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Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca^{2+} mobilization through distinct pathways

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Stimulation of B lymphocytes through their antigen receptor (BCR) results in rapid increases in tyrosine phosphorylation on a number of proteins and induces both an increase of phosphatidylinositol and mobilization of cytoplasmic free calcium. The BCR associates with two classes of tyrosine kinase: Src-family kinase (Lyn, Fyn, Blk or Lck) and Syk kinase. To dissect the functional roles of these two types of kinase in BCR signaling, *lyn*-negative and *syk*-negative B cell lines were established. Syk-deficient B cells abolished the tyrosine phosphorylation of phospholipase C-γ2, resulting in the loss of both inositol 1,4,5-trisphosphate (IP_3) generation and calcium mobilization upon receptor stimulation. Crosslinking of BCR on Lyn-deficient cells evoked a delayed and slow Ca^{2+} mobilization, despite the normal kinetics of IP_3 turnover. These results demonstrate that Syk mediates IP_3 generation, whereas Lyn regulates Ca^{2+} mobilization through a process independent of IP_3 generation.

Key words: B cell receptor/Ca^{2+} mobilization/Lyn/phosphatidylinositol turnover/Syk

Introduction

Engagement of the B cell antigen receptor (BCR) initiates signaling mechanisms that can lead to immune proliferation and differentiation (Cambier and Ransom, 1987). The BCR is a multisubunit complex composed of an antigen recognition component, membrane Ig and associated subunits. Two of these receptor-associated subunits, Igα (mb1) and Igβ (B29), are responsible for cell surface transport, as well as signal transduction of BCR (Costa *et al.*, 1992; Desiderio, 1992; Reth, 1992; Sanchez *et al.*, 1993). These Igα and Igβ chains contain within their cytoplasmic domains a phylogenetically conserved motif (ARH1 motif) bearing two tyrosine residues separated by 10 or 11 residues, which is found in a number of other signal transducer chains of the T cell antigen receptor complex (TCR) and various forms of Fc receptor (Reth, 1989; Cambier and Campbell, 1992; Samelson and Klausner, 1992; Weiss, 1993). Clustering of chimeric receptors bearing this motif initiates an activation cascade, suggesting that this short sequence carries sufficient structural information to activate signaling pathways (Irving and Weiss, 1991; Letourneur and Klausner, 1991; Romeo and Seed, 1991; Romeo *et al.*, 1992; Wegener *et al.*, 1992; Irving *et al.*, 1993).

Stimulation of the BCR initiates a biochemical cascade in which protein tyrosine kinase (PTK) activity is the earliest known event (Campbell and Sefton, 1990; Gold *et al.*, 1990; Brunswick *et al.*, 1991). PTK inhibitors block early events such as calcium mobilization and inositol 1,4,5-trisphosphate (IP_3) generation (Carter *et al.*, 1991; Lane *et al.*, 1991). Numerous cellular proteins become tyrosine phosphorylated following BCR engagement. Although many of them have not been characterized at the molecular level, one known PTK substrate is phospholipase C (PLC)-γ2 (Emori *et al.*, 1989; Hempel and DeFranco, 1991; Coggeshall *et al.*, 1992). Tyrosine phosphorylation of PLC-γ2 is responsible for its increased activity (Nishibe *et al.*, 1990), allowing the conversion of phosphatidylinositol 4,5-bisphosphate into the two second messengers diacylglycerol and IP_3. Diacylglycerol activates protein kinase C (Nishizuka, 1988), and IP_3 is thought to cause Ca^{2+} release from intracellular stores after binding to its receptor (Berridge, 1993a). Indeed, purified IP_3 has been shown to cause an increase of intracellular Ca^{2+} in permeabilized B cells (Ransom *et al.*, 1986).

Since none of the BCR subunits possesses intrinsic PTK activity, it is implicated that cytoplasmic PTKs are associated with BCR complexes. Thus far, the BCR-associated PTKs fall into two classes: Src-family PTKs and recently characterized Syk kinase (Taniguchi *et al.*, 1991). Syk kinase is expressed in B cells (Hutchcroft *et al.*, 1991), and its primary structure is highly homologous to ZAP-70 which is expressed in T and NK cells (Chan *et al.*, 1991, 1992). Among the Src-PTKs, Lyn, Fyn, Blk and Lck kinases have been shown to associate with the BCR (Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1991; Campbell and Sefton, 1992). The mechanism of the observed association has not been established in detail, but *in vitro* binding data suggest that the cytoplasmic domain of the Igα chain may physically associate with Lyn or Fyn (Clark *et al.*, 1992). Although in BCR signaling the requirement of a Src-PTK for activation has not been definitely established, several systems have been developed to address the functional role of the Src-PTK in T cell activation. The most compelling evidence for the direct participation of Lck or Fyn kinase in TCR-mediated signaling comes from studies using Lck-negative mutant T cell lines (Goldsmith and Weiss, 1987; Karnitz *et al.*, 1992; Straus and Weiss, 1992) or mutant mice lacking Fyn or Lck (Appleby *et al.*, 1992; Molina *et al.*, 1992; Stein *et al.*, 1992).

Unlike the Src-PTKs, ZAP-70 and Syk bear two SH2 domains and no N-terminal myristoylation site (Taniguchi *et al.*, 1991; Chan *et al.*, 1992). Based on the analysis of

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association between ZAP-70 and ζ chain through TCR stimulation (Chan *et al.*, 1991, 1992), the attractive hypothesis was proposed that ZAP-70 binds the tyrosine residues of the ζ chain via SH2 domains once ζ is phosphorylated by ligand stimulation (Weiss, 1993). However, in B cells the Syk kinase has been reported to associate with the BCR complex even in the absence of ligand (Hutchcroft *et al.*, 1992). To date, evidence for the importance of Syk kinase has been developed from the study of the chimeric molecule bearing a CD16 extracellular domain and a Syk kinase intracellular domain in T cells (Kolanus *et al.*, 1993). Clustering of CD16/Syk chimeric molecule, but not CD16/Fyn, triggers a calcium mobilization, suggesting a clear distinction between Syk and Src-PTK.

