosphotyrosine Ab, 4G10, was purified from the culture medium of hybridoma 4G10. Anti-ZAP-70 and anti-p85 subunit of PI3K Abs were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-Ig-α Ab was obtained by immunization of the peptide corresponding to the C terminus sequence of mouse Ig-α (GGTYQDVGNLHIGDALEK) to a rabbit.

**Cells**

The mouse B lymphoma IIA1.6 cell line is a FcyR-defective variant of A20 B lymphoma cells, and has the phenotype of quiescent mature B cells expressing surface IgG2a. Cells were maintained in RPMI 1640 supplemented with 10 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 5 mM sodium pyruvate, and 10% FCS. A Syk-deficient mouse B cell clone (C35) was identified from a population of IIA1.6 B lymphoma cells. To obtain Syk-reconstituted clones, the Syk-deficient C35 cells were electroporated with the pNTIIH2 expression vector into which the cDNA encoding the wild-type rat Syk or kinase-inactive mutant of Syk (K395R mutant in the ATP-binding domain) had been inverted, and cultured in the presence of hygromycin B. We checked the reconstitution of Syk in clones by Western blotting with an anti-Syk Ab.

**RT-PCR**

cDNA was prepared from total RNA by reverse transcription with random hexamer-mixed primers, and was subjected to PCR with a set of primers (5′-GAA GCC CGC TGG CCC ATG GAC ACA-3′ and 5′-AAC CCT CAC ATG ACC TTT GC-3′ for GAPDH, 5′-CAG GAA GTC GGG GCG ATC TTC-3′ and 5′-CAG GAA GTC GGG GCG ATC TTC-3′) designed to amplify the DNA fragment corresponding to the region corresponding to nt 1000–1852 of the mouse ZAP-70 mRNA. The primers for ZAP-70 are completely identical with the corresponding human sequences. As a control, we amplified a 813-bp fragment of actin mRNA, by RT-PCR with the same primers and the primers 5′-GGC GAC GAC GGC CAC AGC AAG-3′ and 5′-CTT CAT GGT GTC AGG AGC CAG-3′.

**Analysis of surface expression of BCR and CD19**

Cells were stained with biotin-labeled rat anti-mouse IgG2a F(ab)2 (Southern Biotechnology Associates, Birmingham, AL), followed by DTAF-labeled streptavidin (Immunotech, Marseille, France) for BCR in PBS supplemented with 5% FCS and 0.01% sodium azide. For staining of CD19, rat anti-CD19 (1D3) and Alexa488-labeled goat anti-rat IgG (Molecular Probes, Eugene, OR) Abs were used. Then cells were analyzed on a FACScalibur (BD Biosciences, Le pont de Claire, France).

**Cell activation**

Cells were cultured in RPMI 1640 medium. At a density of ~5 × 10⁸ cells/ml, cells were harvested, washed in RPMI 1640 supplemented with 15 mM HEPES buffer, pH 7.4 (reaction buffer), and incubated at 37°C for 10 min in the reaction buffer at a density of 1 × 10⁵ cells/ml. The suspension was then chilled; 10 μg/ml goat anti-mouse IgG F(ab)2 (ICN Pharmaceuticals, Costa Mesa, CA) was added; and the suspension was kept on ice for 30 min. After centrifugation at 4°C, the cells were resuspended in prewarmed reaction buffer supplemented with 20 μg/ml donkey anti-goat IgG F(ab)2 (Jackson ImmunoResearch Laboratories, West Grove, PA), and incubated at 37°C for the time indicated. The reaction was stopped by adding 10 vol of cold PBS. Lysis of cells and immunoprecipitation of PLC-γ2 (7), or Ig-α, BLNK, and CD19 (11) were performed, as described previously. In the experiment for immunoprecipitation of CD19, cell surface molecules were first biotinylated by 0.2 mg/ml sulfo-NHS-LC-biotin (Pierce, Bevon, France) at 4°C for 15 min in 20 mM HEPES, pH 7.4, 138 mM NaCl, and 5.3 mM KCl. After three washes in RPMI 1640 containing 15 mM HEPES, pH 7.4, and 3 mg/ml BSA, cells were stimu-

