

# Role of the Syk Autophosphorylation Site and SH2 Domains in B Cell Antigen Receptor Signaling

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## Summary

To explore the mechanism(s) by which the Syk protein tyrosine kinase participates in B cell antigen receptor (BCR) signaling, we have studied the function of various Syk mutants in B cells made Syk deficient by homologous recombination knockout. Both Syk SH2 domains were required for BCR-mediated Syk and phospholipase C (PLC)- $\gamma$ 2 phosphorylation, inositol 1,4,5-triphosphate release, and  $\text{Ca}^{2+}$  mobilization. A possible explanation for this requirement was provided by findings that recruitment of Syk to tyrosine-phosphorylated immunoglobulin (Ig)  $\alpha$  and Ig $\beta$  requires both Syk SH2 domains. A Syk mutant in which the putative autophosphorylation site (Y518/Y519) of Syk was changed to phenylalanine was also defective in signal transduction; however, this mutation did not affect recruitment to the phosphorylated immunoreceptor family tyrosine-based activation motifs (ITAMs). These findings not only confirm that both SH2 domains are necessary for Syk binding to tyrosine-phosphorylated Ig $\alpha$  and Ig $\beta$  but indicate that this binding is necessary for Syk (Y518/519) phosphorylation after BCR ligation. This sequence of events is apparently required for coupling the BCR to most cellular protein tyrosine phosphorylation, to the phosphorylation and activation of PLC- $\gamma$ 2, and to  $\text{Ca}^{2+}$  mobilization.

B cells recognize foreign antigen via the B cell antigen receptor (BCR),<sup>1</sup> a multichain structure consisting of the variable Ig heavy and light chains, which are responsible for antigen recognition, and the invariant Ig $\alpha$  and Ig $\beta$  chains (CD79a and b), which couple the BCR to the intracellular signaling machinery (1, 2). The BCR shares a number of important features with the TCR and receptors for IgG Fc (Fc $\gamma$ RI, Fc $\gamma$ RIII) and IgE Fc (Fc $\epsilon$ RI). All of these receptors have similar multisubunit structures, in which the signal-transducing components contain a cytoplasmic sequence motif recently termed immunoreceptor family tyrosine-based activation motif, or ITAM (3), that includes two tyrosines separated from an aliphatic residue by two amino acids [D/E-(X)<sub>7</sub>-D/E-(X)<sub>2</sub>-Y-(X)<sub>2</sub>-L/I-(X)<sub>7</sub>-Y-(X)<sub>2</sub>-L/I] (4). Chimeric receptors containing these ITAMs are

competent to initiate an activation cascade, indicating that the motif carries sufficient structural information to activate early and late signaling events and that all of these receptors function by activating similar biochemical pathways (for reviews see references 5 and 6).

Stimulation of the BCR initiates a biochemical cascade in which protein tyrosine kinase (PTK) activation is the earliest known event. Since the BCR complex does not have any intrinsic kinase activity, cytoplasmic PTKs are implicated in initiation of BCR signaling. Two classes of cytoplasmic PTK have been shown to associate with BCR: the Src-PTKs, including Lyn, Fyn, Lck, and Blk (7–9), and the Syk tyrosine kinase (10, 11). Unlike the Src-PTKs, Syk bears two SH2 domains, no NH<sub>2</sub>-terminal myristylation site, and no negative regulatory tyrosine phosphorylation site (12). PTK activation through BCR results in the rapid tyrosine phosphorylation of several proteins (13, 14), including Ig $\alpha$  and Ig $\beta$  and phospholipase C (PLC)- $\gamma$ 2 (15, 16). Phosphorylation occurs in both tyrosines found in each ITAM (17). Furthermore, mutational analysis indicates that phosphorylation of both tyrosines is necessary for signal propagation through the receptor (17, 18). Ig $\alpha$  and

<sup>1</sup>Abbreviations used in this paper: BCR, B cell antigen receptor; ECL, enhanced chemiluminescence; IP<sub>3</sub>, inositol 1,4,5-triphosphate; ITAM, immunoreceptor family tyrosine-based activation motif; NP, nitrophenyl; PLC, phospholipase C; PtdIns, phosphatidylinositol; PTK, protein tyrosine kinase; PVDF, polyvinylidene difluoride.

Ig $\beta$  ITAM tyrosine phosphorylation serves to propagate signaling by enabling the motif to bind with high affinity to effectors such as Src-family tyrosine kinases (19) and Shc (D'Ambrosio, D., K. Hippen, and J. C. Cambier, manuscript in preparation) via their SH2 domains. In the case of Src-family kinase, this binding causes enzyme activation (19, 20). The tyrosine phosphorylation of PLC- $\gamma$ 2 appears to be responsible for its increased catalytic activity, leading to the generation of inositol lipid-derived second messengers (21).

To dissect the functional roles of these two types of kinase in BCR signaling, we recently established Lyn- and Syk-deficient DT40 B cells (22). Using these mutant cells, it was shown that both Lyn and Syk are required for coupling BCR to increase cellular protein tyrosine phosphorylation, and that, when expressed individually, these enzymes mediate the phosphorylation of at least partially distinct sets of molecules. One substrate whose phosphorylation is Syk dependent is PLC- $\gamma$ 2. Thus, in B cell signaling, Syk couples the BCR to phosphatidylinositol (PtdIns) pathway.