To address the exact role of Syk and Src-PTK in BCR signaling, we have taken a genetic approach. The chicken B cell line DT40 expresses BCR (IgM isotype) on its surface (Buerstedde and Takeda, 1991). Stimulation of this BCR by anti-receptor antibodies evoked tyrosine phosphorylation, an increase of IP_3 and mobilization of intracellular calcium. These early events are similar to those of normal B cells and mouse B cell line WEHI-231 (Cambier and Ransom, 1987). Since targeted integration occurs at high frequency in DT40 cells (Buerstedde and Takeda, 1991), this cell line allows us to investigate directly the functional participation of Syk and Src-PTK in BCR signaling by gene disruption. Here, we generated *lyn*-negative and *syk*-negative DT40 B cell lines and examined the BCR-mediated signaling properties of these mutant cells. Defects of early signaling events were different between *lyn*-negative and *syk*-negative B cells. Surprisingly, cells lacking Lyn showed a delayed and slow Ca^{2+} mobilization despite the normal kinetics of IP_3 generation, whereas both IP_3 generation and Ca^{2+} mobilization was abolished in cells lacking Syk. The BCR-mediated induction of tyrosine phosphorylation on PLC-γ2 was abolished in the *syk*-negative cells but not in the *lyn*-negative cells. Thus, our results demonstrate that not only IP_3 generation but also an IP_3-independent pathway are required for normal BCR-coupled Ca^{2+} mobilization, and that these two pathways are mediated through Syk and Lyn, respectively.

Results

Targeted disruption of lyn and syk

RNA blot analysis of DT40 cells revealed that *lyn* and *syk* are expressed in this cell line. Transcripts of the *src*, *lck*, *fyn*, *blk*, *yes*, *hck* or *zap-70* could not be detected (data not shown), showing that *lyn* and *syk* are expressed dominantly among *src*-PTKs and *syk*-family PTKs, respectively. To disrupt the *lyn* locus, we transfected a targeting construct pLyn-Neo into parental DT40 cells (Figure 1B). Southern blot analysis indicated that two out of 30 G418-resistant clones had sustained a targeted event. The hybridization signal of a rearranged 11 kb band was weaker than the wild-type 9 kb band, suggesting that DT40 contains three alleles of the *lyn* locus (Figure 1D, lane 2). Disruption of the remaining two alleles of the *lyn* locus was accomplished by the sequential transfection of two other targeting constructs, pLyn-His and pLyn-Hyg (Figure 1B). Hybridization with a *neo*, *hisD* or *hygro* probe indicated that the targeted clone

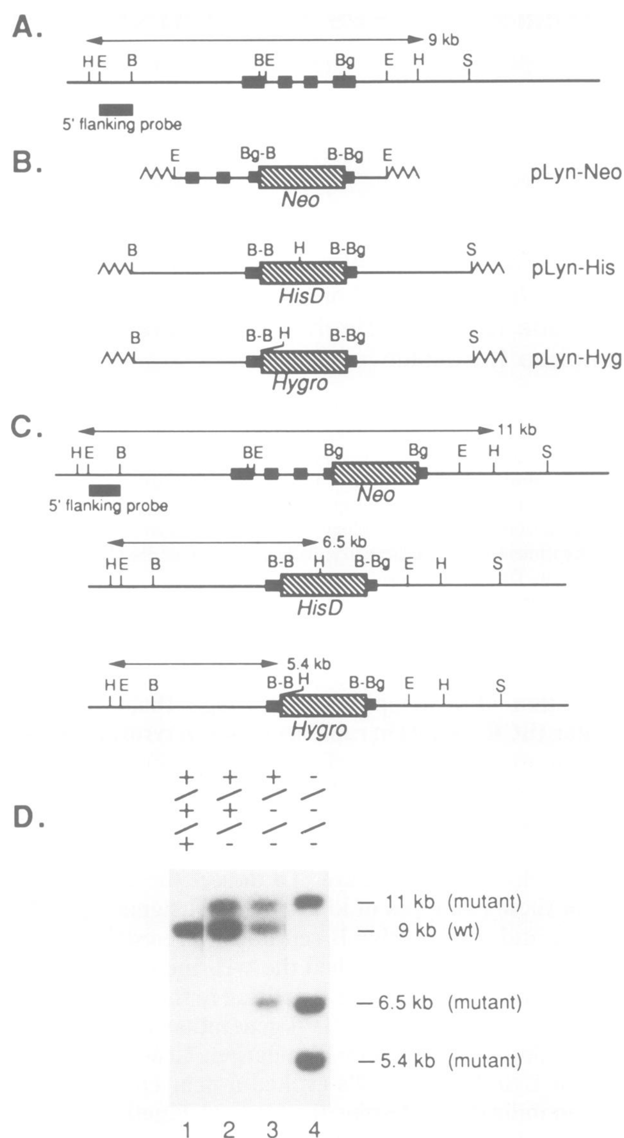

Fig. 1. Homologous recombination at the *lyn* locus. Partial restriction map of the chicken *lyn* gene (**A**), targeting construct (**B**), expected structure of the disrupted alleles (**C**) and Southern blot analysis of genomic DNAs (**D**). Exon 1 (including *Bam*HI site) to exon 4 are shown by closed boxes. The exon including initiation methionine was assigned as exon 1. The restriction endonuclease cleavage sites are abbreviated as A = *Apa*I; B = *Bam*HI; Bg = *Bgl*II; E = *Eco*RI; H = *Hin*dIII; S = *Sac*I; X = *Xba*I. Genomic DNAs were prepared from wild-type (+/+/+, lane 1), *neo*-targeted (+/+/−, lane 2), *neo/hisD*-targeted (+/−/−, lane 3) and *neo/hisD/hygro*-targeted (−/−/−, lane 4) clones, digested with *Hin*dIII and probed using the 5′ flanking region (900 bp *Eco*RI−*Bam*HI fragment).

had incorporated a single copy of each construct (data not shown).

For disruption of the *syk* locus, the targeting construct pSyk-Neo was electroporated into DT40 cells (Figure 2B) and 28 clones were analyzed by genomic Southern blots. The *Xba*I digest allows clear distinction between the 9.3 kb fragment corresponding to the targeted locus and the 13 kb fragment of the non-targeted locus (Figure 2A and C). Of 28 G418-resistant clones screened, four clones showed only the 9.3 kb fragment. The hybridization intensity of the rearranged fragment is almost the same as that of wild-type 13 kb fragment (Figure 2D). These results suggest that *syk*