**Ras assay**

In the case of transient transfection, cells were electroporated with 50 μg of plasmids, and cultured for 48 h. Transfected or non-transfected cells were lysed in lysis buffer (50 mM HEPES, pH 7.4, 2.5 mM MgCl₂, 10% glycerol, 1% Nonidet P-40, 0.1 μM aprotinin, 1 μM leupeptin, 1 mM PMSF) at 4°C. After centrifugation at 15,000 rpm for 15 min, the resulting supernatant was incubated for 45 min at 4°C with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, NJ) precomplexed with GST fused to a Ras-binding domain of Raf (GST-Raf-RBD) protein expressed in Escherichia coli. Then beads were washed three times. Precipitated Ras protein was analyzed by Western blot analysis with an anti-Ras Ab.

**Measurement of inositol trisphosphate content and calcium mobilization**

Cells were suspended in Ringer’s solution (10 mM HEPES, pH 7.4, 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM glucose), incubated at 37°C for 5 min, and stimulated with 30 μg/ml rabbit anti-mouse IgG F(ab)². Then reaction was terminated by addition of 0.2 vol of 20% perchloric acid, and intracellular content of inositol trisphosphate (IP₃) was determined by a n-myo-IP₃-H assay system (Amersham Pharmacia Biotech). For measurement of calcium mobilization, cells were loaded with Fluo-3-AM (Molecular Probes), as described previously (21). Then, cells were resuspended in Ringer’s solution, and intracellular free calcium levels were monitored by flow cytometry with a FACScalibur. Basal Ca²⁺ levels were measured over 35 s, and then either 30 μg/ml rabbit anti-mouse IgG F(ab)² or 10 μM ionomycin was added. The data initially acquired with CellQuest software were converted with the FCS Assistant Program (BD Biosciences).

**IL-2 secretion**

Cells resuspended in fresh RPMI 1640 medium were cultured in a 96-well plate (1 × 10⁵ cells/well) for 2 h and stimulated by incubation with 30 μg/ml rabbit anti-mouse IgG F(ab)². After 24 h, the IL-2 secreted into the medium was determined by sandwich ELISA, using two different anti-IL-2 Abs (BD PharmMingen; 18161D and 18172D) and streptavidin-HRP (Pierce).

**Results**

**Production of tyrosine kinases and BCR in a Syk-deficient mouse B cell line**

We established a Syk-deficient B cell line (C35) from IIA1.6, a variant of A20 B lymphoma cells. Syk protein was undetectable by Western blotting (Fig. 1A), and no Syk mRNA was detected by RT-PCR analysis (Fig. 1B). In contrast, we detected other tyrosine kinases, including Btk and several SFKs, including Lyn, Fyn, Btk, and c-Yes, but not Lck, and the expression of Lyn was somewhat up-regulated in C35 cells (Fig. 1A). We also investigated ZAP-70 expression by Western blotting and RT-PCR analysis, but we detected neither the protein (Fig. 1A) nor the mRNA (Fig. 1B). Syk expression was then reconstituted in Syk-deficient cells with cDNA encoding wild-type Syk (WT8 and WT4) or kinase-dead Syk (K395R mutant) (KD20 and KD3) (Fig. 1C). The WT8 and KD20, and WT4 and KD3 clones were selected because they expressed similar level of Syk protein (Fig. 1C). C35 cells were transfected with insertless vector (V6) (Fig. 1C), and used as control Syk-deficient cells in experiments using the Syk-reconstituted cells. Flow cytometric analysis revealed that all the clones expressed similar levels of BCR at the cell surface (Fig. 1D).

**Impaired BCR-induced tyrosine phosphorylation in Syk-deficient cells**

The engagement of BCR induces the tyrosine phosphorylation of many cellular proteins. Thus, the level of tyrosine phosphorylation peaked within 2.5 min after BCR engagement in Syk-positive parental cells (Fig. 2A), and then gradually decreased (data not shown). In contrast, only a few tyrosine phosphorylations (such as 110-, 63-, 57-, and 55-kDa proteins in Fig. 2A, and 33-kDa protein (data not shown)) were induced by BCR cross-linking in Syk-deficient B cells. The expression of wild-type Syk in C35 cells...
tyrosine phosphorylated after, but not before, BCR cross-linking, and this phosphorylation was independent of Syk function. Therefore, tyrosine kinases such as Lyn upstream of Syk, in BCR signaling, were fully active in Syk-deficient cells.