Here, we focus on mechanisms by which Syk couples the BCR to downstream signaling components. Transfection of mutated Syk into Syk-deficient DT40 cells allowed us to test the structural requirements for Syk function on BCR stimulation. Both NH<sub>2</sub>- and COOH-terminal SH2 mutants of Syk were defective in mediating Syk function in BCR stimulation. Wild-type Syk bound avidly to the phosphorylated Ig $\alpha$  ITAM and somewhat less avidly to phosphorylated Ig $\beta$ , and this binding was severely compromised in both NH<sub>2</sub>- and COOH-terminal SH2 mutants. Taken together, data indicate that recruitment of Syk to phosphorylated Ig $\alpha$  and Ig $\beta$  ITAMs or some other function that requires both SH2 domains is obligatory for receptor-mediated Syk (Y518/519) phosphorylation and coupling the BCR to inositol 1,4,5-trisphosphate (IP<sub>3</sub>) generation and Ca<sup>2+</sup> mobilization. Furthermore, a mutant Syk in which the putative autophosphorylation site was changed to Phe exhibited normal receptor-binding activity but was not able to mediate the phosphorylation of PLC- $\gamma$ 2 upon BCR stimulation, demonstrating that autophosphorylation of Syk (Y518/519) or Syk phosphorylation by another PTK is also critical for the function of Syk in BCR signaling.

## Materials and Methods

**Cell Culture, DNA Transfection, and Antisera.** DT40 cells were cultured in RPMI 1640 with 10% FCS, penicillin, streptomycin, and glutamine. Mutant Syk cDNAs (SH2 mutants and autophosphorylation mutant) were created by PCR and cloned into an EcoRI site of the pApuro expression vector (22). The resulting cDNAs were verified by DNA sequencing. These cDNAs were then transfected into Syk-negative cells by electroporation using gene pulser apparatus (Bio-Rad Laboratories, Richmond, CA) at 550 V, 25  $\mu$ F, and selected in the presence of 0.5  $\mu$ g/ml puromycin. Expression of mutated Syk was assessed by immunoblotting.

J558L $\mu$ m3 cells expressing nitrophenyl (NP)-specific IgM were described previously (23). These cells were cultured in IMDM supplemented with 5% FCS, 1  $\mu$ g/ml mycophenolic acid, penicillin, streptomycin, and glutamine.

The mAb M4, an anti-chicken IgM, was used for stimulation of BCR on DT40 cells (24). Abs against porcine Syk and PLC- $\gamma$ 2 were already described (12, 25). Abs against phosphotyrosine, 4G10 and Ab2, were purchased from Upstate Biotechnology Inc. (Lake Placid, NY) and Oncogene Sciences Inc. (Manhasset, NY), respectively. Polyclonal anti-Ig $\alpha$  and anti-Syk Abs used to analyze IgM coprecipitates from J558L $\mu$ m3 were generously provided by J. Jongstra (Toronto Western Hospital; Toronto, Canada) and E. Clark (University of Washington Medical Center; Seattle, WA), respectively. Immunoprecipitation of mouse mIgM BCR was accomplished using the monoclonal anti- $\mu$  Ab, b-7-6.

**Immunoprecipitation, Immunoblot Analysis, and In Vitro Kinase Assay.** In experiments shown in Figs. 1, 3, and 6, cells were solubilized in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA) containing 50 mM NaF, 10  $\mu$ M molibdate, and 0.2 mM Na<sub>3</sub>VO<sub>4</sub> supplemented with protease inhibitors (1 mM PMSF, 0.5 mM benzamide hydrochloride, 10  $\mu$ g/ml chymostatin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml antipain, 10  $\mu$ g/ml calpastatin 1, 10  $\mu$ g/ml pepstatin, 0.1 mM N- $\alpha$ -p-tosyl-L-lysine-chloromethyl ketone, and 0.1 mM N-1-tosylamide-2-phenylethylchloromethyl ketone). Insoluble material was removed by centrifugation at 12,000 g for 10 min. These clarified cell lysates were incubated sequentially (1 h, 4°C for each incubation) with antibodies and protein A-Sepharose. The immunoprecipitates were washed four times with lysis buffer. Whole-cell lysates were prepared from nonstimulated or M4-stimulated DT40 cells using SDS sample buffer. Whole-cell lysates or immunoprecipitates were fractionated on 6 or 8% SDS-PAGE and transferred to nitrocellulose. The blots were blocked with 5% milk in 25 mM Tris, pH 7.9, and 150 mM NaCl with 0.05% Tween-20, and incubated with primary Ab for 1 h at room temperature. Filters were developed with a goat anti-mouse or donkey anti-rabbit secondary Ab conjugated to horseradish peroxidase using the enhanced chemiluminescence (ECL) detection system (Amersham Corp., Arlington Heights, IL).

In the experiment shown in Fig. 4, in which association of Syk with BCR was studied, J558L $\mu$ m3 cells were either unstimulated or stimulated with NP<sub>7</sub>BSA at 37°C for 1 min and lysed in 1% digitonin lysis buffer (1% digitonin, 150 mM NaCl, 10 mM Tris, pH 8) supplemented with 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 10 mM NaF, 0.4 mM EDTA, and 1  $\mu$ g/ml each of leupeptin, aprotinin, and  $\alpha$ -1 antitrypsin. Lysates were cleared by centrifugation at 12,000 g for 10 min at 4°C, and BCR was immunoprecipitated from lysates at 4°C for 1 h with anti- $\mu$  mAb b-7-6 that was conjugated to Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ). The immunoprecipitates were washed three times with lysis buffer, fractionated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane. Antiphosphotyrosine immunoblotting was carried out by incubating the membrane with mAb Ab2 in 5% BSA in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 2 h at 25°C, followed by goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories) in 5% milk in TBS for 1 h at 25°C. The blot was then developed using ECL (Amersham Corp.). The same blot was stripped in buffer containing 100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, per Amersham Corp. protocol, and reprobed with anti-Syk Ab. The identical blot was further stripped and reprobed with anti-Ig $\alpha$  Ab. When anti-Syk or anti-Ig $\alpha$  were used as primary Ab, horseradish peroxidase-protein A was used as secondary reagent, and the blot was developed with ECL.