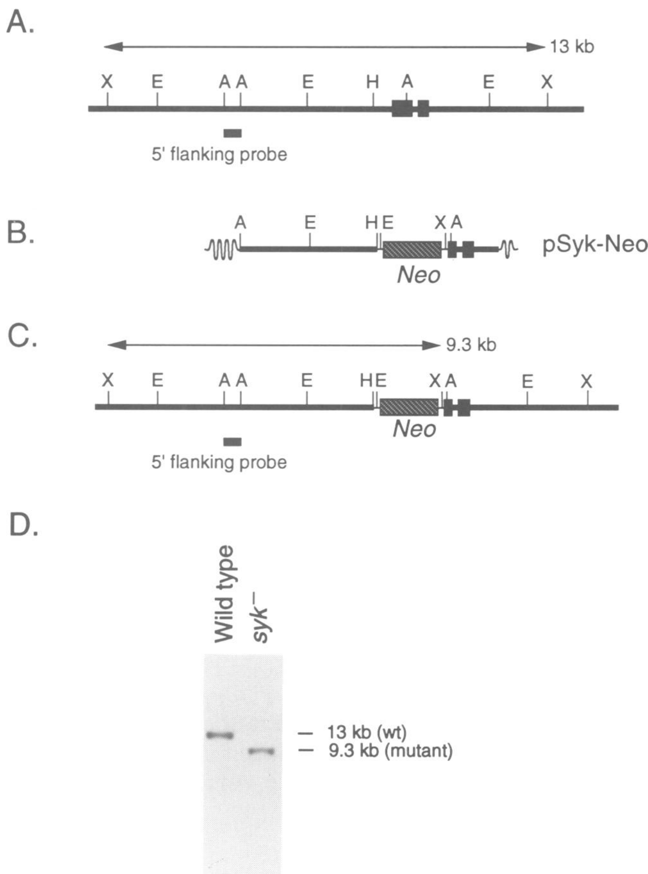

Fig. 2. Homologous recombination at the *syk* locus. Partial restriction map of the chicken *syk* gene (**A**), targeting construct (**B**), expected structure of the disrupted allele (**C**) and Southern blot analysis of genomic DNAs (**D**). Exons 1 and 2 are shown by closed boxes. Abbreviations of restriction endonuclease cleavage sites are given in Figure 1. The exon including initiation methionine was assigned as exon 1 (containing *Apa*I site). Genomic DNA was digested with *Xba*I and probed using the 5′ flanking region (400 bp *Apa*I−*Apa*I fragment).

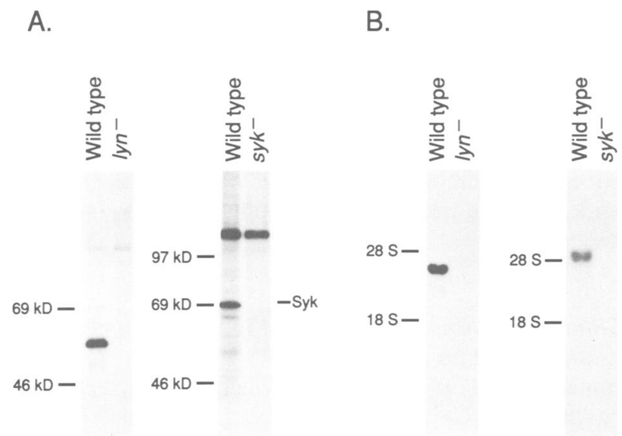

Fig. 3. *In vitro* kinase and Northern analysis for Lyn and Syk. (**A**) Extracts were prepared, immunoprecipitated with anti-Lyn or anti-Syk antibody, and incubated with [γ-^{32}P]ATP in a protein autophosphorylation kinase assay. The radiolabeled protein was identified by autoradiography after electrophoresis on an 8% SDS−PAGE gel. (**B**) 20 μg total RNA was separated in a 1.2% formaldehyde gel, blotted and probed with chicken *lyn* or *syk* cDNA.

has only one allele in DT40, although the possibility that two alleles of *syk* are simultaneously targeted by single DNA transfection is not excluded. The *neo* probe hybridized only the targeted fragment.

To verify that the disruption of *lyn* or *syk* creates a null mutation, we performed *in vitro* kinase assay and blot analysis of RNA from wild-type and targeted DT40 cells. An autophosphorylated protein was present in anti-Lyn or anti-Syk immunoprecipitate from wild-type, but absent from *lyn* or *syk* targeted clone respectively (Figure 3A). Analysis of RNA from *lyn* or *syk* targeted clone failed to reveal a transcript (Figure 3B). The level of cell surface expression of BCR on *lyn* or *syk* targeted clones was essentially the same as that of parental DT40 cells (data not shown).

Both Lyn and Syk are required for coupling BCR to full PTK activity

One of the earliest events following BCR stimulation is the induction of PTK activity (Campbell and Sefton, 1990; Gold *et al.*, 1990; Brunswick *et al.*, 1991). Wild-type, *lyn*-negative and *syk*-negative DT40 cells were stimulated with anti-BCR mAb, M4 (Chen *et al.*, 1982), and the induction of protein tyrosine phosphorylation was analyzed by immunoblotting with anti-phosphotyrosine mAb. Figure 4 demonstrates that parental DT40 cells exhibit a large set of

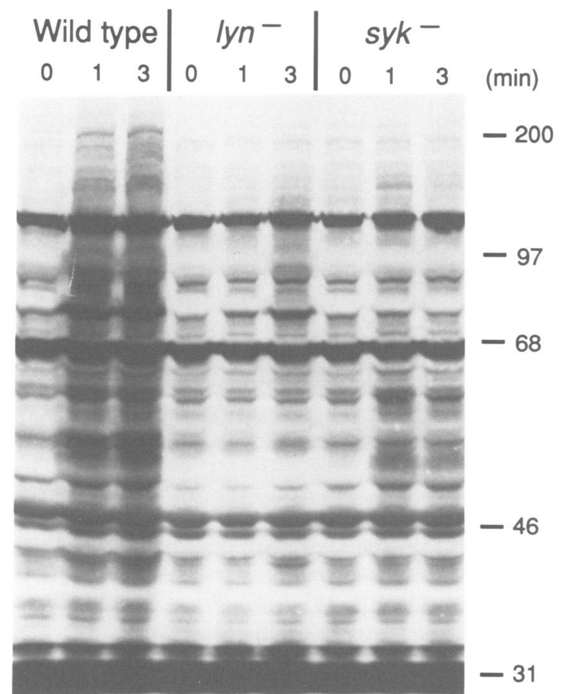

Fig. 4. Tyrosine phosphorylation in wild-type, *lyn*-negative and *syk*-negative DT40 cells following activation with anti-IgM (M4). At the indicated times following the addition of M4 (4 μg/ml), whole-cell lysates prepared from 2.5 × 10^6 cells were loaded onto an 8% SDS−PAGE gel. After transfer to nitrocellulose, the filter was incubated with anti-phosphotyrosine mAb 4G10.

substrates that become rapidly phosphorylated on tyrosine residues after BCR stimulation. In *lyn*-negative cells, the induction of phosphorylation on many of these substrates was abolished, although some proteins were tyrosine phosphorylated (e.g. ~75 kDa protein; Figure 4). Overall phosphorylation of *lyn*-negative cells at 7 and 10 min after stimulation was essentially the same as that at 3 min (data not shown). Compared with *lyn*-negative cells, *syk*-negative cells showed different patterns of tyrosine phosphorylation

induced by BCR stimulation. The induction of phosphorylation on the 75 kDa protein was completely abolished in the *syk*-negative cells, whereas the phosphorylation of several species between 46 and 68 kDa was induced by BCR stimulation. These results suggest that Lyn and Syk have distinct sets of substrates through BCR stimulation and that both kinases are necessary for coupling BCR to full PTK activity.