**Failure of BCR-induced PLC-γ2 activation and calcium mobilization in Syk-deficient cells**

We then investigated the activation of signaling molecules downstream from Syk, such as PLC-γ2, PI3K, and Ras, in C35 B cells. The BCR-induced activation of PLC-γ2, as assessed by the level of tyrosine phosphorylation of this molecule, was lower in Syk-deficient cells than in parental B cells (Fig. 2C). Intracellular content of IP3, an enzymatic product of PLC-γ2, was also measured in these cells, and we found that it was increased by 4.1-fold, reaching at 2.4 pmol/10⁶ cells, 2 min after BCR stimulation in parental cells, whereas there was no significant IP3 production in BCR-stimulated Syk-deficient cells (data not shown). Full tyrosine phosphorylation of PLC-γ2 was restored in transfected cells expressing wild-type Syk, but not in cells expressing kinase-dead Syk mutant (Fig. 2C). BLNK is a critical adaptor protein for full activation of PLC-γ2, forming a signaling complex with Igα/β subunits, Syk, Btk, and PLC-γ2 (8, 24–29). Thus, we investigated the BCR-induced tyrosine phosphorylation of BLNK, and it was revealed to depend on the presence of functional Syk (Fig. 2D), suggesting failure of the complex formation in Syk-deficient cells. The impairment of PLC-γ2 activation suggested a similar calcium signaling defect in Syk-deficient cells. Indeed, intracellular calcium concentration did not increase after BCR stimulation in Syk-deficient cells and kinase-dead Syk-reconstituted cells, whereas the expression of wild-type Syk restored BCR-induced calcium mobilization in these cells (Fig. 2E). Our data were therefore consistent with previous reports, as Syk was absolutely required for BCR-induced intracellular calcium mobilization and PLC-γ2 activation in maturing mouse B cells (18) and in chicken DT40 B cells (7).

**Syk-deficient B cells did initiate PI3K and Ras pathways upon BCR stimulation**

We analyzed the PI3K and Ras pathways in Syk-deficient mouse B cells. We assessed PI3K activation in response to BCR engagement by measuring the level of phosphorylation of the Ser⁷³⁷ residue of Akt because this phosphorylation depends on PI3K activity (12) (data not shown). BCR engagement clearly induced the phosphorylation of Akt in Syk-deficient cells and parental cells (Fig. 3A). However, the level of Akt phosphorylation was higher in wild-type (WT8) than in kinase-dead (KD20) Syk-expressing cells (Fig. 3A), suggesting that overexpressed Syk participated to PI3K activation.

Ras activation was detected by a pull-down assay, using GST-Raf-RBD that associates with the active form of Ras, as demonstrated by transient transfection with a construct encoding a dominant-positive mutant of Ras (Ras G12V) (Fig. 3B). Ras was immediately activated by BCR stimulation, remained active for a few minutes, and was then inactivated within 5 min in Syk-positive parental cells (Fig. 3C). As shown in Fig. 3D using Syk-deficient and Syk-reconstituted cells, we found that the activation of Ras occurred independently of the activity of Syk tyrosine kinase. Therefore, Syk plays a critical role in the PLC-γ2 pathway, but it was not necessary to turn on PI3K and Ras pathways. Nevertheless, Syk might participate in the activation of PI3K and Ras because the levels of Akt phosphorylation and activated Ras were higher in WT8 cells (which express 3-fold higher amount of Syk than parental cells) than in KD20 or Syk-deficient V6 cells (Fig. 3, A and D). These results suggest that overexpression of Syk may facilitate the activation of PI3K and Ras.
Syk is not absolutely required for the activation of MAPKs upon BCR stimulation.