For in vitro kinase assay (Fig. 1), the immunoprecipitates were washed with 20 mM Hepes, pH 8, and 150 mM NaCl after washing with lysis buffer. Added to each sample was 50  $\mu$ l kinase

buffer (20 mM Hepes, pH 8, 150 mM magnesium acetate, 10 mM  $MnCl_2$ ) in the absence or presence of presence of ATP (1  $\mu M$ ). The reactions were allowed to incubate at 30°C for 10 min and terminated by the addition of sample buffer.

**Analysis of Calcium Mobilization and Phosphoinositide Hydrolysis.** Measurements of intracellular free calcium were performed using fura-2/AM. Cells ( $5 \times 10^6/ml$ ) were washed once and loaded with 3  $\mu M$  fura-2/AM in PBS containing 20 mM Hepes, pH 7.2, 5 mM glucose, 0.025% BSA, and 1 mM  $CaCl_2$ . After incubation for 45 min at 37°C, cells were washed twice and diluted to  $10^6$  cells/ml. Fluorescence of cell suspension was continuously monitored with a fluorescence spectrophotometer (model F-2000; Hitachi Limited, Tokyo, Japan) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Intracellular free calcium,  $[Ca^{2+}]_i$ , was calibrated and computed as described (26).

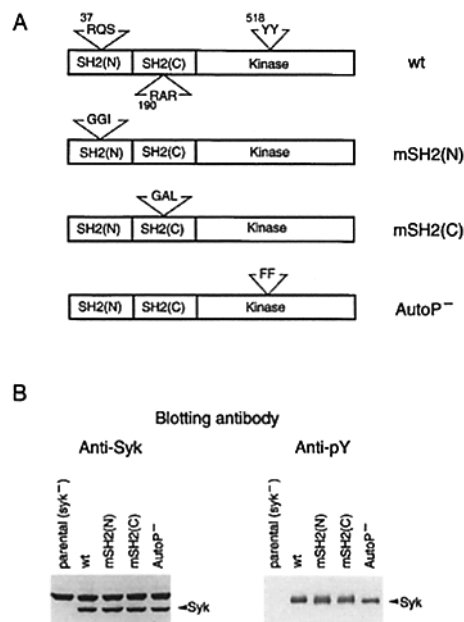
For measurements of  $IP_3$ , cells were labeled with myo- $[^3H]$ inositol (10  $\mu Ci/ml$ ) for 6 h in inositol-free RPMI 1640 supplemented with dialyzed FCS. The labeled cells were pre-equilibrated at 37°C and stimulated with mAb M4 in the presence of 10 mM LiCl. The soluble inositol phosphates were extracted with TCA and applied to 1 ml of AG 1-X8 (formate form) ion exchange columns (Bio-Rad Laboratories) pre-equilibrated with 0.1 mM formic acid. After loading the samples, columns were washed with 10 ml  $H_2O$  and 10 ml 60 mM ammonium formate–5 mM sodium tetraborate, and elution was performed with increasing concentrations of ammonium formate (0.1–0.7 M).

**ITAM-binding Protein Analysis.** Synthetic peptides corresponding to murine  $Ig\alpha$  ITAM (ENLY<sup>182</sup>EGLNLDLDDCSMY<sup>193</sup>EDI),  $Ig\alpha$  ITAM phosphorylated at residues Y182 and Y193,  $Ig\beta$  ITAM (DHTY<sup>195</sup>EGLNIDQTATY<sup>206</sup>EDI), or  $Ig\beta$  ITAM phosphorylated at residues Y195 and Y206 were produced using an Fmoc chemistry with 2-(1H-benzotriazol-1-yl)-1,1,3,3-trimethyluronium hexafluorophosphate active esters. Phosphotyrosine (Novabiochem USA, La Jolla, CA) was substituted for tyrosine as necessary to construct the doubly phosphorylated ITAMs. Peptides were deprotected by incubation for 90 min in 90% TFA, 2.5% aminole, and 2.5% ethane dithiol, purified by HPLC on C18, and analyzed by mass spectrometry to ensure predicted mass. Each peptide was then coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech, Inc.) per manufacturer's instructions at 2 mg peptide per milliliter of packed beads. In all cases, coupling efficiency, based on HPLC analysis of effluent from the coupled gel, was >90%.

Lysates ( $2 \times 10^7$  cells/ml) were prepared as described above and incubated with 10  $\mu l$  of peptide-coated beads overnight at 4°C with constant mixing by inversion. After adsorption, the beads were washed three times in 1 ml NP-40 lysis buffer and eluted by resuspension in 50  $\mu l$  of reducing SDS-PAGE sample buffer and boiling for 5 min. Eluates were resolved by electrophoresis on 8% SDS-polyacrylamide gels and transferred to PVDF membranes. Syk was detected by immunoblotting as described above.

