Title
Role of Src-family tyrosine kinase, Lyn, in B cell antigen receptor signalling

Author
Chan, Vivien Wai-Fan

Publication Date
1996

Peer reviewed|Thesis/dissertation

Role of Src-Family Tyrosine Kinase, Lyn, in B Cell Antigen Receptor Signaling

by

Vivien Wai-Fan Chan

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

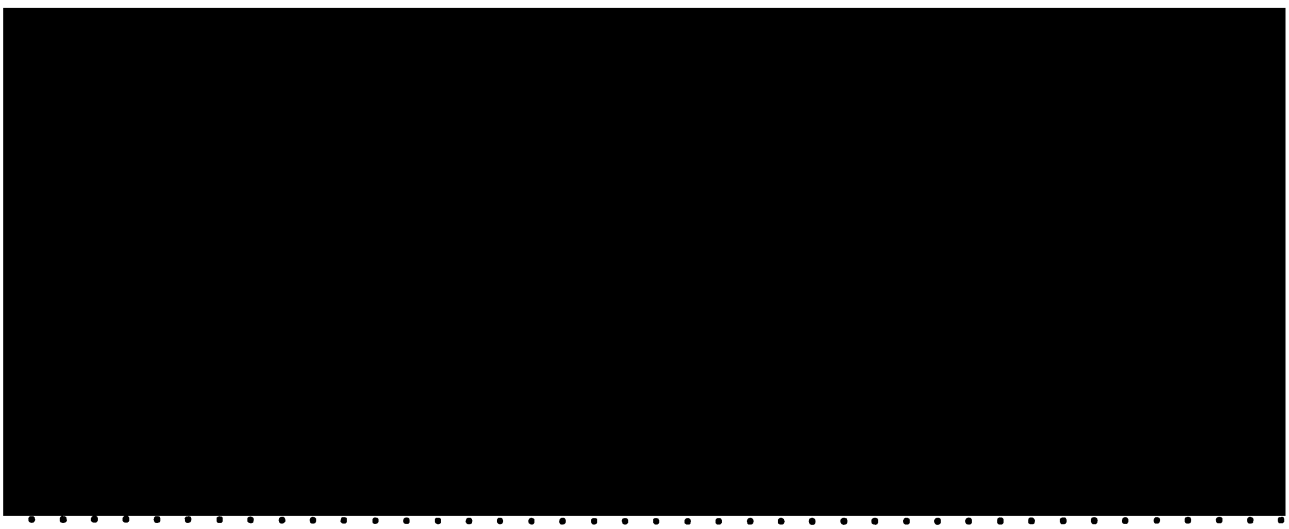
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



. . . . . .

Date **University Librarian**

Degree Conferred: . . . . . .

ACKNOWLEDGMENTS

I wish to thank my thesis advisor Tony DeFranco for his support and intellectual guidance. His insights and resourcefulness have been a tremendous help throughout my graduate career. I am indebted to current and past members of the DeFranco lab who have so generously shared their reagents and taught me many techniques. In particular, I wish to thank Mike Gold for his guidance, especially during my first year in the DeFranco lab, and to Trish Roth for teaching me flow cytometric analysis. Thanks to Steve Weinstein, Julie Hambleton, Stacey Harmer and Mary Crowley for all the helpful discussions, be it related to science, computers, or life. Special thanks to the members of my thesis committee, Tony DeFranco, Mike Bishop and Henry Bourne for their time in reviewing this thesis and their invaluable suggestions at my thesis committee meetings. I am also grateful to Cliff Lowell for a very productive collaboration in the characterization of the *lyn* knockout mice and to Debbie Law for the collaboration in examining kinase association with the antigen receptor chimeras. Thanks to Monte Borders for introducing me to the field of biochemistry. Last but not least, I wish to thank my parents for their understanding and encouragement.

During the first five years of my graduate career, I was supported by a predoctoral fellowship from the Howard Hughes Medical Institute.

Figure 1.3 in this thesis was modified from:
Law, D. A., Chan, V. W. F., Datta, S. K., and DeFranco, A. L. (1993). B-cell antigen receptor motifs have redundant signaling capabilities and bind the tyrosine kinases PTK72, Lyn and Fyn. Curr. Biol. 3, 645-57.

Role of Src-Family Tyrosine Kinase, Lyn, in B Cell Antigen Receptor Signaling

by Vivien Wai-Fan Chan

ABSTRACT

The Src-family tyrosine kinase Lyn has been implicated by a variety of biochemical experiments to participate in signal transduction by the B cell antigen receptor (BCR). Experiments described herein demonstrated that Lyn can phosphorylate Ig-α *in vitro*. Lyn was also found to be associated, albeit at low stoichiometry, with crosslinked intact BCR complexes as well as with crosslinked chimeric receptors bearing the cytoplasmic domains of Ig-α or Ig-β. In addition, anti-Ig treatment of B cells induced a decrease in the mobility of Lyn on SDS-polyacrylamide gels, corresponding to an increase in apparent molecular weight of about 6 kD. This response was rapid and appeared to be specific to BCR signaling. In addition to biochemical evidence for a role of Lyn in BCR signaling, genetic studies by other groups using B cell lines have also provided support for this role. I have extended those studies by characterizing the B cell populations in mice deficient for Lyn and by examining the biochemical and biological responses of B cells from these mice. Lyn-deficient mice exhibited a number of B cell abnormalities, including reduced numbers of peripheral B cells and a greater proportion of immature cells. Peripheral B cells from these mice had a higher than normal turnover rate, also suggesting an inhibition of normal maturation. In contrast, aged *lyn*$^{-/-}$ mice developed splenomegaly, produced autoantibodies and had an expanded population of B lymphoblasts, likely to be of the B1 lineage. Splenic B cells from young *lyn*$^{-/-}$ mice were capable of initiating early BCR signaling events, although in a delayed fashion. Unexpectedly, *lyn*$^{-/-}$ B cells exhibited a considerable enhancement in MAP kinase

activation and an increased proliferative response to BCR engagement. Stimulation of *lyn*$^{-/-}$ B cells with intact and F(ab')$_2$ fragments of anti-IgM revealed at least two defects in mechanisms that negatively regulate BCR signaling, one of which involves the FcγRIIb1. Thus, while other Src-family tyrosine kinases may partially compensate for Lyn in signal initiation, the function of Lyn in the negative regulation of BCR signaling appears to be non-redundant.

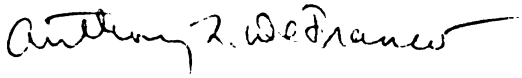


TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

A functional immune system has to maintain the ability to recognize a large diversity of foreign antigens while minimizing self-reactivity. The activation of B cells during an immune response and the elimination of self-reactive B cells are both dependent on antigen binding. Therefore, the study of the molecular events triggered by antigen-binding is important for understanding how the BCR can mediate such different biological consequences.

Biochemical events triggered by the BCR

The BCR consists of membrane Ig complexed with one or more Ig-α/Ig-β dimers, with the former providing antigen recognition, and the latter facilitating the surface expression of the BCR complex (Venkitaraman et al., 1991, Matsuuchi et al., 1992) and mediating signaling functions (Reth, 1994). The biochemical events triggered by BCR engagement include the increased phosphorylation of cellular proteins, the increased hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), the elevation of intracellular calcium, the activation of phosphatidylinositol 3-kinase (PI-3 kinase) and the activation of Ras (Gold et al., 1990, Campbell and Sefton, 1990, Lane et al., 1991, Brunswick et al., 1991, Fahey and DeFranco, 1987, LaBaer et al., 1986, Gold et al., 1992a, Lazarus et al., 1993) with the most rapid response being increased protein tyrosine phosphorylation. BCR-activation of these signaling events was inhibited by the pre-treatment of B cells with tyrosine kinase inhibitors such as herbimycin and genistein (Lane et al., 1991, Mizuguchi et al., 1992, Kawauchi et al., 1994), suggesting that protein tyrosine phosphorylation is responsible for the initiation of all the subsequent signaling events. The findings that PIP_2 breakdown and the calcium response

are affected by mutations in tyrosine kinases (Syk, Btk, Lyn) and phosphatases (SHP-1, CD45) provide further evidence that these proximal events depend on protein tyrosine phosphorylation and dephosphorylation (Takata et al., 1994, Takata and Kurosaki, 1996, Cyster and Goodnow, 1995b, Justement et al., 1991).