BCR-mediated Ca^{2+} mobilization is differentially regulated by Lyn and Syk

Stimulation of BCR induced a rapid increase of cytoplasmic free calcium ($[Ca^{2+}]_i$) in wild-type DT40 cells. EGTA incubation decreased the BCR-mediated Ca^{2+} mobilization ~2-fold (data not shown), suggesting that some fraction of $[Ca^{2+}]_i$ increase is derived from intracellular pools. In *lyn*-negative cells, a rapid increase in $[Ca^{2+}]_i$ was not detected following stimulation of the BCR; however, a delayed and slow $[Ca^{2+}]_i$ increase was observed. In contrast to *lyn*-negative cells, no increase in $[Ca^{2+}]_i$ was detected in *syk*-negative cells (Figure 5A). Transfection of *lyn* or *syk* cDNA into the *lyn*-negative or *syk*-negative DT40 cells, respectively, restored normal BCR-mediated Ca^{2+} mobilization (data not shown and Figure 8), confirming that this abnormal Ca^{2+} mobilization is due to Lyn or Syk deficiency.

Syk, not Lyn, couples BCR to the phosphatidylinositol (PtdIns) pathway

Previous studies have suggested that at least some of the $[Ca^{2+}]_i$ increase through BCR stimulation is derived from IP_3-dependent release from intracellular pools (Cambier and Ransom, 1987). We therefore examined the ability of anti-BCR mAb to induce PtdIns breakdown in wild-type, *lyn*-negative or *syk*-negative DT40 cells. Despite the delayed and slow calcium response in *lyn*-negative cells after receptor stimulation, crosslinking of BCR on these cells resulted in somewhat higher generation of all three inositol phosphate fractions than that of wild-type cells. As shown in Figure 5B, temporal kinetics of these inositol phosphates in *lyn*-negative cells were similar to those in wild-type cells. These data indicate that the BCR-coupled delayed and slow $[Ca^{2+}]_i$ increase in *lyn*-negative cells cannot be accounted for simply by IP_3 accumulation kinetics, suggesting that Lyn is required for normal BCR-coupled calcium mobilization, in addition to IP_3. In contrast to *lyn*-negative DT40 cells, *syk*-negative cells abolished the generation of all three inositol phosphate fractions following BCR stimulation (Figure 5B). These results demonstrate that Syk, not Lyn, couples BCR to the PtdIns pathway.

To test for the involvement of PLC-γ2 in the observed IP_3 generation, we assayed the effect of anti-BCR mAb M4 on PLC-γ2 tyrosine phosphorylation in wild-type, *lyn*-negative or *syk*-negative DT40 cells. Anti-PLC-γ2 immuno-

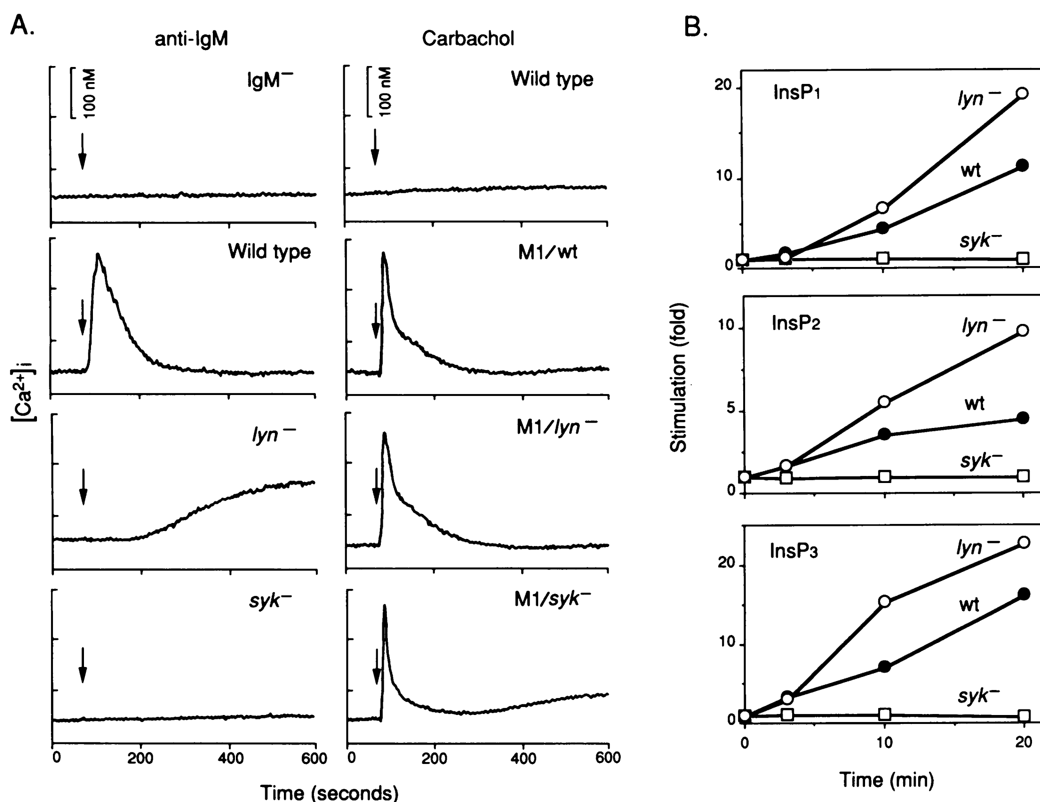

Fig. 5. Calcium mobilization and phosphoinositide hydrolysis in wild-type, *lyn*-negative and *syk*-negative cells following activation with anti-IgM (M4). (**A**) Cells from surface IgM-negative (Buerstedde and Takeda, 1991), wild-type, *lyn*-negative and *syk*-negative cells were loaded with fura-2/AM. After equilibration at 37°C, the samples (1×10^6 cells/ml) were stimulated with M4 (2 μg/ml). Muscarinic M1 receptor cDNA was transfected into the wild-type, *lyn*-negative or *syk*-negative DT40 cells, selected in the presence of puromycin and screened by the QNB binding assay. Typical clones expressing M1 receptor (M1/wt, M1/*lyn*⁻, M1/*syk*⁻) were stimulated by 500 μM carbachol. (**B**) Wild-type, *lyn*-negative and *syk*-negative DT40 cells (2×10^6 cells/ml) were stimulated with M4 (4 μg/ml). Soluble inositol was extracted and separated by the AG 1-X8 ion exchange columns. We titered M4 concentrations for Ca^{2+} mobilization and IP_3 generation. Data using 0.5 μg/10^6 cells and 8 μg/10^6 cells were essentially the same as those shown in this figure.

precipitates were prepared from the BCR-stimulated wild-type, *lyn*-negative or *syk*-negative B cells, electrophoresed and immunoblotted with anti-phosphotyrosine mAb (Figure 6). Induction of tyrosine phosphorylation on PLC-γ2 was observed in wild-type and *lyn*-negative cells following BCR crosslinking. No stimulation of tyrosine phosphorylation on PLC-γ2 was detected in *syk*-negative cells, indicating good correlation between IP_3 generation and induction of PLC-γ2 phosphorylation.