## Results

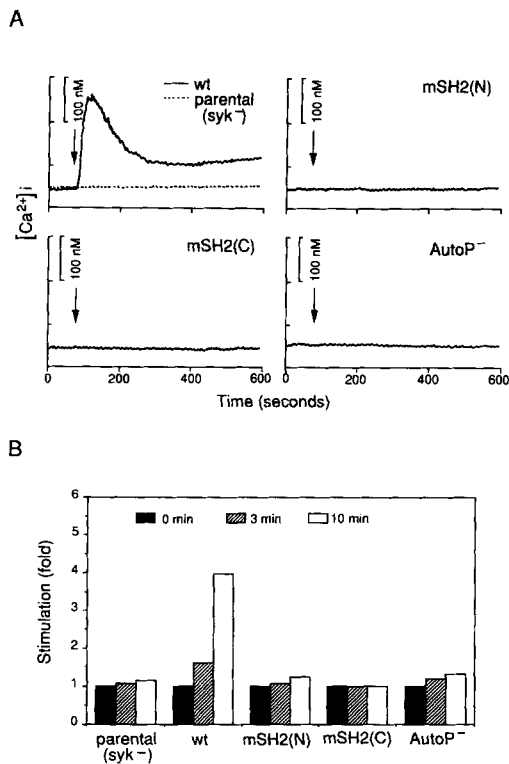
To study the functional importance of the two SH2 domains of Syk in BCR signaling, we introduced mutations within the highly conserved residues of  $NH_2$ - or  $COOH$ -terminal SH2 domains of Syk (Fig. 1 A). DNA was transfected into Syk-deficient DT40 B cells, and puromycin-resistant clones were selected. The expression of mutated Syk was monitored by immunoblotting and in vitro kinase assay (Fig. 1 B). Cell surface expression of BCR by these



**Figure 1.** Schematic representation of Syk cDNA constructs (A) and expression of mutant Syk in Syk-negative DT40 cells (B). DT40 cells ( $0.5 \times 10^6$  cells/lane) expressing indicated constructions were lysed with NP-40 buffer and subjected to electrophoresis on an 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anti-porcine Syk Ab (B, left), NP-40 lysates ( $2 \times 10^6$  cells/lane) were immunoprecipitated by anti-Syk Ab, and in vitro kinase reactions were carried out in the presence of ATP. These samples were also run on an 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using mAb 4G10 (B, right). In the absence of ATP, in vitro kinase samples of these immunoprecipitates showed no significant tyrosine phosphorylation.

transfected cells was assayed by FACS<sup>®</sup> and demonstrated essentially the same level as wild-type DT40 and Syk-deficient DT40 cells (data not shown).

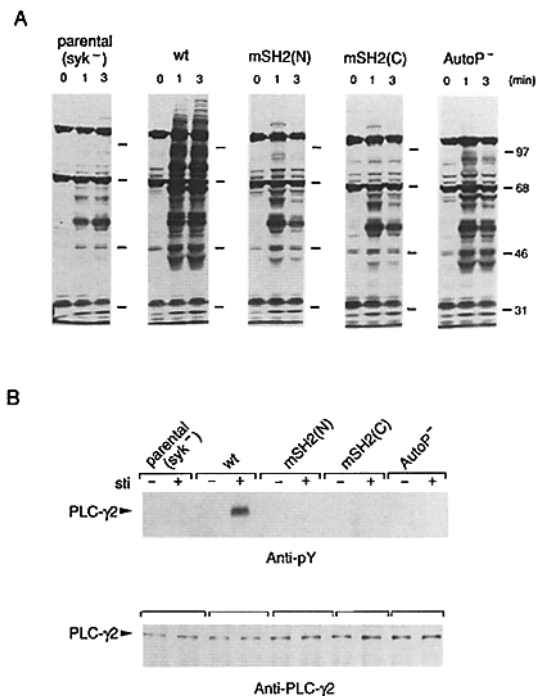
In previous studies, we have shown that Syk is essential for coupling the BCR to PtdIns pathway (22). Thus, as a functional complementation assay, we first analyzed whether Syk mutants mSH2(N) or mSH2(C) were able to restore  $IP_3$  generation response as well as calcium mobilization normally seen upon BCR stimulation. In contrast to wild-type Syk, DT40 cells expressing either of the SH2 mutants of Syk did not sustain the generation of  $IP_3$  after BCR stimulation (Fig. 2 B). Consistent with  $IP_3$  generation data, BCR-induced calcium mobilization was not detected in cells reconstituted with mSH2(N) or mSH2(C) (Fig. 2 A). These data indicate that both SH2 domains of Syk are essential for coupling the BCR to PtdIns pathway. To test whether the uncoupling receptor to  $IP_3$  generation caused by these SH2 mutations is due to the loss of BCR-induced tyrosine phosphorylation of PLC- $\gamma_2$ , anti-PLC- $\gamma_2$  immunoprecipitates were prepared from the BCR-stimulated cells and immunoblotted with antiphosphotyrosine and anti-PLC- $\gamma_2$  Abs. In DT40 cells expressing wild-type Syk, tyrosine phosphorylation of PLC- $\gamma_2$  was induced. However, in cells expressing mSH2(N) or mSH2(C) mutants, this BCR-induced tyrosine phosphorylation of PLC- $\gamma_2$  was abolished (Fig. 3 B).



**Figure 2.** Calcium mobilization (A) and IP<sub>3</sub> generation (B) upon BCR stimulation of DT40 cells. Cells were loaded with fura-2/AM, and the samples (10<sup>6</sup> cells/ml) were stimulated with anti-chicken IgM (M4, 1 μg/ml), with analysis of [Ca<sup>2+</sup>]<sub>i</sub> by fluorimetry. As a control, cells were stimulated with thapsigargin (10 μg/ml), which induced similar calcium mobilization in all clones (data not shown). For measurements of phosphoinositide hydrolysis, cells (2 × 10<sup>6</sup> cells/ml) were loaded with [<sup>3</sup>H]inositol and stimulated with anti-IgM (M4, 1 μg/ml). After 3 or 10 min of incubation, soluble inositol phosphates were extracted and separated by AG 1-X8 ion exchange columns.