Tyrosine kinases in BCR signaling

A large number of cellular proteins are tyrosine phosphorylated upon BCR engagement and many of them have been identified. Increased tyrosine phosphorylation coincides with an alteration of enzymatic activity in some cases, or creates new binding sites for protein-protein interactions in others. The receptor components Ig-α and Ig-β, are among the first substrates to be tyrosine phosphorylated upon BCR crosslinking (Gold et al., 1991). Mutational studies of Ig-α have demonstrated the importance of the tyrosine residues within the ITAM for effective activation of tyrosine kinases as well as biological responses to BCR signaling (Flaswinkel and Reth, 1994, Yao et al., 1995). We have found that receptor crosslinking leads to increased amounts of Lyn, Fyn and Syk kinase activities associated with the intact BCR complex as well as with receptor chimeras bearing the cytoplasmic domains of Ig-α and Ig-β (Chapter I and Law et al., 1993). These tyrosine kinases can bind to phosphorylated ITAM peptides via their SH2 domains and this binding has been shown to cause the activation of the kinases (Pleiman et al., 1994a, Johnson et al., 1995, Rowley et al., 1995).

The cytoplasmic tyrosine kinases mentioned above fall into two families: Syk belongs to the Syk/ZAP-70 family while Lyn and Fyn belong to the Src-family. Syk and several members of the Src-family kinases (Lyn, Fyn, Lck, Blk and Fgr) have been shown to be phosphorylated and activated upon BCR signaling (Hutchcroft et al., 1992, Burg et al., 1994, Yamanashi et al., 1991, Burkhardt et al.,

1991, Wechsler and Monroe, 1995). These two kinase families are probably responsible for the majority of the BCR-induced tyrosine phosphorylations since the simultaneous genetic disruption of Lyn and Syk in DT40, a chicken B cell line in which Lyn is the only Src-family tyrosine kinase expressed, resulted in an almost complete abolition of BCR-induced phosphorylation of cellular proteins (Kurosaki et al., 1995). The BCR-induced signaling events are likely to be important for B cell development since Lyn-deficient mice have reduced numbers of mature B cells (Chapter II, Hibbs et al., 1995, Nishizumi et al., 1995), and Syk-deficient mice exhibit an almost complete lack of mature B cells (Cheng et al., 1995, Turner et al., 1995).

A third family of cytoplasmic tyrosine kinases, known as the Tec family, is also found in B cells. One of the members of this family, Btk, is also phosphorylated and activated by BCR signaling (Saouaf et al., 1994, Aoki et al., 1994, de Weers et al., 1994). Mutations of Btk cause defects in B cell development and activation in mice (Khan et al., 1995) and has been linked to the human immunodeficiency disease, X-linked agammaglobulinemia (XLA), and murine X-linked immunodeficiency (XID) (Tsukada et al., 1994). To date, Btk has not been reported to associate with the BCR complex after anti-Ig crosslinking, suggesting that the BCR-induced activation of Btk may be indirect. Based on co-expression studies, it is currently thought that the Src-family kinases are responsible for the activation of Btk (Rawlings et al., 1996). Genetic disruption of Btk in the DT40 cell line did not significantly affect the general tyrosine phosphorylation pattern induced by BCR crosslinking, but its absence led to a less robust induction of PLC-γ2 phosphorylation and severely impaired PIP_2 breakdown and calcium response (Kurosaki et al., 1995). The PLC pathway is also altered in DT40 cells lacking Lyn and severely impaired in cells lacking Syk (Takata et al., 1994). Thus,

while these three families of kinases may have distinct subsets of substrates in B cells, they all appear to participate in the regulation of the PLC pathway.

The results described above, as well as work examining the kinetics of activation of the three kinase families (Saouaf et al., 1994) are consistent with a multi-step model that has been proposed for BCR signaling (DeFranco, 1995). In unstimulated cells, the Src-family kinases are weakly associated with the BCR, perhaps by the phosphotyrosine-independent, low-affinity interaction that has been reported between the N-terminal unique domains of the Src-family kinases and a four amino acid sequence (DCSM) in Ig-α (Pleiman et al., 1994a, Clark et al., 1994). Upon receptor crosslinking, the Src-family kinases are activated by the dephosphorylation of a negative regulatory tyrosine near the C-termini and the auto-phosphorylation of an activating tyrosine residue within the kinase domain (Mustelin and Burn, 1993). The predicted optimal substrate recognition sequences for the Src-family kinases, Lyn, Blk (Schmitz et al., 1996) and Lck (Zhou et al., 1995), resemble the sequences flanking the tyrosines in the Ig-α and Ig-β ITAMs. In fact, Blk caused increased tyrosine phosphorylation of Ig-α or Ig-β chimeras when they are co-expressed in COS cells (Saouaf et al., 1995). Thus, the Src-family kinases are thought to phosphorylate the ITAMs upon B cell stimulation, creating binding sites for the SH2 domains of the Src-family kinases (Clark et al., 1994), as well as for Syk (Kurosaki et al., 1995). Phosphorylated ITAMs have been shown to directly stimulate Lyn activity (Johnson et al., 1995), as well as the autophosphorylation of Syk, resulting in as much as a 10-fold increase in Syk activity (Rowley et al., 1995). As a result of the activation of the Syk and Src-family kinases, other kinases such as Btk are activated and together they contribute to the phosphorylation and/or activation of downstream effectors.

BCR-induced PIP_2 hydrolysis

Phospholipase C (PLC) is responsible for the breakdown of PIP_2, generating second messengers diacylglycerol and inositol trisphosphate, which lead to the activation of protein kinase C (PKC) and the elevation of cytosolic free calcium, respectively. In anti-Ig stimulated B cells, this involves the tyrosine phosphorylation and activation of PLC-γ1 (Carter et al., 1991) and PLC-γ2 (Hempel et al., 1992, Coggeshall et al., 1992). The BCR-induced tyrosine phosphorylation events can be bypassed by using pharmacological agents to directly stimulate PKC and elevate calcium. For example, in the WEHI-231 B cell line, the BCR-induced growth arrest can be induced by the treatment of cells with a combination of a phorbol ester and a calcium ionophore (Page and DeFranco, 1988). The importance of the phosphoinositide pathway in the induction of long term biological responses, such as growth arrest, is further illustrated by the finding that 4 out of 7 IgM-resistant WEHI-231 mutants have normal levels of tyrosine phosphorylation but decreased PIP_2 breakdown upon BCR engagement (Page et al., 1991). The decreased effectiveness of BCR signaling to cause growth arrest in these mutants is likely to be due to lowered levels of second messenger generation. When one of these mutants were transfected with cDNA encoding the human muscarinic receptor I (HMI), a G-protein coupled receptor which causes PIP_2 hydrolysis upon agonist binding, the cells effectively undergo growth arrest when co-stimulated with anti-IgM and HMI agonist (V. Chan, unpublished results). A likely explanation for the restoration of the growth arrest response is that the additional second messengers provided by HMI signaling supplemented the amount generated by anti-IgM in these mutants to reach a critical threshold required for the biological response. As will be discussed later, mutations of a variety of signaling molecules have been shown to

alter the BCR signaling threshold, resulting in profound changes in B cell maturation and survival.

Other BCR-induced tyrosine phosphorylations

The BCR-mediated activation of the Ras pathway is also dependent on protein tyrosine phosphorylations, based on studies using tyrosine kinase inhibitors (Kawauchi et al., 1994). BCR-crosslinking triggers the tyrosine phosphorylation of signaling components such as Shc and GTPase-activating protein for Ras (RasGAP) (Saxton et al., 1994, Nagai et al., 1995, Gold et al., 1993). It has also been demonstrated that tyrosine phosphorylated Shc participate in the formation of a Shc/Grb2/Sos complex (Saxton et al., 1994, Kumar et al., 1995). Since Sos is a guanine nucleotide exchange factor for Ras, the formation of this complex is thought to recruit Sos for the activation of Ras observed upon B cell activation (Lazarus et al., 1993, Harwood and Cambier, 1993). Likewise, the BCR-induced phosphorylation of RasGAP is also thought to contribute to the Ras pathway.

The activation of Ras initiates a kinase signaling cascade involving Raf and MEK, resulting in the phosphorylation and activation of the MAP kinases, Erk1 and Erk2 (Maruta and Burgess, 1994). The activation of MAP kinases has been demonstrated in anti-Ig or antigen-stimulated B cells (Gold et al., 1992b, Cyster et al., 1996). The duration of MAP kinase activation has been shown in other cell types to be critical for cellular responses to different growth factors (Marshall, 1995). As will be discussed in Chapter II, the magnitude and duration of MAP kinase activation also correlates with anti-IgM-induced B cell proliferation and is altered in the absence of the Src-family kinase, Lyn.