Lack of Lyn or Syk does not affect IP_3 generation and Ca^{2+} mobilization through muscarinic acetylcholine receptor

To address whether the abnormal kinetics of calcium mobilization in *lyn*-negative cells and complete abolishment of this mobilization in *syk*-negative cells are restricted to the BCR-coupled pathway, we transfected muscarinic acetylcholine receptor subtype-1 (M1) into wild-type, *lyn*-negative and *syk*-negative cells, yielding M1/wt, M1/*lyn*$^-$ and M1/*syk*$^-$ clones, respectively. M1, M3 and M5 muscarinic receptors evoke IP_3 generation through G protein-coupled PLC-β activation by agonist stimulation (Bonner, 1989; Smrcka *et al.*, 1991; Berridge, 1993a). The muscarinic receptor agonist, carbachol, stimulated IP_3 generation in M1/wt, M1/*lyn*$^-$ and M1/*syk*$^-$ cells. The accumulation kinetics of all three inositol phosphate fractions was similar in these three clones (data not shown), indicating that M1-coupled IP_3 generation is independent of Lyn or Syk. Recently, data that both IP_3 generation and an additional component are required for full M3-operated Ca^{2+} mobilization were reported (Felder *et al.*, 1992). Thus, the analysis of carbachol-induced Ca^{2+} mobilization on these clones allows us to examine the possibility that this additional component required for full Ca^{2+} mobilization is affected by Syk or Lyn deficiency. The carbachol stimulated Ca^{2+} mobilization in M1/wt, M1/*lyn*$^-$ and M1/*syk*$^-$ cells with similar kinetics (Figure 5A). These data show that not only M1-coupled IP_3 generation but also normal M1-operated Ca^{2+} mobilization is not affected by loss of the *lyn* or *syk* gene product.

Functional compensation by other Src-PTK members

To address functional redundancy among Src-PTKs, we transfected other *src*-PTKs, *src*, *fyn* or *lck* cDNA, into the *lyn*-negative DT40 cell line. Expression of Fyn or Lck restored the normal Ca^{2+} mobilization and activation of tyrosine phosphorylation via BCR stimulation, whereas expression of Src, even at very high levels (data not shown), did not reconstitute these functions (Figure 7). These findings demonstrate that the function of Lyn can be replaced by Fyn or Lck, but not by Src, implicating some functional specificity among Src-PTKs in signal transduction through BCR.

Kinase activity of Syk is indispensable for BCR-mediated IP_3 generation and Ca^{2+} mobilization

To establish that the defect of IP_3 generation and calcium mobilization in *syk*-negative cells was directly due to loss

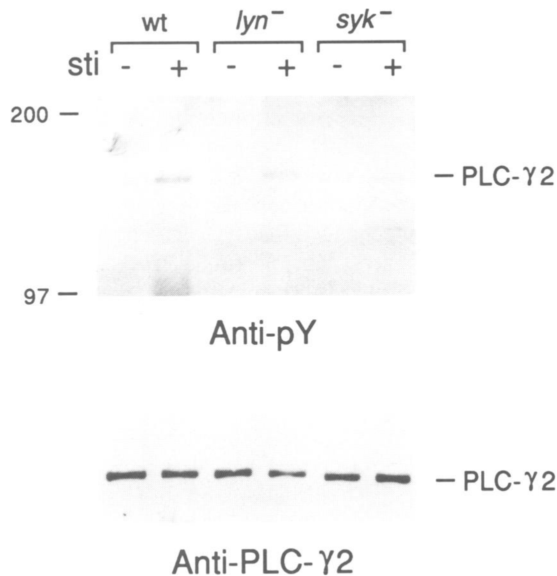

Fig. 6. Tyrosine phosphorylation of PLC-γ2 in wild-type, *lyn*-negative and *syk*-negative DT40 cells following anti-IgM (M4) stimulation. PLC-γ2 was immunoprecipitated from cells (2×10^6 cells/lane) subjected to M4 stimulation (4 μg/ml) for 3 min, and the immunoprecipitates were separated on a 6% SDS–PAGE gel, transferred to nitrocellulose and probed with anti-phosphotyrosine mAb 4G10. After deprobing, the same membrane was reprobed with anti-PLC-γ2 Ab.

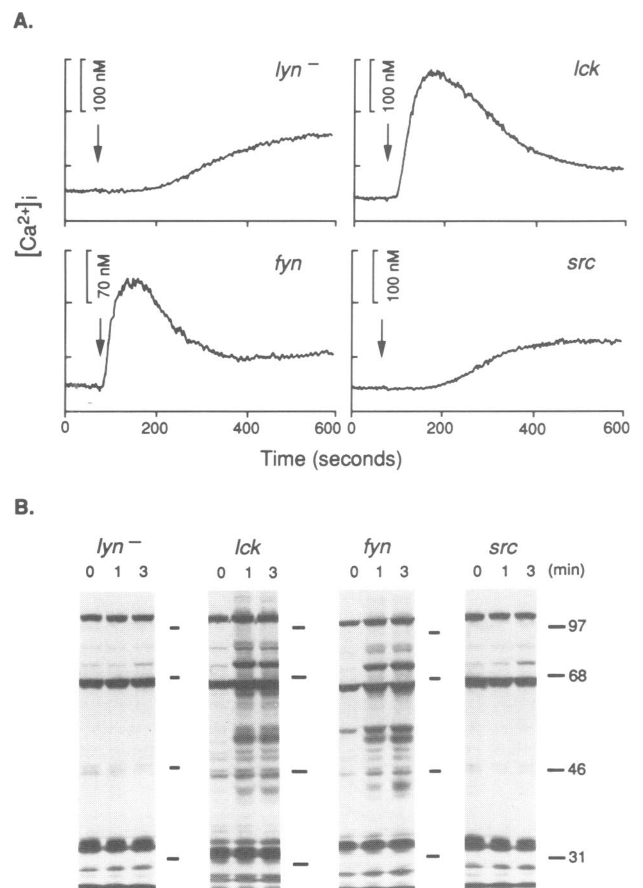

Fig. 7. Functional compensation by other Src-PTKs. Increase of $[Ca^{2+}]_i$ (**A**) and activation of tyrosine phosphorylation (**B**) following anti-IgM (M4) stimulation. Human *lck*, mouse *fyn* and chicken *src* cDNAs were transfected into *lyn*-negative DT40 cells and selected in the presence of 0.5 μg/ml puromycin. The expression of Lck, Fyn and Src was checked by Western blot analysis. Particularly for the *src* transformant, we confirmed that the expression level of *src* is more than *fyn* or *lck* by Northern blot analysis. Stimulation conditions with M4 were the same as described in the legends to Figures 4 and 5.