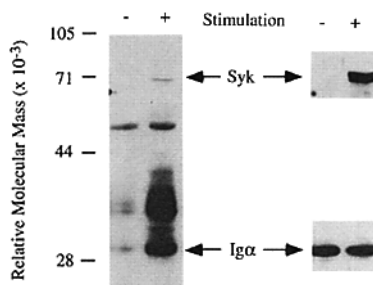
To examine whether these SH2 mutations affect only phosphorylation of PLC-γ2 upon BCR stimulation, DT40 cells expressing these mutants were stimulated by anti-BCR mAb M4, and whole lysates were analyzed by antiphosphotyrosine mAb. Although wild-type Syk rescued the phosphorylation pattern as seen in wild-type DT40 cells (22), neither the mSH2(N) nor mSH2(C) mutant rescued the BCR response (Fig. 3 A). These results suggest that both SH2 domains of Syk are necessary for coupling BCR to Syk-dependent manifestations of signal transduction.

A possible explanation for the phenotype of these SH2 mutants is that, like ZAP70 and TCR components (27, 28), Syk is recruited to phosphorylated Igα and Igβ during receptor signaling, and that both SH2 domains are crucial for this recruitment. Since BCR-induced tyrosine phosphorylation of Igα is weak in DT40 cells (data not shown), we examined the recruitment of Syk to BCR upon antigen (NP<sub>7</sub>BSA) stimulation in J558Lμm3 myeloma cells (23). These cells express NP-specific IgM BCR by virtue of transfection. After stimulation for 1 min and detergent lysis, a tyrosine-phosphorylated protein of ~70 kD was noticeably coprecipitated with IgM (Fig. 4, left). Sequential im-



**Figure 3.** Tyrosine phosphorylation of whole-cell proteins (A) and anti-PLC-γ2 (B) after DT40 cell activation. At the indicated times after the stimulation of cells with anti-IgM (M4, 2 μg/ml), whole-cell lysates were prepared from 2.5 × 10<sup>6</sup> cells in 100 μl and loaded on an 8% SDS-PAGE. After transfer to nitrocellulose, the phosphotyrosine-containing proteins were detected by immunoblotting with mAb 4G10. For detection of PLC-γ2 tyrosine phosphorylation, cells (2 × 10<sup>6</sup> cells/ml) were stimulated for 3 min with anti-BCR mAb (M4, 2 μg/ml), lysed, and immunoprecipitated with anti-PLC-γ2. Samples were divided, subjected to 6% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine Ab 4G10 (B, top) or with anti-PLC-γ2 (B, bottom).

munoblotting identified this phosphoprotein as Syk (Fig. 4, upper right). Since anti-Igα immunoblotting of the same membrane showed a similar amount of Igα in each immunoprecipitate (Fig. 4, lower right), the increase in Syk coprecip-



**Figure 4.** Receptor ligation-induced association of Syk with BCR in J558Lμm3 cells. J558Lμm3 cells (4 × 10<sup>7</sup> in 400 μl) were either unstimulated (-) or stimulated (+) with NP<sub>7</sub>BSA (40 μg/sample) for 1 min and lysed with 1% digitonin buffer. BCR was immunoprecipitated using anti-μ mAb (b-7-6, 15 μg/15 μl beads/sample)-conjugated Sepharose beads, fractionated by 10% SDS-PAGE, and transferred to PVDF membrane. The membrane was immunoblotted using antiphosphotyrosine mAb Ab2 (left) and subsequently reprobed with polyclonal anti-Syk (upper right) Ab, and then with anti-Igα Ab (lower right).

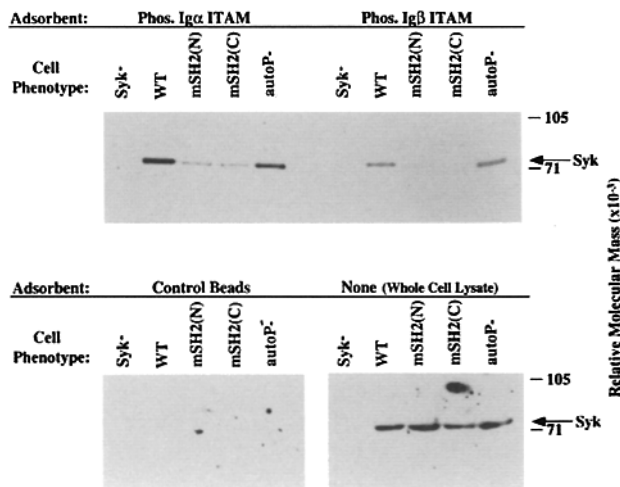
itated with stimulated receptors reflects Syk recruitment to BCR upon antigen stimulation. This Syk recruitment also correlated with inductive tyrosine phosphorylation of Ig $\alpha$  (Fig. 4, *left*). The apparently inducibly tyrosine-phosphorylated bands above Ig $\alpha$  and below the 44-kD marker probably reflect the more heterogeneous Ig $\beta$  (29).