Another group of proteins that have been found to be phosphorylated or activated by BCR signaling are enzymes that utilize phospholipids as substrates. In addition to PLCγ discussed above, an inositol polyphosphate-5-phosphatase, p145*ship*, is also tyrosine phosphorylation upon BCR engagement (Crowley et al., 1996, Chacko et al., 1996). p145*ship* belongs to a class of phosphatase that removes the phosphate from the 5 position of inositol phospholipids, but requires its substrates to be phosphorylated at the 3 position of the inositol, which would correspond to the products of PI-3 kinase (Jackson et al., 1995). BCR crosslinking also leads to an increase in anti-phosphotyrosine-precipitable PI-3 kinase activity (Gold et al., 1992a, Yamanashi et al., 1992) and an increased accumulation of PI-3 kinase products (Gold and Aebersold, 1994). Both p145*ship* and PI-3 kinase have been implicated in growth regulation in other cell systems (van der Geer and Hunter, 1993, Lioubin et al., 1996). The prevention of BCR-mediated growth inhibition of the RL human B lymphoma cell line by the PI-3 kinase inhibitor, wortmannin, suggests that PI-3 kinase also participates in growth regulation in B cells (Beckwith et al., 1996). Whether p145*ship* is also involved in BCR-mediated biological responses in B cells remains to be demonstrated. However, the fact that PI-3 kinase products serve as substrates for p145*ship* suggests that these two pathways are likely to act in concert in growth regulation.

Other proteins known to be phosphorylated upon BCR engagement include transmembrane proteins CD19 (Chalupny et al., 1993), FcγRIIb1 (Muta et al., 1994), CD22 (Schulte et al., 1992), a cytoplasmic SH2-containing tyrosine phosphatase which has recently been renamed SHP-1 (D'Ambrosio et al., 1995, Adachi et al., 1996) and two other cytoplasmic proteins Vav (Bustelo and Barbacid, 1992) and HS1 (Yamanashi et al., 1993). A common feature among CD19, FcγRIIb1, CD22 and SHP-1 is that they modulate BCR signaling. As will be discussed below, mutations of the genes encoding each of these proteins in

mice all have dramatic effects on their B cell responses. The mechanisms by which Vav and HS1 participate in BCR signaling is relatively less well understood. Nevertheless, the reduction of peripheral B cell numbers in Vav-deficient RAG chimeric mice and the decreased proliferative response to anti-IgM in Vav-deficient (Tarakhovsky et al., 1995, Zhang et al., 1995) as well as HS1-deficient B cells (Taniuchi et al., 1995) suggest that these two proteins are likely to play important roles in B cell development and/or activation.

Positive and negative regulation of BCR signaling

A protein that positively regulates BCR signaling is CD19. This is a B cell-specific surface protein with two extracellular Ig-like domains and a long cytoplasmic tail. A fraction of CD19 is pre-associated with the BCR in unstimulated B cells (Pesando et al., 1989). BCR signaling has been shown to induce tyrosine phosphorylation of CD19 (Chalupny et al., 1993), and the phosphorylated cytoplasmic tail of CD19 associates with Lyn, Fyn, PI-3 kinase and Vav (van Noesel et al., 1993, Chalupny et al., 1995, Weng et al., 1994). The co-ligation of CD19 with the BCR lowers the threshold for anti-Ig-induced B cell proliferation by two orders of magnitude (Carter and Fearon, 1992), perhaps due to the additional recruitment of the CD19-associated kinases to the BCR complex. The physiological significance of this increased sensitivity to BCR signaling may be related to the association of CD19 with the complement receptor type 2, CD21 on mature B cells (Bradbury et al., 1992). CD21 binds fragments of C3, which are generated by the complement cascade and covalently attached to antigens. Thus, the CD21/C3/antigen complex may serve as a bridge to bring together CD19 and membrane-bound Ig. Indeed, antigens expressed as a fusion to C3 fragments are much more potent as *in vitro* B cell activators and as *in vivo*

immunogens than the corresponding monomeric antigens (Dempsey et al., 1996). The importance of CD19 in B cell responses is best illustrated by the finding that CD19-deficient mice have impaired B cell responses to T cell-dependent antigens (Rickert et al., 1995, Engel et al., 1995), a class of antigens that does not cause extensive crosslinking of membrane Ig and may require signal amplification by CD19.

In contrast to CD19, the two other transmembrane proteins mentioned above that become tyrosine phosphorylated upon anti-Ig treatment are thought to dampen B cell signaling. FcγRIIb1 is the B cell receptor for the Fc region of IgG. The negative regulatory role of FcγRIIb1 was demonstrated by the finding that while $F(ab')_2$ fragments of anti-Ig antibodies effectively induce B cell proliferation, intact IgG antibodies (with the Fc portion) inhibit this proliferation (Phillips and Parker, 1983). The coligation of FcγRIIb1 and membrane Ig by intact anti-Ig antibodies mimics the late stages of an immune response, when antigen/IgG complexes may form a similar bridge between the two receptors. It is hypothesized that this coligation serves as a signal to B cells that sufficient antibodies have been produced. Indeed, mice deficient for FcγRII display a slightly elevated antibody response after immunization (Takai et al., 1996). In addition to inhibiting BCR-induced proliferation, the coligation of FcγRIIb1 with BCR *in vitro* also inhibits anti-Ig induced PIP_2 breakdown (Bijsterbosch and Klaus, 1985) and transmembrane calcium influx (Choquet et al., 1993).

Another transmembrane protein that is rapidly tyrosine phosphorylated upon BCR engagement is CD22, a B cell-specific molecule that has been found to associate with the BCR with low stoichiometry in unstimulated cells (Schulte et al., 1992, Peaker and Neuberger, 1993, Leprince et al., 1993). The inhibitory effect of CD22 on BCR signaling was first demonstrated by studies showing that the prevention of CD22 co-aggregation with the BCR lowers the threshold for BCR-

induced proliferation by two orders of magnitude (Pezzutto et al., 1987, Doody et al., 1995). Recently, mice deficient in CD22 have been generated (O'Keefe et al., 1996). B cells from these mice exhibit enhanced antibody responses upon immunization, as well as a lowered threshold for anti-Ig-induced calcium elevation and proliferation *in vitro,* thus reinforcing the notion that CD22 acts as a negative regulator of BCR signaling.

Another common feature between the two transmembrane proteins that negatively regulate BCR signaling is that both FcγRIIb1 and CD22 recruit the binding of the cytoplasmic SH2-containing tyrosine phosphatase, SHP-1, to their tyrosine phosphorylated cytoplasmic domains (Muta et al., 1994, D'Ambrosio et al., 1995, Doody et al., 1995, Law et al., 1996). It has been found that the phosphatase activity of SHP-1 is dramatically increased by binding to phosphorylated peptides from CD22 (Doody et al., 1995) and FcγRIIb1 (D'Ambrosio et al., 1995). The affinity of SHP-1 for peptide substrates is also enhanced by the deletion of the N-terminal SH2 domain in SHP-1 (Townley et al., 1993). These results suggest that SHP-1 may be regulated by an autoinhibitory mechanism which is relieved upon the binding of its SH2 domains to tyrosine phosphorylated sites.

The association of CD22 and FcγRIIb1 with SHP-1 and the activation of SHP-1 by phosphorylated peptides derived from these two proteins suggest that their inhibitory effects are mediated via SHP-1. There is evidence that such may be the case, at least for FcγRIIb1. The ability of FcγRIIb1 to inhibit the BCR-mediated calcium influx is dependent on the phosphorylation of a tyrosine within a 13 amino acid region in its C-terminal (Muta et al., 1994) and a phosphorylated peptide corresponding to this region was found to associate with SHP-1 (D'Ambrosio et al., 1995). Doses of intact anti-Ig that were not mitogenic to wild type B cells was able to induce the proliferation of B cells derived from FcγRII-

deficient mice (Takai et al., 1996). A similar effect was observed when intact anti-Ig was use to stimulate B cells from mice with mutations in SHP-1, despite coligation with FcγRIIb1 (D'Ambrosio et al., 1995, Pani et al., 1995) (see Table I). These results suggest that FcγRIIb1 is unable to exert its inhibitory effects in the absence of functional SHP-1. The exact mechanism by which SHP-1 downregulates the BCR signal is not yet clear. Presumably, its recruitment to the BCR via CD22 or FcγRIIb1 leads to the dephosphorylation and inactivation of certain key components involved in BCR signaling.

SHP-1 is not the only tyrosine phosphatase that has been demonstrated to regulate BCR signaling. The finding that BCR-induced intracellular calcium elevation in a cell line requires the expression of the transmembrane tyrosine phosphatase, CD45, suggests that this phosphatase is a positive regulator of the BCR signal (Justement et al., 1991), in contrast to SHP-1. The positive role of CD45 is further support by the finding that B cells from mice that are deficient for all isoforms of CD45 failed to exhibit any proliferative response by normally mitogenic doses of anti-Ig antibodies (Byth et al., 1996). Based on studies in T cells, which also require CD45 for T cell receptor signaling, it is thought that CD45 positively regulates BCR signaling by removing the negative regulatory tyrosine near the C-termini of Src-family kinases (Thomas, 1994). As discussed above, the activation of the Src-family kinases is thought to be the first step in the initiation of the BCR signal.