of the kinase activity of Syk, and not to the loss of the association of this molecule with signal transduction elements in the BCR activation process, we created the kinase-negative mutant cDNA of porcine *syk*. Abrogating phosphotransferase activity of the kinase-negative mutant was confirmed by the deficiency of *in vitro* kinase activity (data not shown). Wild-type or kinase-negative porcine *syk* cDNA was transfected into the *syk*-negative DT40 cell line, selected with puromycin and the expression of porcine Syk was determined by Western blotting. Wild-type Syk restored the signal transduction defect in both IP_3 generation and Ca^{2+} mobilization following BCR stimulation, whereas kinase-negative Syk, at the comparable level of expression to wild-type porcine Syk, did not reconstitute these signaling events (Figure 8). This finding indicates that the kinase activity of Syk is required for its ability to couple the BCR to early signaling events.

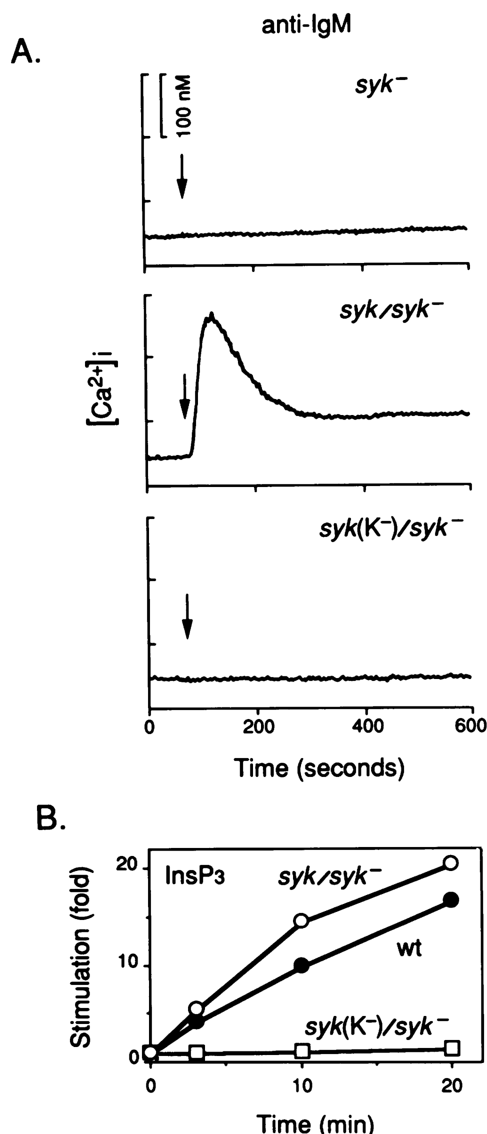

Fig. 8. Requirement of kinase activity of Syk for functional reconstitution. Increase of $[Ca^{2+}]_i$ (**A**) and IP_3 generation (**B**) following anti-IgM (M4) stimulation. Porcine wild-type and kinase-negative *syk* cDNAs were transfected into *syk*-negative DT40 cells and selected. The expression was checked by Western blot analysis. Stimulation conditions with M4 were the same as described in the legend to Figure 5.

Discussion

Both Lyn and Syk are involved in BCR-induced PTK activity

The work presented here demonstrates that crosslinking of BCR in *syk*-negative and *lyn*-negative B cells induces different patterns of tyrosine phosphorylation (Figure 4). These data, together with those obtained with CD16/Syk and CD16/Fyn chimeras (Kolanus *et al.*, 1993), suggest that Syk and Lyn mediate tyrosine phosphorylation on different intracellular substrates through BCR stimulation. However, the activation of these two kinases through BCR stimulation may not be fully independent of each other. For instance, the drastic changes in the phosphotyrosyl protein pattern seen in the wild-type cells seem not to be accounted for by a simple addition of the phosphorylation induced in the *lyn*-negative and *syk*-negative cells. Second, although Syk is a prerequisite for BCR-mediated phosphorylation of the 75 kDa protein, the kinetics and the stimulation level of phosphorylation on this protein in the *lyn*-negative (*syk*-positive) cells were slow and reduced compared with wild-type cells (Figure 4). These observations provoke several possibilities. Coexpression of Src-PTK with ZAP-70 or Syk leads to a remarkable increase in net tyrosine phosphorylation, whereas Src-PTK, ZAP-70 or Syk alone induces only marginal phosphorylation in COS cells (Chan *et al.*, 1992; Kurosaki *et al.*, 1994). Thus, Lyn may activate the Syk and/or vice versa in BCR signaling. Since Lyn and Syk have one or two SH2 domains, it is also possible that these enzymes may be recruited to the tyrosine phosphorylated Igα or Igβ chain following receptor stimulation, resulting in the activation of Syk and Lyn.

Syk, not Lyn, mediates BCR-induced PLC-γ2 phosphorylation

The different substrate specificity by Lyn and Syk in the BCR signaling was highlighted by the PLC-γ2 phosphorylation in the *lyn*-negative and *syk*-negative cells (Figure 6). These data provide good agreement with the previous results that CD16/Syk chimera increased the tyrosine phosphorylation of PLC-γ1 through receptor stimulation, whereas cross-linking CD16/Fyn was not able to produce this increase (Kolanus *et al.*, 1993). The correlation between stimulation of PLC-γ2 phosphorylation and IP_3 generation in DT40 cells supports the mechanism that tyrosine phosphorylation of PLC-γ1 or -γ2 is responsible for its increased activity (Nishibe *et al.*, 1990), resulting in the generation of IP_3. Thus, in B cell signaling Syk, not Lyn, couples BCR to the PtdIns pathway. Recent reconstitution data using COS cells indicate that Fyn is required for coupling the TCR to the PLC-γ1 activation and, moreover, that this PLC-γ1 activation does not require ZAP-70 (Hall *et al.*, 1993). At a first glance these data seem to contradict our results. However, as Hall *et al.* mentioned in their report, it is still possible that COS cells may express endogenous Syk. One explanation of their results is that the activity of endogenous Syk may be enhanced by the overexpression of transfected Fyn.