To further elucidate the mechanism by which Syk is recruited to BCR, we used beads derivitized with synthetic peptides corresponding to nonphosphorylated and doubly phosphorylated ITAMs of Ig $\alpha$  and Ig $\beta$  as affinity matrices to assess the binding of Syk from DT40 cell lysates by immunoblotting. Although binding of wild-type Syk to non-phosphorylated ITAMs was not detectable (data not shown), binding to phosphorylated Ig $\alpha$  and Ig $\beta$  ITAMs was easily seen. Interestingly, the enzyme bound more strongly to Ig $\alpha$  than Ig $\beta$ . As shown in Fig. 5, Syk binding to Ig $\beta$  pITAM is approximately one third (by densitometry) the binding seen to Ig $\alpha$  pITAM. Syk mutant mSH2(N) or mSH2(C) binding to Ig $\alpha$  pITAM and Ig $\beta$  pITAM was greatly diminished compared with wild-type Syk. Thus, both SH2 domains are required for efficient binding of Syk to doubly phosphorylated ITAMs.

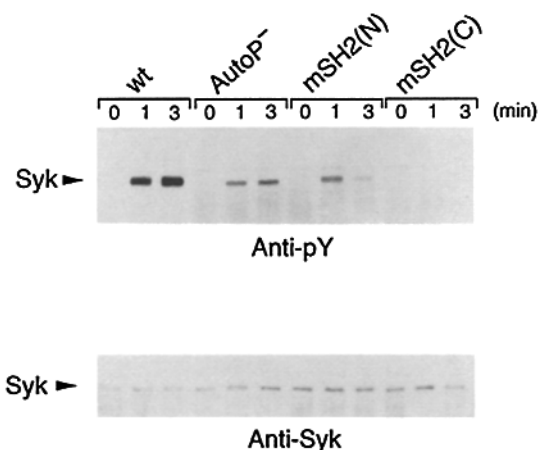
We previously showed that BCR-induced tyrosine phosphorylation of Syk is dependent on and possibly mediated directly by Lyn (30). To examine whether this Lyn-dependent tyrosine phosphorylation of Syk is affected by SH2 mutations, we stimulated DT40 cells expressing Syk SH2 mutants with anti-BCR mAb (M4) and measured tyrosine phosphorylation of Syk. Cell lysates were immunoprecipitated with anti-Syk Ab and analyzed by antiphospho-

phosphotyrosine mAb. Detectable tyrosine phosphorylation of mSH2(N) Syk was induced by BCR stimulation, although the kinetics were slower and the extent of phosphorylation much reduced compared with wild-type Syk. In contrast to mSH2(N) Syk, BCR-induced tyrosine phosphorylation of mSH2(C) Syk was completely abolished (Fig. 6). Thus, these results suggest that the recruitment of Syk to phosphorylated Ig $\alpha$  and Ig $\beta$  may be a prerequisite for tyrosine phosphorylation of Syk induced by receptor ligation. Significant phosphorylation of the NH<sub>2</sub>-terminal SH2 mutant despite the failure to detect binding (Fig. 5) may reflect relative insensitivity of the binding assay; that is, although it is not detectable, the NH<sub>2</sub>-terminal SH2 mutant may bind receptor *in vivo*.

Previous data suggest that the major autophosphorylation site of Src-PTK, such as Lck 394, is required for full activation in TCR signaling (31). Tyrosine 518 or 519 of Syk is presumed to be the major autophosphorylation site based on sequence homology between Syk and Src-PTKs (32). Thus, we wished to test the possibility that these tyrosines are sites of phosphorylation and are pivotal for BCR-induced signaling. Syk carrying tyrosine to phenylalanine substitutions at positions 518 and 519 (autoP-Syk) was expressed in Syk-deficient DT40 cells (Fig. 1 A). Cell lines expressing similar amounts of autoP-Syk to that of wild-type Syk were selected and further characterized. To determine whether autoP-Syk is phosphorylated *in vitro*, anti-Syk immunoprecipitates were incubated in the presence or absence of ATP, subjected to electrophoresis, and blotted with antiphosphotyrosine mAb. In the absence of ATP, tyrosine phosphorylation of neither wild-type Syk nor Syk mutants could be observed (data not shown). Even though the amount of immunoprecipitated autoP-Syk was equivalent to that of wild-type Syk (data not shown), the tyrosine phosphorylation of autoP-Syk was only ~30% of that of



**Figure 5.** Binding of wild-type and mutant Syk to phosphorylated Ig $\alpha$  and Ig $\beta$  ITAMs and to underivatized beads. Bead-bound peptides corresponding to phosphorylated Ig $\alpha$  or Ig $\beta$  ITAM (20  $\mu$ g peptide on 10  $\mu$ l beads) were used to adsorb Syk from DT40 cell lysates ( $2 \times 10^7$  cell equivalents in 1 ml) expressing various forms of porcine Syk. Adsorbates were washed, eluted, resolved on SDS-PAGE, transferred to PVDF membrane, and immunoblotted with polyclonal anti-Syk Ab. Whole-cell lysates were analyzed to control for expression. Detection was by protein A-horseradish peroxidase and ECL; exposure time was equivalent in all panels.



**Figure 6.** BCR-induced tyrosine phosphorylation of Syk mutants. DT40 cells ( $2 \times 10^6$  cells/ml) expressing various mutants were stimulated with anti-BCR mAb (M4, 4  $\mu$ g/ml) and immunoprecipitated with anti-Syk Ab (4  $\mu$ g/sample). Samples were divided, subjected to 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the antiphosphotyrosine Ab 4G10 (*top*) or anti-Syk (*bottom*).

wild-type Syk (Fig. 1 *B*). In other experiments, immunoprecipitates from cells expressing kinase-negative Syk did not exhibit tyrosine phosphorylation even in the presence of ATP (data not shown), indicating that a nonspecifically associated kinase activity is probably not involved in phosphorylation of autoP-Syk *in vitro*. Thus, these results suggest that autoP-Syk may still be competent to autophosphorylate a site other than tyrosines 518 and 519 under the *in vitro* conditions used.