Src-family kinases in BCR signaling

Among the Src-family kinases, B cells have been found to primarily express Lyn, Blk, Fyn and Fgr (Bolen et al., 1992, Law et al., 1992). Mice deficient in each of these kinases have been generated. Disruption of Blk (S. Tarakhovsky,

Table I. Phenotypes of mice or cell lines deficient in proteins that regulate BCR signaling.

	in vitro B cell assays					*in vivo* effects			
Mutation	Basal tyr-P	BCR-induced tyr-P	BCR-induced proliferation	BCR-induced Ca^{2+} influx	BCR-induced B7-2 ↑*	Antibody response	AutoAb production	Convenional B cell #	B1 B cell #
$Lyn^{-/-}$[a]	⇓	⇓	⇑	ND	⇔	⇑[b]	⇑[a,b,c]	⇓	⇑
$CD22^{-/-}$[d]	⇓	⇔	⇑	⇑	⇑	⇑	⇑	⇓	ND
$Fc\gamma RII^{-/-}$[e]	ND	ND	⇑	ND	ND	⇑	ND	⇔	ND
$SHP\text{-}1^{-/-}$	⇑[f]	ND	⇑[g]	⇑[h]	ND	ND	⇑[i]	⇓[i]	⇑[i]
$CD19^{-/-}$	ND	ND	⇓[j]	ND	ND	⇓[k]	ND	⇔[j,k]	⇓[j,k]
$CD45^{-/-}$[l]	ND	ND	⇓	⇓[m]	ND	⇓	ND	⇑	ND

ND = not determined; ⇑ = increased; ⇓ = decreased; ⇔ = no change as compared to wild type

* % B7-2+ B cells vs anti-Ig dose (⇑ indicates an increased sensitivity, i.e. responds to lower anti-Ig concentration).

[a] work described in Chapter II of this thesis except [b] (Nishizumi et al., 1995) and [c] (Hibbs et al., 1995)

[d] (O'Keefe et al., 1996)

[e] (Takai et al., 1996)

[f] (Shultz et al., 1993); [g] (Pani et al., 1995); [h] (Cyster and Goodnow, 1995); [i] (Shultz and Green, 1976, Sidman et al., 1986)

[j] (Engel et al., 1995); [k] (Rickert et al., 1995)

[l] (Byth et al., 1996) except [m] (Justement et al., 1991)

personal communication) and Fgr (Lowell et al., 1994) did not result in any B cell abnormalities. B cells from mice deficient in Fyn^T, the hematopoietic isoform of Fyn, also had no demonstrable defect in BCR signaling, but were found to have defective IL-5 receptor signaling (Appleby et al., 1995, Sillman and Monroe, 1994)(Sillman and Monroe, 1994; Appleby et al., 1995). The inability to detect BCR signaling defects had been attributed to functional redundancy among Src-family kinases, since a number of them are activated and associate with the BCR upon activation. However, based on results from *in vitro* kinase assays and immunoblotting, Lyn appears to be the most abundant Src-family tyrosine kinase in splenic B cells. Thus, the absence of Lyn would be predicted to have a more dramatic effect on BCR signaling than deficiencies in the other Src-family kinases. Indeed, as will be described in Chapter II, mice deficient in Lyn have multiple B cell abnormalities. Moreover, biochemical characterizations of the splenic B cells from these mice suggest that Lyn participates in BCR signal initiation as well as downregulation.

CHAPTER I: Activation-Induced Association of Lyn with the BCR and the Covalent Modification of Lyn.

INTRODUCTION

The BCR complex consists of membrane Ig and two non-covalently associated molecules, Ig-α and Ig-β (Reth, 1992). Antigen recognition by B cells is mediated by the Ig heavy and light chains. However, since the cytoplasmic domain of the IgM heavy chain consists of only three amino acids, the signal generated upon receptor ligation is presumably mediated by the associated proteins, Ig-α and Ig-β (with cytoplasmic domains of 61 and 48 amino acids respectively). Indeed, studies using chimeric transmembrane proteins have shown that conserved sequences, known as the immunoreceptor tyrosine-based activation motif (ITAM), within the cytoplasmic domains of Ig-α and Ig-β are responsible for transducing BCR intracellular signaling events (Law et al., 1993, Kim et al., 1993, Sanchez et al., 1993).

Since tyrosine phosphorylation of cellular proteins is the earliest biochemical event detectable upon BCR engagement, my initial experiments were aimed at studying the possible involvement of Src-family tyrosine kinases in BCR signaling. Lyn was found to associate with the intact BCR with very low stoichiometry and this association was extremely weak, requiring the use of mild detergent conditions. Subsequent experiments utilized chimeric receptors that were fusions of the extracellular domain of IgM to the intracellular domains of Ig-α and Ig-β. Upon crosslinking of the chimeric receptors, we found an increased amount of Lyn and Fyn kinase activity associated with the chimeric receptor, while no increase could be demonstrated for Blk under the same conditions (Law et al., 1993).

BCR engagement also caused an increased level of tyrosine phosphorylation of Lyn. Moreover, upon anti-IgM stimulation in B cell lines as well as primary splenic B cells, a small fraction of Lyn shifted to higher apparent molecular weights (≥ 6 kD shift). A series of experiments were done in an attempt to characterize these higher MW forms of Lyn and to identify the modification(s) which caused this mobility shift.