MAPKs are critical targets of Syk and turn on the transcription of many genes in B cells. We therefore investigated the activation of ERK2, JNK1, and p38 MAPKs following BCR engagement in parental and Syk-deficient B cells. In both cells, ERK2 was strongly activated after 2.5 min. A second wave of ERK2 phosphorylation occurred after 30 min, but only in parental cells and cells reconstituted with wild-type Syk (WT8) (Fig. 4A). The observed Syk-independent ERK activation was consistent with the activation of Ras (Fig. 3C), a critical regulator of the Ras-Raf-MAPK kinase-ERK pathway, in Syk-deficient cells. The activation

**FIGURE 2.** Impairment in BCR-induced tyrosine phosphorylation and PLC-γ2 pathway activation in Syk-deficient cells. A, BCR-induced tyrosine phosphorylation of cellular proteins. Cells were stimulated by BCR cross-linking for 2.5 min and lysed. The lysate was analyzed by Western blotting using the anti-phosphotyrosine Abs PY-20 and 4G10. B–D, BCR-induced tyrosine phosphorylation of Ig-α/β subunits, PLC-γ2, and BLNK. Cells were stimulated by BCR cross-linking for the time indicated (B) or 2.5 min (C and D), and lysed. The lysate was subjected to immunoprecipitation with anti-Ig-α (B), anti-PLC-γ2 (C), or anti-BLNK (D) Abs. The amount of tyrosine-phosphorylated proteins in the immunoprecipitates was analyzed by Western blotting with anti-phosphotyrosine Abs (top). The precipitated Ig-α, PLC-γ2, and BLNK were also detected with specific Abs to them (bottom). E, BCR-induced intracellular calcium mobilization. Cells loaded with Fluo-3-AM cell-permeable calcium indicator were stimulated by BCR cross-linking (filled line) or the addition of 10 μM ionomycin (gray line), and changes in fluorescence in FL1 were monitored with a FACS Calibur.

**FIGURE 3.** BCR stimulation did induce the activation of PI3K and Ras pathways in mouse Syk-deficient B cells. A, BCR-induced activation of PI3K pathway. Cells were stimulated by BCR cross-linking for the time indicated and lysed. The lysate was analyzed by Western blotting using an anti-phospho-Akt (Ser473) Ab (top). The total amount of Akt in the lysate was also determined with an anti-Akt Ab (bottom). B, GST-Raf-RBD specifically associated with the activated form of Ras in mouse B cells. Parental cells transfected with or without pcDNA-H-Ras (wild type) or pcDNA-H-Ras (G12V) were lysed, and the lysate was incubated with GST-Raf-RBD-immobilized beads for 1 h. The beads were washed, and the Ras bound to the beads was detected by Western blotting with an anti-Ras Ab (left panel). Ras protein was also detected in the lysates (right panel). The level of Ras production in transfected cells was ∼100 higher than that of endogenous Ras (data not shown). C and D, BCR-mediated Ras activation. Parental cells were stimulated by cross-linking BCR for the indicated time and lysed (C). D, Syk-deficient and Syk-reconstituted cells were also analyzed. The amount of the active form of Ras and the total amount of Ras in the lysate were determined, as described in B.
of JNK1 (Fig. 4B) and p38 (Fig. 4C) was also observed in both parental and Syk-deficient cells, although these two proteins were phosphorylated to a lesser extent in Syk-deficient cells than in parental cells. Given the inability of mouse Syk-deficient B cells to activate PLC-γ2 and BLNK (Fig. 2, C–E), this BLNK-PLC-γ2-independent activation of MAPKs is consistent with the results obtained in mouse B cells lacking BLNK, which plays a critical role in the PLC-γ2 activation, in which normal activation of JNK and p38 was observed after BCR stimulation (30). The expression of wild-type Syk, but not of kinase-dead Syk, enhanced the activation of JNK1 and p38 (Fig. 4, B and C, respectively). These results indicate that Syk was not absolutely required for the activation of MAPK activation, although its overexpression can increase the phosphorylation of these kinases.