Distinct requirement of the Src-PTK for BCR and TCR signaling

Our results may explain the potential difference in the mechanism through which the BCR and TCR induce PTK activity. Mutant T cells lacking Lck abolished both TCR-

mediated Ca^{2+} mobilization and IP_3 generation (Goldsmith and Weiss, 1987; Straus and Weiss, 1992), similar to the phenotype of *syk*-negative but not *lyn*-negative DT40 B cells. This may suggest the distinct participation of the Src-PTK between TCR and BCR signaling. In TCR signaling it is hypothesized that ligand-dependent tyrosine phosphorylation of TCR ζ chain by Lck is a crucial process for recruitment of ZAP-70 to the receptor complex and its subsequent activation (Chan *et al.*, 1992; Weiss, 1993). Thus, Lck appears to be essential for coupling TCR to ZAP-70 activation.

In contrast to TCR signaling, Syk was reported to associate with the BCR complex even in the absence of ligand (Hutchcroft *et al.*, 1992). Moreover, our data indicate that Syk mediates the BCR-coupled phosphorylation of PLC-γ2 even in the absence of Lyn. Recently, it has been demonstrated that CD16/Syk chimera alone induces the phosphorylation of PLC-γ1, whereas CD16/ZAP-70 is ineffective. However, co-crosslinking CD16/ZAP-70 plus CD16/Fyn stimulates the phosphorylation of PLC-γ1 (Kolanus *et al.*, 1993). Thus, the distinct role of Src-PTK in BCR and TCR signaling may be accounted for by the different requirement of Src-PTK for Syk and ZAP-70 activation.

Functional redundancy of Src-PTK members

The *lyn*-negative cells allow us to test directly the redundancy among Src-PTK members in the BCR signaling. Lck or Fyn was able to compensate the function of Lyn, whereas Src was not, suggesting some specificity among each Src-PTK member. Lyn, Lck and Fyn kinases are expressed in lymphoid cells, whereas Src is expressed dominantly in platelets and neurons, but not in lymphoid cells (Bolen *et al.*, 1992). This tissue distribution may reflect these functional differences. Consistent with our results, recent *in vitro* studies indicate the clear distinction between Lyn and Src; Lyn is capable of binding for PLC-γ2 or Igα, whereas Src is not (Pleiman *et al.*, 1993).

A second factor is required for normal BCR-induced Ca^{2+} mobilization in addition to IP_3

Rapid Ca^{2+} mobilization following BCR stimulation is thought to be critical for B cells to enter from G_0 to G_1 phase (Yamada *et al.*, 1993). The rise in free calcium is derived by both influx from the extracellular environment and release from intracellular stores (Cambier and Ransom, 1987). This Ca^{2+} mobilization from intracellular stores is shown to be mediated by the second messenger IP_3 (Berridge, 1993a). Because Syk-deficient cells abolished Ca^{2+} mobilization as well as IP_3 generation upon receptor crosslinking, IP_3 appears to be the essential mediator for Ca^{2+} mobilization through BCR. Stimulation of BCR on *lyn*-negative cells evoked a delayed and slow increase of $[Ca^{2+}]_i$ despite the normal kinetics of IP_3 generation. Thus, normal BCR-mediated Ca^{2+} mobilization requires not only IP_3, but also an additional factor which is mediated by Lyn.

To account for this additional factor, we propose three possibilities. (i) Ligand sensitivity or Ca^{2+} mobilizing kinetics of IP_3 receptor are regulated through Lyn-dependent pathways. As one of the mechanisms to modulate the function of IP_3 receptor (Berridge, 1993a), post-translational modifications, such as phosphorylation, are well known (Supattapone *et al.*, 1988). (ii) Another component, for instance the Ca^{2+} influx channel located in plasma membrane, is regulated by Lyn. In fact, electrophysiological analysis indicates the existence of membrane Ca^{2+} channels and Ca^{2+} currents regulated by surface receptors in T cells (Lewis and Cahalan, 1989, 1990). (iii) Lyn is involved in the IP_3-independent Ca^{2+} regulatory pathway. Recently, cyclic ADP-ribose-dependent Ca^{2+} regulatory pathway was demonstrated to operate, probably through ryanodine receptor, in non-excitable cells, suggesting the presence of an IP_3-independent Ca^{2+} regulatory mechanism (Berridge, 1993b; Mészáros *et al.*, 1993; Takasawa *et al.*, 1993). Although we cannot at this point make a statement as to which of these three possibilities is the most likely, our data clearly suggest that Lyn regulates normal BCR-mediated Ca^{2+} mobilization through a process independent of IP_3 generation which is mediated by Syk.

Our results prompt the conclusion that Syk and Lyn mediate discrete signaling functions through the kinase activity. B cell lines lacking Lyn or Syk will provide the tools for dissecting a more detailed biochemical mechanism of the signal transduction pathways to which these kinases uniquely contribute.

Materials and methods

Cells, expression vector and antisera

DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin and glutamine. To construct chicken expression vector (pApuro) harboring the chicken actin promoter and puromycin-resistant gene, a *Pst*I–*Pst*I 1.1 kb fragment containing the Moloney murine leukemia virus long terminal repeat (LTR) was deleted from the pBabe Puro vector (Morgenstern and Land, 1990), and an *Eco*RI cloning site of pBabe Puro was modified by T_4 DNA polymerase treatment, resulting in deletion of the *Eco*RI site. Then a *Xho*I–*Xho*I 1.0 kb fragment containing the chicken actin promoter of pAGS-3 vector (Miyazaki *et al.*, 1989) was inserted into a *Sal*I site of the modified pBabe Puro, yielding pApuro vector. Human *lck* (Koga *et al.*, 1986), mouse *fyn* (Cooke and Perlmutter, 1989), chicken *src* (Takeya and Hanafusa, 1983), porcine *syk* (Taniguchi *et al.*, 1991) and porcine muscarinic M1 receptor (Kubo *et al.*, 1986) cDNAs were inserted into the *Eco*RI site of the pApuro vector. A point mutation (395Lys→Arg) in the ATP binding site of porcine *syk* cDNA was created by PCR. These cDNAs were transfected by electroporation using Gene pulser apparatus (Bio-Rad Laboratories) at 550 V, 25 μF, and selected in the presence of 0.5 μg/ml puromycin. Expression of transfected cDNAs was confirmed by Western blot analysis (*lck*, *fyn*, *src* or *syk*) or binding assay (M1 muscarinic receptor).

The mAb used for the stimulation of BCR was M4, which recognizes chicken IgM (Chen *et al.*, 1982). Antisera were generated against the GST fusion protein containing the N-termini of chicken Lyn or porcine Syk. A mAb, 4G10 (Upstate Biotechnology), was used for the detection of phosphotyrosine-containing proteins. Antisera against the rat PLC-γ2 were already described (Homma *et al.*, 1990). Anti-Fyn polyclonal antibody was purchased from Oncogene Science.