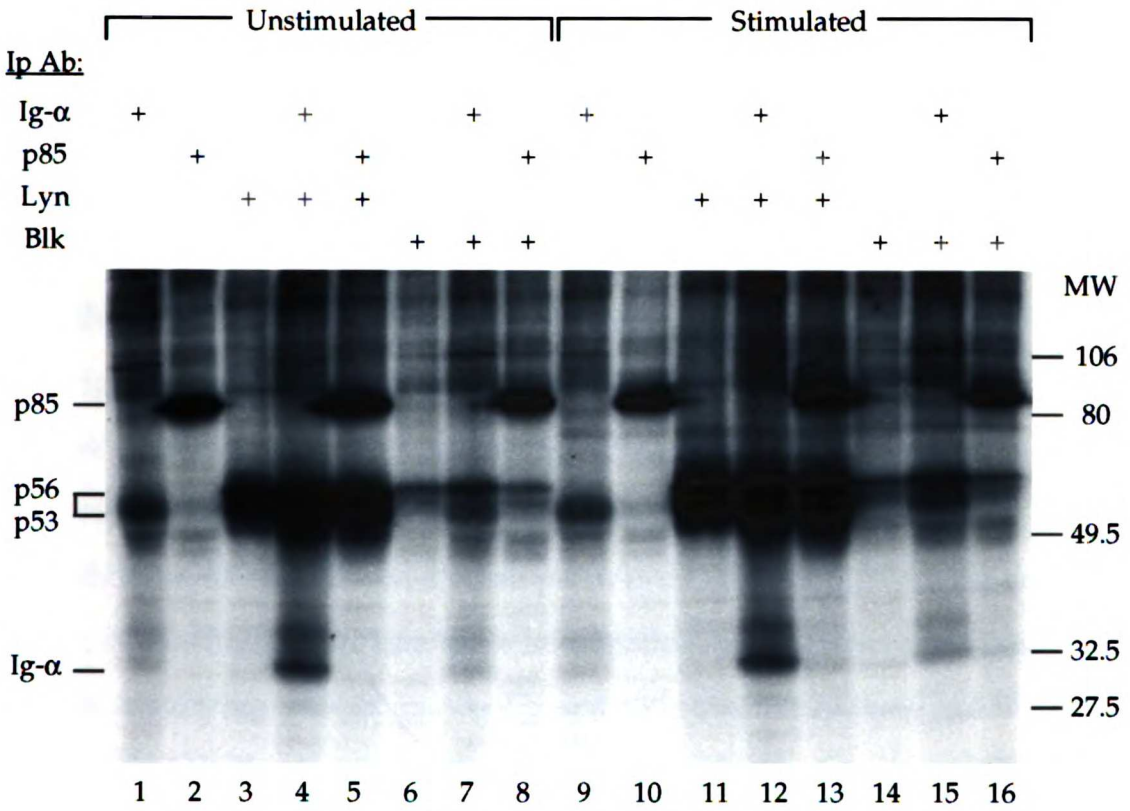
RESULTS AND DISCUSSION

Lyn can phosphorylate Ig-α *in vitro*

It has been postulated that the BCR signaling cascade is initiated by the increased tyrosine phosphorylation of the cytoplasmic tails of the BCR complex by the Src-family tyrosine kinases. The cytoplasmic domain of Ig-α contains a sequence DENLYEGLN (part of the ITAM motif) which closely resembles the predicted substrate specificities for both Lyn and Blk (Schmitz et al., 1996). To test whether Ig-α can be phosphorylated *in vitro* by Lyn and Blk, these proteins were immunoprecipitated from unstimulated or anti-IgM-stimulated lysates of the B lymphoma cell line WEHI-231. The kinases were either immunoprecipitated alone or in combination with Ig-α and the immunoprecipitates were incubated with ^{32}P-γ-ATP under conditions that are permissive for protein kinase activity (*in vitro* kinase assay). As shown in Figure 1.1, Ig-α appeared to associate with a small amount of kinase activity which led to a low level of Ig-α phosphorylation *in vitro* (lanes 1, 9). If Lyn was co-immunoprecipitated with Ig-α, the degree of phosphorylation on Ig-α was considerably increased (lanes 4, 12) indicating that Ig-α can be phosphorylated *in vitro* by Lyn. However, a similar increase in Ig-α phosphorylation was not observed when Blk was co-immunoprecipitated with Ig-α (lanes 7, 15). This may be because less Blk kinase activity (as judged by autophosphorylation) was immunoprecipitated from the same amount of lysate as compared to Lyn kinase activity (lanes 3, 11 vs. lanes 6, 14). Lyn immunoprecipitated from stimulated cell lysates appeared to be more active when compared to Lyn from unstimulated cell lysates (lane 3 vs 11) while Blk activity increased only slightly (lane 6 vs 14). The increase in kinase activities of Lyn and Blk from stimulated lysates was also reflected in the slightly

Figure 1.1

In vitro kinase assays of Lyn or Blk in combination with Ig-α or p85/PI-3 kinase. The proteins were immunoprecipitated in the indicated combinations from lysates of WEHI-231 that were untreated or stimulated with 40 μg/ml goat anti-IgM at 37°C for 3 minutes. The immunoprecipitates were subjected to *in vitro* kinase assays. The samples were resolved on a 10% SDS-polyacrylamide gel and visualized by autoradiography. The p53/p56 bracket on the left indicate the positions where $p53^{lyn}$, $p55^{blk}$ and $p56^{lyn}$ run on this gel.


Unstimulated
Stimulated
Ip Ab:
Ig-α
p85
Lyn
Blk
MW
106
80
49.5
32.5
27.5
p85
p56
p53
Ig-α
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

enhanced phosphorylation of Ig-α *in vitro* (lanes 4 vs. 12; 7 vs. 15).

Stimulation of B cells with anti-IgM has been reported to increase the amount of PI-3 kinase activity associated with Lyn (Yamanashi et al., 1992). PI-3 kinase consists of two subunits, a non-catalytic p85 subunit and a catalytic p110 subunit. B cell activation was reported to cause an increased association of Lyn with the p85 subunit (Yamanashi et al., 1992). To test whether Lyn and Blk can phosphorylate p85 *in vitro*, p85 was immunoprecipitated alone or in combination with the kinases, similar to the experiment described above. The p85 subunit was highly phosphorylated *in vitro*, even when immunoprecipitated alone (Figure 1.1, lanes 2, 10). This is likely to be caused by the p110 catalytic subunit that was associated with p85. However, no significant increase in phosphorylation was observed when p85 was co-immunoprecipitated with Lyn or Blk, even when the proteins were immunoprecipitated from stimulated lysates, suggesting that p85 may not be a direct substrate of these two Src-family kinases. In fact, it is now thought that the SH3 domains of the Src-family tyrosine kinases regulate PI-3 kinase activity by binding to a proline-rich sequence in the p85 subunit (Pleiman et al., 1994b).

Association of Lyn with the BCR

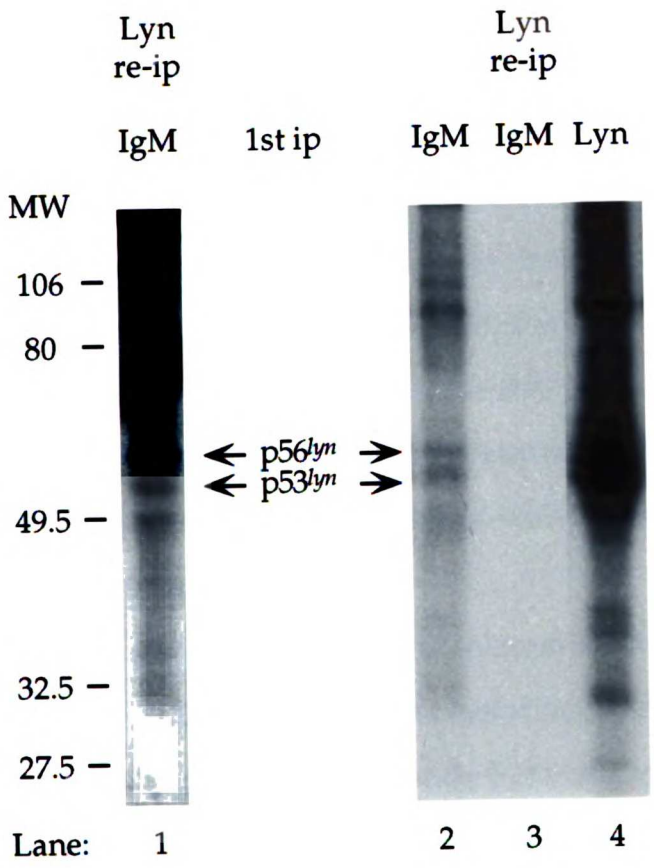
The experiment in Figure 1.1 suggested that Ig-α may be phosphorylated by Lyn. To test whether there is an induced association between the kinase and the substrate upon B cell activation, the association of Lyn and Blk with the BCR was next examined. This line of experiment was complicated by the fact that the components of the BCR complex are easily dissociated by detergents like Triton X-100 and NP-40. Therefore, mild detergents, such as digitonin and CHAPS, were used in order to preserve the BCR complex. One drawback in using these

detergents was that a smaller proportion of the BCR complex could be solubilized. In order to visualize the kinases that co-immunoprecipitated with the receptor, IgM immunoprecipitates were subjected to *in vitro* kinase assays, and the kinases were dissociated from the receptor and re-immunoprecipitated with specific antibodies. Using this technique, Lyn was found to associate with IgM immunoprecipitated from 1% CHAPS lysates of stimulated WEHI-231 cells (Figure 1.2). The amount of Lyn that was associated with IgM was estimated to be approximately 0.6% of the total cellular pool of Lyn, while no Blk was detected to associate with IgM under similar conditions (data not shown). This association between Lyn and IgM was stimulation-dependent (data not shown). The low stoichiometric association between Lyn and the BCR was consistent with results published by other groups (Yamanashi et al., 1991, Burkhardt et al., 1991, Campbell and Sefton, 1992, Lin and Justement, 1992, Burg et al., 1994).

Since chimeric proteins of the cytoplasmic domains of Ig-α and Ig-β have been shown to induce all the signaling events initiated by the intact receptor, we next tested whether the Src-family tyrosine kinases associate with these chimeras (Law et al., 1993). This series of experiments was performed with an $IgM^{-}IgG^{+}$ B cell line, 2PK3, that was transfected with either the wild type IgM heavy chain or chimeric proteins of the IgM extracellular domain fused to the transmembrane domain of CD8 and portions of the cytoplasmic domains of Ig-α or Ig-β. The chimera of the IgM extracellular domain with the cytoplasmic tail of CD8 was used as a negative control as this molecule does not associate with Ig-α or Ig-β and has no detectable signaling ability (Blum et al., 1993). Anti-IgM was used to immunoprecipitate the wild type or chimeric IgM receptors from 1% Brij-96 lysates of stimulated or unstimulated cells and the immunoprecipitates were subjected to *in vitro* kinase assays. The kinases that co-immunoprecipitated with the receptors and were labeled during the *in vitro* kinase assays were dissociated

Figure 1.2

Low stoichiometric association of Lyn with IgM in stimulated WEHI-231 cells. 1% CHAPS solubilized lysates (2 mg of proteins) of stimulated WEHI-231 cells were immunoprecipitated with anti-IgM and subjected to an *in vitro* kinase assay. The beads were washed after the kinase assay and subsequently divided into two equal portions. One portion was boiled in SDS-sample buffer for direct analysis (lane 2) and the other was boiled in TX lysis buffer + 0.5% SDS to elute the bound proteins. The eluate was then subjected to an anti-Lyn immunoprecipitation (lane 3). For comparison, Lyn was also directly immunoprecipitated from 1 mg of 1% CHAPS lysates of stimulated WEHI-231, and subjected to an *in vitro* kinase assay (lane 4). Lanes 2-4 were from a 4.5 hour exposure of the resolving gel. Since no proteins were visible in lane 3, the gel was exposed for 80 times longer and the same region corresponding to lane 3 was shown in lane 1.