**BCR-induced CD19 pathway was initiated by Syk**

Mouse B cells apparently have Syk-independent mechanism(s) to initiate some signalings upon BCR stimulation. The Syk-independent signalings could be mediated via B cell coreceptor CD19, because: 1) tyrosine residues at the cytoplasmic tail of CD19 are phosphorylated upon BCR stimulation, resulting in recruitment of various signaling molecules, including p85 subunit of PI3K (11, 31–35); 2) the phosphorylation of CD19 is shown to be eliminated in Lyn-deficient B cells (35) and is not affected by overexpression of a dominant-negative form of Syk (11); 3) chicken CD19 molecule has not been found yet. Therefore, we investigated the BCR-induced tyrosine phosphorylation of CD19 molecule and recruitment of p85 subunit of PI3K to it in Syk-deficient cells. The equal cell surface expression of CD19 molecule on the clones was confirmed by flow cytometric analysis (Fig. 5A). In the experiment for immunoprecipitation of CD19 molecule, cell surface molecules including CD19 were first biotinylated, and then cells were stimulated and lysed. After immunoprecipitation with anti-CD19 Ab, CD19 molecule was revealed as a 100-kDa protein on Western blot analysis with streptavidin-HRP (Fig. 5B). Unexpectedly, Western blotting of the immunoprecipitated CD19 with anti-phosphotyrosine Abs showed that BCR-induced tyrosine phosphorylation of CD19 occurred in parental cells and WT8 cells, but not in C35, V6, and KD20 cells (Fig. 5B). In addition, p85 subunit of PI3K was communoprecipitated with the CD19 molecule after BCR stimulation only in the presence of functional Syk (Fig. 5B). In contrast with previous work using dominant-negative mutant of Syk in A20 cells (11, 35), Syk-deficient cells indicate that Syk plays a pivotal role for initiation of BCR-induced CD19 pathway. Thus, the Syk-independent mechanism to activate PI3K, Ras, and MAPKs in BCR signaling must involve other mechanism(s) from the CD19 pathway.

**Inhibition of the BCR-induced NF-κB pathway and IL-2 secretion in Syk-deficient cells**

These results indicated the critical role of Syk for PLC-γ2 activation and led us to investigate the effects of Syk tyrosine kinase on gene expression in B cells after BCR engagement. PLC-γ2 activation is required for the BCR-mediated activation of NF-κB (36), a transcription factor that is maintained in an inactive state in the cytosol by binding to I-κB. The degradation of I-κB induces the translocation of NF-κB to the nucleus and NF-κB-dependent gene transcription. In cells expressing functional Syk (parental cells and WT8), I-κB degradation occurred rapidly after BCR stimulation (5 min) (Fig. 6A). In contrast, in Syk-deficient cells (C35 and V6) and cells reconstituted with kinase-dead Syk, I-κB degradation remained detectable after 30 min of stimulation. Therefore, the impairment of PLC-γ2 activation in Syk-deficient mouse B cells may lead to a defect in I-κB degradation, preventing the translocation of NF-κB to the nucleus to induce gene transcription. However, many known or unknown transcription factors may control gene expression in B cells. We investigated the effect of Syk on the induction of gene expression after BCR stimulation by measuring IL-2 secretion. This secretion acts as a measure of IL-2 gene expression because unstimulated cells did not secrete IL-2 (Fig. 6B) or transcribe the IL-2 gene (data not shown). Syk-deficient cells (C35, V6) and cells producing kinase-dead Syk (KD20) produced IL-2 upon BCR stimulation. However, the level of IL-2 production was 40–60% lower in these cells than in parental cells or cells reconstituted with wild-type Syk. Therefore, Syk is not absolutely required to induce a signaling cascade leading to gene activation.
upon BCR engagement in mouse IIA1.6/A20 B cells. Although it is required to investigate in other types of mouse B cells, our results led us to propose a new model of BCR-mediated signal transduction in mouse B cells in which Syk-dependent and Syk-independent pathways participate to drive the activation of mature B cells.

Discussion
Syk tyrosine kinase plays a key role in the initiation of Ag-dependent B lymphocyte activation. Thus, disruption of the syk gene impairs B cell differentiation in mice and BCR signaling in the chicken B cell line, DT40. In this study, we established a Syk-deficient mouse B cell line and investigated the activity of signaling molecules involved in BCR-mediated cell activation. With this new model, we were able to demonstrate clearly that the BCR-induced activation of PI3K, Ras, and MAPKs (ERK2, JNK1, and p38) was not strictly dependent on Syk expression in mouse IIA1.6/A20 B cells. However, our results do confirm the crucial function of Syk in the activation of PLC-γ2, calcium signaling, and NF-κB-dependent regulation of gene transcription in B cells.