Generation of lyn-negative and syk-negative DT40 cells

Chicken spleen cDNA and genomic DNA libraries were obtained from Clontech. Chicken cDNA library was screened by the human *lyn* cDNA (obtained from ATCC) and porcine *syk* cDNA. Several chicken cDNA isolates were sequenced to confirm the identification. Genomic library was screened by the chicken *lyn* and *syk* cDNAs. After subcloning the genomic clones of chicken *lyn* and *syk*, the targeting constructs were made. The *neo*, *hisD* and *hygro* cassettes for these constructions were described previously (Santerre *et al.*, 1984; Hartman and Mulligan, 1988; Takeda *et al.*, 1992). For pLyn-Neo, the *neo* cassette was introduced into the *Bgl*II site in exon 4 of the 2.9 kb *Eco*RI–*Eco*RI genomic sequence. Two other constructs (pLyn-His and pLyn-Hyg) were designed for *hisD* and *hygro* cassettes to replace a 1.9 kb *Bam*HI–*Bgl*II fragment containing exon 1–4. The *hisD* and *hygro* cassettes were flanked by 2.8 and 3.4 kb of *lyn* sequence on the 5′ and 3′ side, respectively. For construction of pSyk-Neo harboring 3.1 kb (5′ side) and 0.9 kb (3′ side) of *syk* sequence, a *Hin*dIII–*Apa*I 1.0 kb fragment containing exon 1 was replaced by the *neo* cassette. pLyn-Neo or pSyk-Neo was linearized and transfected into DT40 cells by electroporation (550 V, 25 μF). 24 h after DNA transfection, 2 mg/ml G418

was added to the culture medium and was selected for ~14 days. Genomic DNAs were isolated from several G418-resistant clones, digested with appropriate restriction enzymes and hybridized with the chicken *lyn* or *syk* genomic DNA fragment. For isolation of the null mutant of *lyn*, pLyn-His was transfected into the *neo* targeted clone and selected in the presence of 2 mg/ml G418 and 1 mg/ml histidinol. After obtaining the *neo/his* targeted clone, pLyn-Hyg was transfected, selected (2 mg/ml G418, 1 mg/ml histidinol and 2 mg/ml hygromycin) and analyzed. Cell surface expression of BCR was analyzed by FACS using M4 mAb. A single *lyn* or *syk* targeted clone was extensively analyzed, although some critical experiments were carried out using three different clones.

Northern blot analysis

RNA was prepared from wild-type and mutant DT40 cells using the guanidium thiocyanate method. Total RNA (20 μg) was separated in 1.2% formaldehyde gel, transferred to Hybond-N membrane (Amersham) and probed with ^{32}P-labeled cDNAs. Probes used were cDNA fragments of chicken *src*, chicken *lck* (Strebhardt *et al.*, 1987; Chow *et al.*, 1992), mouse *fyn*, mouse *blk* (Dymecki *et al.*, 1990), chicken *yes* (Sudol *et al.*, 1988), mouse *hck* (Ziegler *et al.*, 1987), chicken *zap-70*, chicken *lyn* and chicken *syk*. Chicken *lck* and *zap-70* cDNAs were obtained during cDNA cloning for chicken *lyn* and *syk*, respectively. Identification of chicken versions of *lck* or *zap-70* was with the sequencing data of these clones. In the cases of probes derived from species other than chicken, hybridization was performed in both high and low stringency conditions.

Immunoprecipitation and in vitro kinase assay

Cells were solubilized in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA) containing 50 mM NaF, 10 μM molibrate, 0.2 mM vanadate (Sabe *et al.*, 1992) supplemented with protease inhibitors (1 mM PMSF, 0.5 mM benzamidine hydrochloride, 10 μg/ml chymostatin, 0.1 mM TLCK, 0.1 mM TPCK, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml calpastatin I and 10 μg/ml pepstatin) (Taniguchi *et al.*, 1991). Insoluble material was removed by centrifugation at 13 000 *g* for 10 min. Cell lysates were sequentially incubated (1 h, 4°C for each incubation) with antibodies and protein A–Sepharose. The immunoprecipitates were washed four times with lysis buffer. For *in vitro* kinase assay, after washing with lysis buffer the immunoprecipitates were washed with 20 mM HEPES (pH 8) and 150 mM NaCl. Added to each sample was 50 μl kinase buffer (20 mM HEPES, pH 8, 150 mM NaCl, 10 mM magnesium acetate, 10 mM $MnCl_2$) containing 10 μCi [γ-^{32}P]ATP (>3000 Ci/mmol, Amersham). The reactions were allowed to incubate at 30°C for 10 min and terminated by the addition of sample buffer.

Immunoblot analysis

Whole lysates were prepared from non-stimulated or M4-stimulated cells using SDS sample buffer. Whole lysates or immunoprecipitates were separated on 6 or 8% SDS–PAGE gels and transferred to nitrocellulose. The blots were blocked with 5% milk in 25 mM Tris (pH 7.9), 150 mM NaCl with 0.05% Tween-20, and incubated with primary antibodies for 1 h at room temperature. Filters were developed with a goat anti-mouse or donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase using the Enhanced Chemiluminescence (ECL) detection system (Amersham). Deprobing was performed according to the manufacturer's instructions.

Calcium analysis

Measurements of intracellular free calcium levels were performed with fura-2/AM. Cells (5×10^6 ml^{-1}) were washed once and loaded with 3 μM fura-2/AM in PBS containing 20 mM HEPES (pH 7.2), 5 mM glucose, 0.025% BSA and 1 mM $CaCl_2$. After 45 min of incubation at 37°C, cells were washed twice and diluted to 10^6 cells/ml with the same buffer. Fluorescence of the stirred cell suspension was continuously monitored with a fluorescence spectrophotometer Hitachi F-2000 at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calibrated and computed as described (Grynkiewicz *et al.*, 1985).

Phosphoinositide analysis

Cells (10^6 ml^{-1}) were labeled with myo-[^3H]inositol (10 μCi/ml, Amersham) for 6 h in inositol-free RPMI 1640 supplemented with 10% dialyzed fetal calf serum. The cells (5×10^6 ml^{-1}) were pre-equilibrated at 37°C and sequentially 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) pre-equilibrated with 0.1 M 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) (Berridge *et al.*, 1983).

Binding assay for M1 muscarinic receptor expression

Transfected clones were assayed for expression of muscarinic receptor essentially as described (Goldsmith *et al.*, 1989). Briefly, intact cells (10^6 cells/sample) were incubated for 90 min with the muscarinic receptor antagonist [^3H]quinuclidinyl benzilate ([^3H]QNB, 100 pM, 47 Ci/mmol, Amersham). All incubations were performed in duplicate and background binding activity was determined in the presence of 10 μM atropine. Then cells were collected on a Whatman GF/B membrane, washed extensively and bound radioactivity was determined by a liquid scintillation counter.

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