DT40 cells expressing autoP-Syk were stimulated with anti-BCR mAb M4, and the induction of protein tyrosine phosphorylation was analyzed by immunoblotting with antiphosphotyrosine mAb. BCR-induced tyrosine phosphorylation in cells expressing autoP-Syk was significant but reduced, compared with DT40 cells expressing wild-type Syk (Fig. 3 *A*). Thus, autoP-Syk not only appears to have some kinase activity detectable *in vitro*, but it supports a partial inductive tyrosine phosphorylation response *in vivo*. Tyrosines 518 and/or 519 are apparently not absolutely essential for partial participation of Syk in BCR signaling. As shown in Fig. 5, autoP-Syk bound phosphorylated ITAMs equivalently to wild-type Syk. Since BCR-induced tyrosine phosphorylation of PLC- $\gamma$ 2 is dependent on Syk, we examined next whether autoP-Syk could support the tyrosine phosphorylation of PLC- $\gamma$ 2 after BCR cross-linking. As shown in Fig. 3 *B*, BCR-induced phosphorylation of PLC- $\gamma$ 2 was not observed in DT40 cells expressing autoP-Syk. Consistent with these data, BCR stimulation in these cells resulted in no increase in IP<sub>3</sub> generation and no calcium mobilization (Fig. 2, *A* and *B*). These results demonstrate that tyrosine phosphorylation at 518 and/or 519 is required for coupling Syk to the PtdIns pathway, but not for pITAM binding.

Amino acid homology suggests that Syk tyrosines 518/519 are likely sites of autophosphorylation and perhaps transphosphorylation of Syk, yet some tyrosine phosphorylation of this autoP-Syk mutant is seen *in vitro*. To determine whether non-Y518/519 tyrosine phosphorylation of Syk occurs in BCR signaling, we compared receptor-mediated Syk tyrosine phosphorylation in wild-type and autoP-Syk transfectants. As shown in Fig. 6, BCR-induced tyrosine phosphorylation of autoP-Syk was significant, but approximately fivefold lower than in wild-type Syk-transfected cells. Binding of autoP-Syk to phosphorylated Ig $\alpha$ / $\beta$  ITAMs was the same as that of wild-type Syk (Fig. 5), consistent with the possibility that autoP-Syk may be phosphorylated by other receptor-associated kinases such as Lyn. Thus, this observation demonstrates that 518 and/or 519 tyrosines are important sites of Syk phosphorylation upon BCR stimulation, but some inductive phosphorylation clearly occurs at other sites. However, this limited phosphorylation of autoP-Syk does not support BCR-mediated phosphorylation and activation of PLC- $\gamma$ 2. Finally, since SH2 domain mutants do not associate with phosphorylated ITAMs or become phosphorylated themselves, yet the autoP-Syk mutant associates with pITAMs but does not become phosphorylated, pITAM binding may be an essential prerequisite for Y518/519 phosphorylation and signal propagation.

## Discussion

The important role of SH2 domains in signal transmission is widely appreciated. SH2 domains mediate intermolecular interactions by binding to phosphotyrosine-containing sequences with high affinity (33, 34). Although previous studies have demonstrated that both SH2 domains of ZAP70 are necessary for binding to phospho- $\zeta$  (28) and Syk binding to Fc $\epsilon$ RI $\gamma$  (35), studies reported here are the first to show that both NH<sub>2</sub>- and COOH-terminal SH2 domains of Syk are indispensable for BCR signal transduction. This demonstrates the functional importance of these domains. The BCR-mediated protein tyrosine phosphorylation response of DT40 cells expressing Syk SH2 mutants appeared only modestly increased over that of Syk-deficient cells, and far less than wild-type Syk-expressing cells (Fig. 3 *A*), indicating that both SH2 domains are essential for most Syk-dependent protein tyrosine phosphorylation. *In vitro* kinase assay of these SH2 mutants (Fig. 1 *B*), together with findings in COS cells that the overall tyrosine phosphoprotein patterns of cells overexpressing these SH2 mutants and Fyn was similar to that with wild-type Syk and Fyn (data not shown), indicates that Syk SH2 mutants have enzymatic activity both *in vitro* and in COS cells. Thus, our functional results suggest that Syk SH2 domains provide a localization function that is necessary for coupling the BCR to PLC $\gamma$ .

It has been shown that an essential intermediary event in BCR coupling to downstream events is phosphorylation of Ig $\alpha$  and Ig $\beta$  ITAMs (17, 18). As shown here, BCR ligation leads to recruitment of Syk to the receptor (Fig. 4). Thus, it seemed possible that the signaling deficit of Syk SH2 mutants might relate to failed interaction with phosphorylated ITAMs. To explore this possibility, we conducted an analysis of binding of Syk to phosphorylated or nonphosphorylated Ig $\alpha$  and Ig $\beta$ . Unstimulated receptors and nonphosphorylated ITAM peptides of Ig $\alpha$  and Ig $\beta$  did not bind significantly to wild-type Syk. Phosphorylation of Ig $\alpha$  and Ig $\beta$  ITAMs increased their binding of wild-type Syk. Phosphorylated Ig $\alpha$  binding to Syk was much more readily detected than binding to phosphorylated Ig $\beta$ . Thus, Syk prefers to bind to Ig $\alpha$ . The difference in the binding activity of phosphorylated Ig $\alpha$  and Ig $\beta$  ITAMs may explain the observations that Ig $\alpha$  cytoplasmic tails are more competent than Ig $\beta$  cytoplasmic tails in chimeric receptor coupling to protein tyrosine phosphorylation (18, 36). Binding of Syk to phosphorylated ITAMs was dependent on both SH2 domains, but in some experiments the COOH-terminal SH2 domain of Syk appeared somewhat more critical for binding to phosphorylated ITAMs than the NH<sub>2</sub>-terminal SH2 domain (data not shown). These findings are consistent with the recent report of Shiue et al. (35), which demonstrated that both SH2 domains are necessary for optimal Syk association with the tyrosine-phosphorylated Fc $\epsilon$ RI $\gamma$  ITAM *in vitro*, and that the COOH-terminal SH2 exhibits higher affinity for this ITAM than the NH<sub>2</sub>-terminal SH2. These findings suggest that recruitment of Syk to phosphorylated ITAMs via its SH2 domains is required for BCR coupling to Syk function.