The involvement of Syk in BCR signaling has been clearly established using Syk-deficient chicken B cells. However, the phenotype of Syk-deficient B cells differs between chicken DT40 cells and mouse A20/IIA1.6 cells. Thus, almost all BCR signaling events were abolished in Syk-deficient DT40 cells, whereas PI3K, Ras, and MAPKs remained active after BCR stimulation in mouse A20/IIA1.6 Syk-deficient cells. One key issue to be addressed is the molecular basis of these differences. Syk-deficient DT40 cells were obtained by inactivation of the syk gene (7), whereas Syk-deficient C35 cells were obtained, without genetic manipulation or mutagenesis, by screening the A20 subclones obtained by the limiting dilution method. Thus, there is a possibility that C35 cells could express an undetectable amount of Syk mRNA or Syk protein, which thus allowed the activation of certain signalings. However, this is unlikely to be the case for three main reasons. First, a defect in PLC-γ2 activation was observed in C35 cells (Fig. 2, C–E) and in DT40 cells; second, PI3K, Ras, and MAPKs were activated in C35 cells overexpressing kinase-dead Syk (Fig. 3, A and D, and Fig. 4); third, the overexpression of dominant-negative mutant of Syk, containing the two Src homology 2 domains, did not change the phenotype of the C35 cells (data not shown). Although these two inactive forms of Syk should compete with undetectable amounts of endogenous Syk for binding to phosphorylated ITAMs, we observed no change in Ras and PI3K activation in these conditions. We cannot completely exclude the other possibility that the slight increased expression of Lyn in C35 cells (as in Fig. 1A) might give some effects in the phenotype of Syk signaling in part; however, BCR cross-linking did apparently initiate PI3K and Ras pathways in the absence of Syk. This suggests that the function of Syk is restricted to the activation of PLC-γ2 pathway leading to calcium signaling, because C35 cells overexpressing kinase-dead Syk were not able to activate PLC-γ2, but efficiently induced other signaling pathways such as PI3K or Ras pathways, which both participate to activate ERK, as recently shown by Jacob et al. (37), using PI3K inhibitors in mouse B cells. In relation to this, we investigated the effect of wortmannin on
ERK activation in Syk-deficient cells (data not shown). As compared with ERK activation in Syk-positive parental cells, Syk-deficient C35 cells were found to be more sensitive to this inhibitor. This result implies that Syk activity is capable of compensating, in some degree, the inhibition of PI3K pathway to ERK activation, and suggests that Ras, PI3K, and Syk-dependent PLC-γ2 pathways complexly and cooperatively regulate ERK activation like the model of Jacob et al. (37).

One possibility to be considered could be the difference of stages at which DT40 cells and A20/IIA1.6 cells have matured: immature-like chicken DT40 cells have an IgM as membrane Ig (mlg) of BCR, while memory-like mouse A20/IIA1.6 cells express a BCR of class IgG2a. mlgM has the short cytoplasmic tail (KVKK), but in the case of mlgG, the sequence is extended by 25 residues to form a cytoplasmic tail containing a YxxM sequence ("x" means any amino acid), which resembles the tyrosine-based endocytosis signal (38), or the binding motif of p85 subunit of PI3K if phosphorylated. In fact, the mlg tail is suggested to contribute to BCR-mediated responses (39, 40). Nevertheless, some evidences showing differences in BCR signaling between IgM-bearing immature mouse B cells and chicken DT40 cells have been accumulated. For example, BCR-mediated activations of ERK, JNK, and p38 MAPks are almost lost in BLNK-deficient DT40 cells (13), but nearly intact in BLNK-deficient mouse B cells (30). BLNK or Btk deficiency results in complete loss of BCR-induced calcium mobilization in DT40 cells (13, 41), but partial or slight impairment in mouse B cells (20, 33, 42–44). It is possible that DT40 cells lack a Syk-independent pathway(s) for BCR signaling, SFKs can themselves activate various signaling pathways without Syk. Although DT40 cells express Lyn, they do not express several SFKs (7), which are normally expressed in mouse B cells (Fig. 1A) (45–47) and might be required to induce the activation of PI3K and/or Ras in the absence of Syk. Thus, Syk seems to be absolutely required for BCR signaling in DT40 cells, whereas mouse B cells may have developed an alternative pathway(s) to initiate B cell activation, despite the difference of class of mlg.