Lyn
re-ip
IgM
1st ip
Lyn
re-ip
IgM
IgM
Lyn
MW
106
80
49.5
32.5
27.5
p56lyn
p53lyn
Lane:
1
2
3
4

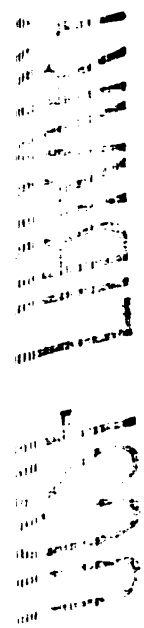
from the receptor and re-immunoprecipitated with antibodies specific for the kinases. As shown in Figure 1.3A, Lyn kinase activity was found to associate with both the Ig-α and the Ig-β chimeras upon receptor crosslinking while the amount of kinase associated prior to stimulation was undetectable. By immunoprecipitating Lyn from 1 and 5% of the amount of lysates used in the experiment and comparing the resulting kinase activities to that which was associated with the chimeras, it was estimated that between 1 to 5% of the total cellular pool of Lyn was induced to associate with the chimeras upon activation. This may be an overestimation since it is possible that the chimera-associated Lyn have a higher activity than average Lyn activity in the lysate. Unlike the result shown in Figure 1.2, the association of Lyn with the wild type IgM receptor was barely detectable in this system.

A similar experiment was then carried out to examine another Src-family tyrosine kinase, Fyn. Fyn kinase activity was found to associate with the wild type IgM complex as well as the Ig-α and the Ig-β chimera even prior to stimulation (Figure 1.3B). Moreover, the amount of kinase activity was increased upon receptor crosslinking. This could reflect an increase in the activity of pre-bound Fyn, an increase in the amount Fyn bound to the BCR and chimera following stimulation, or both. Again, the amount of Fyn that associated with the wild type IgM complex or the chimeras was estimated to be less than 5% of the total cellular pool of Fyn. Interestingly, an induced association of Blk with the BCR or with the chimeras was not detected under similar conditions (Figure 1.3C).

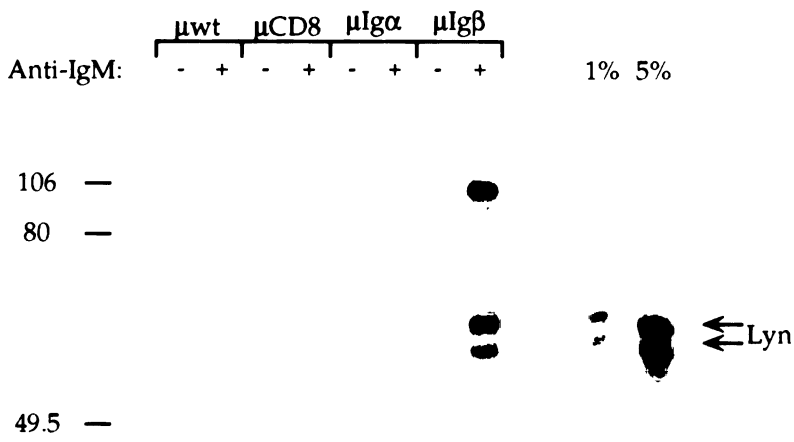
Results from the above experiments are consistent with the model that the Src-family tyrosine kinases initiate the BCR signaling cascade by associating with the receptor complex and phosphorylating the cytoplasmic domains of Ig-α and Ig-β. Further support for this model comes from kinetic studies which indicate

Figure 1.3

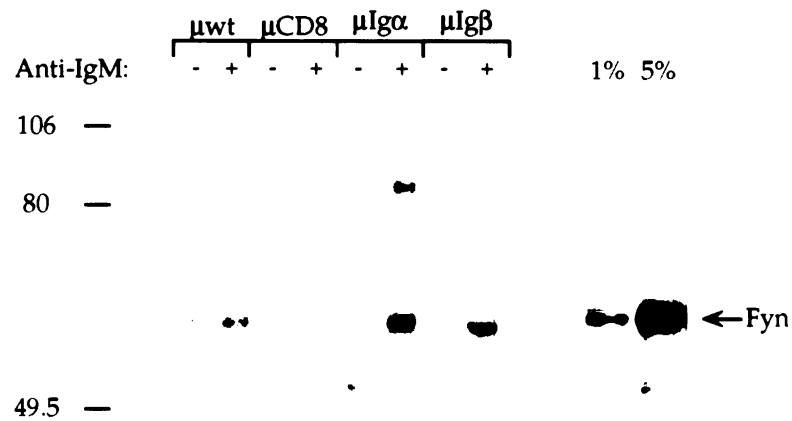
Association of Lyn and Fyn with chimeric proteins containing the intracellular domains of Ig-α and Ig-β. 2PK3 transfectants were stimulated with 10 μg/ml of biotinylated goat anti-IgM for 3 minutes at 37°C. Stimulated and untreated cells were lysed in 1% Brij-96 lysis buffer. The IgM complex or the chimeric receptors were immunoprecipitated from 1 mg of soluble lysates by the biotinylated goat anti-IgM antibody plus avidin-agarose and subjected to an *in vitro* kinase assay. Anti-Lyn (A), Fyn (B) and Blk (C) antibodies were used to re-immunoprecipitate the kinases from the proteins eluted from the immunoprecipitates after the kinase assay. For comparison, each of those kinases were immunoprecipitated from 10 μg (1%) or 50 μg (5%) of stimulated lysates and processed in parallel with the anti-IgM immunoprecipitates. Samples were resolved on an 8% SDS-polyacrylamide gel and visualized by autoradiography.



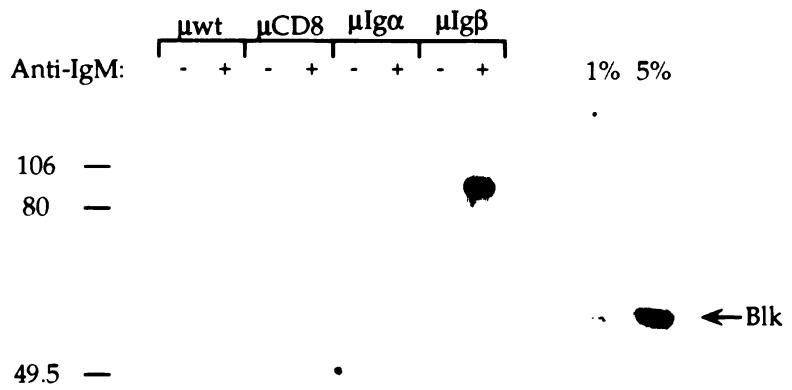
(A) Lyn



(B) Fyn



(C) Blk



that the Src-family tyrosine kinases are the first to be activated upon BCR engagement (Saouaf et al., 1994). Perhaps the most direct evidence for a role of Src-family tyrosine kinases in B cell signaling comes from studies carried out with B cells from mice genetically disrupted in their active *lyn* locus (described in Chapter II), as well as studies with a Lyn-deficient chicken B cell line generated by gene-targeting (Takata et al., 1994). These studies have demonstrated that in the absence of Lyn, the tyrosine phosphorylation of some cellular substrates were either delayed or diminished. A surprising finding from the studies of Lyn-deficient primary murine B cells was that in addition to participating in BCR signal initiation, Lyn also appeared to be required for the downregulation of the BCR signal (see Chapter II).

Covalent Modification of Lyn upon BCR engagement

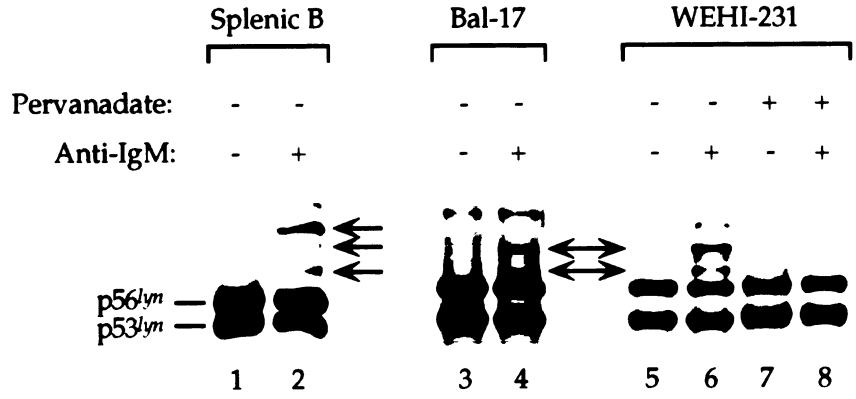
We and others have demonstrated that BCR signaling leads to an increased level of tyrosine phosphorylation of Lyn (Burkhardt et al., 1991 and Figure 2.4A in Chapter II). The low stoichiometry by which Lyn associates with the BCR prompted a series of experiments to examine whether Lyn might be associated with other proteins in B cells. While those experiments did not yield any positive results (data not shown), an interesting observation was made during the course of these studies.