The question of how interaction of the Syk SH2 domains with phosphorylated Ig $\alpha$  and Ig $\beta$  ITAMs could affect Syk function arises. Phosphorylation of the ITAMs could result in Syk binding induced allosteric activation. Recent studies indicate that this is in fact the case (37, 38). Fc $\epsilon$ R1 $\gamma$ , Ig $\alpha$  and Ig $\beta$  ITAM phosphopeptides were shown to stimulate phosphorylation and activation of Syk in vitro. It is noteworthy, however, that in studies in our laboratory, pITAMs activate Lyn (19) but were ineffective activators of Syk both in vitro and in permeabilized cells (Johnson, S., and J. Cambier, manuscript submitted for publication). The reason for this inconsistency is not known but may reflect different assay conditions.

In studies reported here, binding of autoP-Syk to phosphorylated ITAMs was the same as that of wild-type Syk; however, autoP-Syk did not sustain PLC- $\gamma$ 2 phosphorylation and activation, indicating that in addition to Syk interaction with ITAMs, Y518/519 phosphorylation is required for coupling Syk to PLC- $\gamma$ 2 phosphorylation and activation. This could reflect a necessity for Syk phosphorylation to achieve full activity. However, it may not reflect a deficit in Syk activation but rather a deficit in its ability to recruit substrates via interaction between phospho Y518/519 sites and SH2-containing proteins such as PLC- $\gamma$ 2.

In earlier studies, we demonstrated that Lyn coexpression with Syk was necessary to achieve Syk activation (30). Further, Iwashima et al. (28) have shown using CD8- $\zeta$  chimeric receptors and kinase-inactive ZAP70 that ligand-activated ZAP70 phosphorylation does not depend on ZAP70 kinase activity. Taken together, these data indicated that Syk/ZAP70 kinases can be activated by (p)<sub>2</sub>ITAM-induced autophosphorylation or by phosphorylation mediated by an Src-family kinase. The latter may normally only occur when (p)<sub>2</sub>ITAMs trigger activation of the Src-family kinase and bring the two kinases together.

The question of how phosphorylated ITAMs activate Syk arises. It is possible that recruitment of Syk to phosphorylated Ig $\alpha$ /Ig $\beta$  may lead to activation by dimerization-driven Syk transphosphorylation. This possibility may be supported by the observation that aggregation of CD16/Syk chimeric receptor bearing a CD16 extracellular domain and an Syk

kinase intracellular domain induces tyrosine phosphorylation upon receptor aggregation (39). However, the ability of monomeric (p)<sub>2</sub>ITAMs to activate Syk in immunoprecipitates (38) is inconsistent with a dimerization-driven mechanism. Further, since both Syk SH2 domains are required for (p)<sub>2</sub>ITAM binding, it is unlikely that (p)<sub>2</sub>ITAMs could dimerize Syk. Resolution of Syk regulation after BCR ligation awaits further study.

The requirement for two functional SH2 domains for Syk phosphorylation and later events may reflect in part a need for the molecule to interact via these domains with phosphotyrosine on molecules other than Ig $\alpha$  and Ig $\beta$ . It seems likely that such tyrosine residues would be constitutively phosphorylated and therefore constitutively associated with Syk, or they may be inducibly phosphorylated even in the absence of Syk by, for example, Src-family kinases. To date, no constitutive association of Syk with tyrosine phosphoproteins has been reported. Candidate inducibly phosphorylated proteins include those seen in Fig. 3 A in stimulated Syk-deficient cells. The ability of Syk SH2 domains to bind to proteins inducibly tyrosine phosphorylated in the absence of Syk is currently under study.

Studies to date have only begun to dissect biochemical steps for BCR signal transmission. Our working hypothesis is as follows: (a) Although the identity of the first kinase activated upon BCR cross-linking is unknown, it appears almost certain that activation of Ig $\alpha$ -associated Lyn is among the earliest steps (40–42); (b) available evidence is most consistent with Lyn-mediated phosphorylation of several molecules including Ig $\alpha$  and Ig $\beta$  (17); (c) Syk and additional Lyn are recruited to the phosphorylated Ig $\alpha$  and Ig $\beta$  via their SH2 domains (Figs. 4 and 5; 19, 20, 43); (d) recruited Lyn and Syk are activated by this binding (19, 37, 38); (e) subsequently, Syk is phosphorylated by autophosphorylation or Lyn-mediated transphosphorylation at the 518 and/or 519 tyrosine sites; (f) activated Syk phosphorylates its own substrates apparently including PLC- $\gamma$ 2; and (g) tyrosine-phosphorylated PLC- $\gamma$ 2 mediates phosphoinositide hydrolysis leading to Ca<sup>2+</sup> mobilization. Thus, both recruitment of Syk and its phosphorylation appear essential for BCR signaling.

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