What is the Syk-independent mechanism to initiate PI3K and Ras in mouse B cells? Interestingly, Pleiman et al. (48) demonstrated the BCR-induced activation of PI3K in mouse B cells by its association with Fyn, as well as Lyn (48, 49), which implies that the additional expression of SFKs in mouse B cells may result in not only redundancy among these kinases, but also quantitatively additional activation of PI3K. According to the recent model of Jacob et al. (37), Ras and PI3K cross-regulate their activations each other in mouse B cells. In fact, active Ras is capable of activating PI3K pathway in mouse B cells (37), most likely by the direct association of p110 catalytic subunit of PI3K with Ras, as described in fibroblasts (50–52). Although the mechanism for the BCR-mediated Ras activation is still not yet understood, one proposed mechanism is a direct recruitment of Shc, the adaptor protein for Grb2-Sos Ras guanine nucleotide exchange complex, to tyrosine-phosphorylated Ig-α (at Tyr182) and Ig-β (at Tyr152) subunits (4, 6). The critical role of Shc in BCR-induced Ras-ERK pathway has been already shown in mouse B cells (37), although this adaptor molecule is not required in chicken DT40 cells (9).

Given the evidences that BCR-induced tyrosine phosphorylation of Ig-α/β subunits does not require Syk (18) (Fig. 2B) and that Shc phosphorylation, which leads to further recruitment of the Grb2-Sos complex, depends on the action of Lyn in mouse B cells (23), this pathway could account for the activation of Ras in Syk-deficient mouse B cells.

The other new finding is the Syk-dependent initiation of CD19 pathway upon BCR stimulation. Because Lyn-deficient B cells showed no tyrosine phosphorylation of CD19, it is thought that Lyn is the dominant tyrosine kinase that phosphorylates CD19 upon BCR stimulation (35). However, our results showed the Syk-dependent phosphorylation of CD19 (Fig. 5B). Because Lyn is also implicated in phosphorylation of ITAMs in Ig-α/β subunits, followed by activation of Syk (23), collaboration of both Lyn and Syk may be required for the maximal tyrosine phosphorylation of CD19 upon BCR stimulation. Another group using dominant-negative mutant of Syk did not obtain similar result, suggesting that this mutant did not completely inhibit Syk function in A20 cells (11). During preparation of manuscript, it was reported that BCR-induced phosphorylation of Akt is not reduced in the absence of CD19 (20), suggesting that the Syk-dependent recruitment of PI3K to CD19, as seen in Fig. 5B, might not play a central role in the BCR-induced PI3K activation.

Is it possible to speculate relationship between the BCR signalings and B cell functions? We have shown that Syk-activated PLC-γ2 pathway may link to NF-κB-dependent regulation of gene transcription in mouse B cells. Strictly regulated Syk-dependent BCR signaling has been reported to be important in the developmental program leading to the maturation of the B cell lineage (17, 18). Interestingly, mice lacking Bik, BLNK, PLC-γ2, both the Rel and RelA components of NF-κB, or IκB kinase α, all of which are molecules acting downstream from Syk, also show a similar defect, although less severe than Syk-deficient mice, in B cell development in the spleen at the stage at which mature B cells are formed (43, 44, 53–58). This may again confirm the role of Syk to turn on the PLC-γ2/NF-κB pathway in mouse B cell development. Oppositely, given the evidences that immature Syk+/− B cells are able to differentiate into Ab-secreting plasma cells (17) and that down-regulation of BCR at the cell surface does not require Syk either (18, 59), Syk-independent pathways, such as those involving the activation of PI3K, Ras, and/or MAPKs, may be involved in these processes. We are currently. E. A. Clark. We are currently carrying out further analysis of B cell functions in mouse Syk-deficient cells.

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