Immunoblotting of whole cell lysates of unstimulated B cells using affinity purified anti-Lyn antibodies can detect both the p53 and the p56 forms of Lyn. In lysates of anti-IgM-stimulated B cells, additional bands with higher apparent MW were detectable by anti-Lyn immunoblotting (Figure 1.4A). The mobility shift corresponded to a ≥6 kD change in apparent MW and was presumed to be due to a covalent modification of Lyn. This phenomenon was observed in

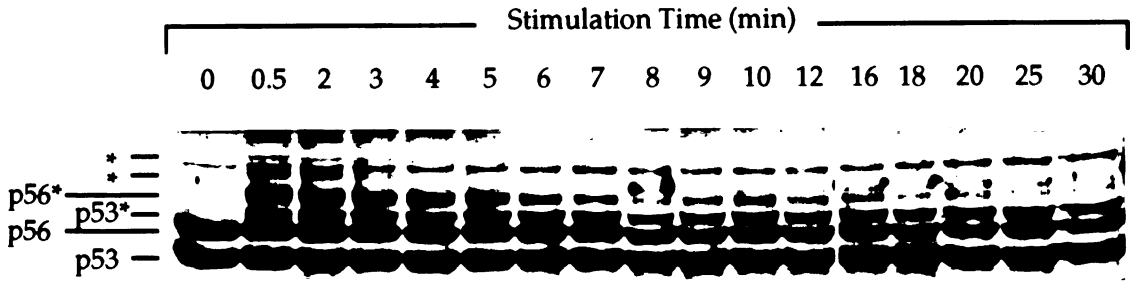
Figure 1.4

Anti-IgM induced the formation of higher apparent molecular weight forms of Lyn. (A) Anti-Lyn immunoblot of TX/DC lysates of membranes prepared from primary murine splenic B cells (6×10^6 cell equivalents), Bal-17 (2×10^6) or WEHI-231 (2×10^6) treated with media alone (lanes 1,3,5,7) or stimulated with 10 μg/ml goat anti-IgM for 1 min at 37°C (lanes 2,4,6,8). The WEHI-231 cells in lanes 7 and 8 were pretreated with pervanadate (0.1 mM Na_3VO_4, 2 mM H_2O_2) for 15 minutes at 37°C prior to stimulation. (B) WEHI-231 cells were stimulated with 10 μg/ml goat anti-IgM for the indicated length of time and permeabilized with 0.2% TX lysis buffer. The resulting cell pellets, enriched in membrane proteins, were lysed with TX/DC lysis buffer. Lysates from 1×10^6 cell equivalents were separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with the anti-Lyn antibody (ab2) that recognizes both $p53^{lyn}$ and $p56^{lyn}$.

(A)


Splenic B
Bal-17
WEHI-231
Pervanadate: - - - - - - + +
Anti-IgM: - + - + - + - +
p56lyn
p53lyn
1 2 3 4 5 6 7 8

(B)


Stimulation Time (min)
0 0.5 2 3 4 5 6 7 8 9 10 12 16 18 20 25 30
p56*
p56
p53*
p53

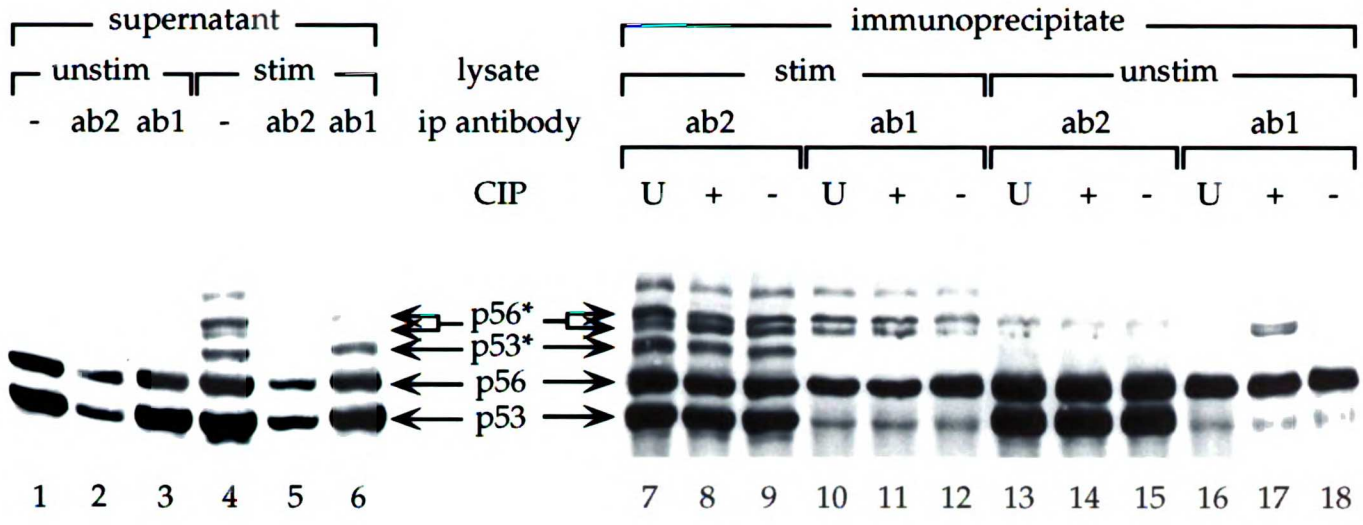
stimulated lysates from primary murine splenic B cells, as well as B cell lines such as Bal-17 and WEHI-231. A similar shift was not observed for Blk under the same conditions (data not shown). The induction of these higher MW forms of Lyn was detectable within 30 seconds of anti-IgM treatment (Figure 1.4B). Interestingly, one of these forms (denoted by p53* in Figure 1.4B) appeared to persist even after 30 minutes of stimulation, while others were greatly decreased after 5 minutes of stimulation.

To confirm that these were in fact modified forms of Lyn and not proteins that crossreacted with the anti-Lyn antibody (ab2) used for immunoblotting in Figure 1.4, another antibody (ab1) raised against a domain unique to $p56^{lyn}$ and therefore recognizes $p56^{lyn}$ but not $p53^{lyn}$, was utilized. As shown in Figure 1.5, three new bands were detected by anti-Lyn immunoblotting upon anti-IgM stimulation (lane 1 vs. lane 4). The higher MW doublet along with p56 were partially immunodepleted by the anti-Lyn antibody ab1 (lane 6). This antibody, which is specific for $p56^{lyn}$, did not immunodeplete $p53^{lyn}$ and the band directly above p56. On the other hand, the antibody (ab2) that recognizes both $p53^{lyn}$ and $p56^{lyn}$ depleted all the higher MW bands as well as $p53^{lyn}$ and $p56^{lyn}$ (lane 5). These results were mirrored when the immunoprecipitates were examined (lanes 7, 10). Thus, it appeared that the band directly above p56 (denoted by p53*) was a modified form of $p53^{lyn}$, while the higher MW doublet were modified forms of $p56^{lyn}$ (denoted by p56*). As a further confirmation that these induced bands were modified forms of Lyn, they were undetectable in anti-IgM stimulated splenic B cells from Lyn-deficient mice (data not shown).

The induction of these higher MW forms of Lyn appeared to be specific for the BCR signaling pathway since they were not induced by treatment with 2 μg/ml of LPS or anti-RP105 (up to 10 μg/ml) (data not shown), even though these two stimuli induce strong proliferation of primary B cells, as does anti-IgM

Figure 1.5

The higher apparent MW forms of Lyn induced by anti-IgM can be immunoprecipitated by two different anti-Lyn antibodies and are not sensitive to calf intestinal phosphatase (CIP) treatment. Membrane fraction of unstimulated or anti-IgM stimulated WEHI-231 cells were lysed in TX/DC lysis buffer. The lysates from 30 x 10^6 cell equivalents were subjected to immunoprecipitations with either an antibody (ab2) that recognizes both $p53^{lyn}$ and $p56^{lyn}$ or one that recognizes only $p56^{lyn}$ (ab1). The immunoprecipitating antibodies were immobilized on Sepharose beads by covalent coupling to prevent the heavy chain of the antibodies from obscuring the 50 kD region in the separating gel. *Left panel:* Portions of the undepleted lysates (-) or the supernatants after the immunoprecipitations by ab1 or ab2 are shown in lanes 1-6. Each of these lanes represent 2 x 10^6 cell equivalents. *Right panel:* The immunoprecipitates were washed with lysis buffer and divided into three equal portions. The samples were either boiled immediately in SDS sample buffer (lanes 7,10,13,16) and analyzed, or washed in CIP assay buffer and treated with CIP (lanes 8,11,14,17) or mock-treated (lanes 9,12,15,18) for 1 hour at 37°C. All samples were resolved on the same 8% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with an anti-Lyn antibody (ab2).


supernatant
immunoprecipitate
unstim
stim
lysate
stim
unstim
- ab2 ab1 - ab2 ab1
ip antibody
ab2
ab1
ab2
ab1
CIP
U + - U + - U + - U + -
p56*
p53*
p56
p53
Lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

treatment (data in Chapter II and Miyake et al., 1994, Yamashita et al., 1996).

Another line of experiment was also performed involving the treatment of B cells with pervanadate which inhibits tyrosine phosphatases and causes extensive tyrosine phosphorylation of cellular proteins (Gordon, 1991). Interestingly, pervanadate treatment not only did not cause the appearance of these modified forms of Lyn (lane 7 in Figure 1.4A), it inhibited the anti-IgM induction of these bands (lane 8 in Figure 1.4A). Tyrosine phosphatases like CD45, are thought to activate the Src-family tyrosine kinases by dephosphorylating the negative regulatory tyrosine at their C-termini (Thomas, 1994). Thus, one attractive hypothesis would be that the activation of Lyn by the C-terminal dephosphorylation is a prerequisite for this IgM-induced modification.

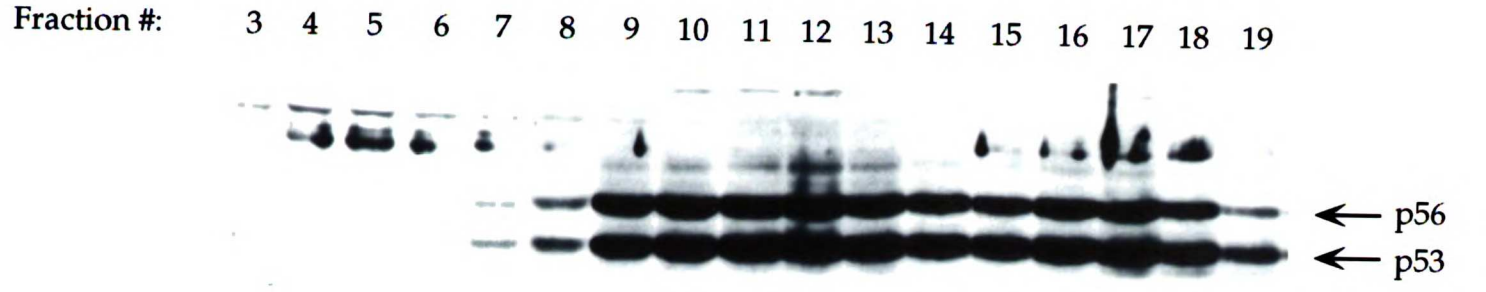
To examine the subcellular localization of the modified forms of Lyn, homogenates of unstimulated and anti-IgM stimulated cells were loaded on a sucrose step gradient (Bole et al., 1986). Anti-Lyn immunoblotting of the fractions collected from this gradient revealed that the majority of Lyn was localized to the fractions containing plasma membrane and smooth endoplasmic reticulum (ER) (Figure 1.6A, fractions 10-13). A considerable amount of Lyn was also found in fractions 15-17, which contained the rough ER and lysosomes. As shown in Figure 1.6B, most of the higher MW forms of Lyn co-localized with $p53^{lyn}$ and $p56^{lyn}$ in the plasma membrane/smooth ER fractions, consistent with the formation of these modified forms of Lyn being a proximal event in BCR signaling. The appearance of some of the higher MW forms of Lyn in fractions 15-17 (Figure 1.6B) could be due to some internalization that occurred upon anti-IgM stimulation.

Subsequent experiments were carried out in an attempt to identify the nature of the anti-IgM-induced modification(s) on Lyn. Anti-IgM stimulated WEHI-231 cells were homogenized and loaded onto a sucrose step gradient similar to that

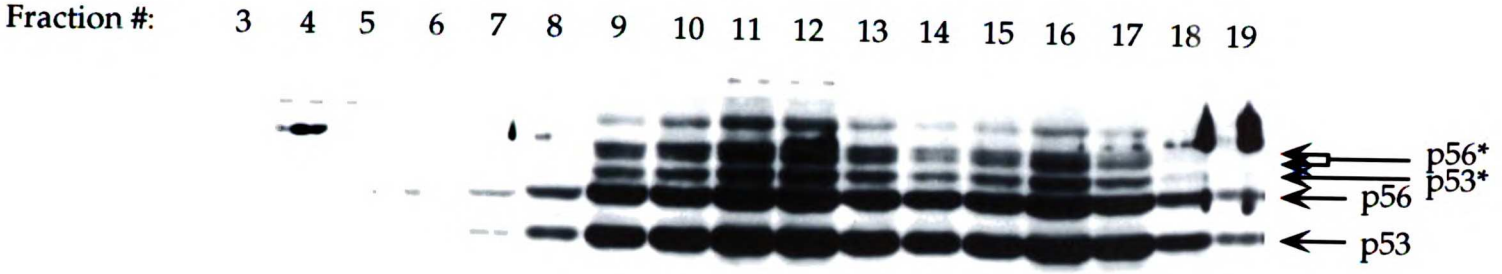
Figure 1.6

Subcellular localization of modified forms of Lyn. Membrane preparations from untreated (A) or anti-IgM stimulated (B) WEHI-231 cells (400 x 10^6 cells each) were separated on discontinuous sucrose step gradients. Fractions collected from the gradients were analyzed on 8% thick SDS-polyacrylamide gels and Lyn was detected by immunoblotting with the anti-Lyn antibody ab2.

(A) Unstimulated



(B) Anti-IgM stimulated



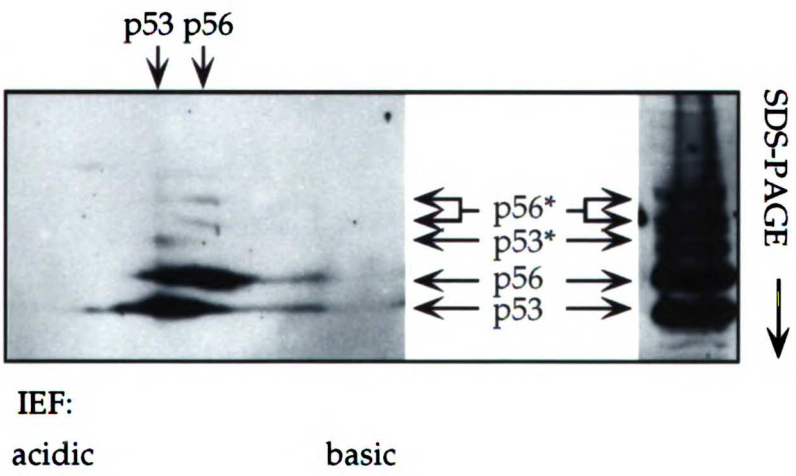
described in Figure 1.6. A fraction enriched in Lyn was separated on a two-dimensional (IEF/SDS-PAGE) gel to determine whether the higher apparent MW forms of Lyn have pI's different from their respective forms of Lyn in the unstimulated state. An internal control for the separation on the IEF dimension was the resolution of $p53^{lyn}$ and $p56^{lyn}$. $p56^{lyn}$ differs from $p53^{lyn}$ by a 21-amino acid insertion within the N-terminal unique domain. Since the insertion contains 7 charged residues (4 arginines, 1 lysine and 2 aspartates), $p56^{lyn}$ is predicted to differ from $p53^{lyn}$ by 3 net charges. However, the IEF separation of these two forms of Lyn may be complicated by their *in vivo* phosphorylation states. Nevertheless, the hypothesis was that if changes in phosphorylation were to cause a mobility shift of ≥6 kD, it would be due to multiple phosphorylations and should be manifested as a significant change on the IEF dimension. Such was not the case. As shown in Figure 1.7, the higher apparent MW forms of Lyn have pI's very similar to their respective forms of Lyn in the unstimulated state.

To directly test whether the mobility shift was due to phosphorylation, immunoprecipitated Lyn was subjected to treatment with calf intestinal phosphatase (CIP) which removes tyrosine, serine and threonine phosphorylations. As shown in Figure 1.5, the mobilities of the higher apparent MW forms of Lyn did not change upon CIP treatment (lane 8 vs. 9; lane 11 vs. 12), even though the level of tyrosine phosphorylation on Lyn was reduced by the CIP treatment (data not shown). Other attempts to identify the nature of the modification using antibodies (anti-PSer, anti-PThr and anti-ubiquitin), as well as hydroxylamine treatment (for fatty acid modification) have also yielded only negative results (data not shown).

The magnitude of the mobility shift, the rapidity by which these modified forms appear and the apparent specificity of the induction of these modified forms of Lyn to BCR signaling all suggest that the identification of the

Figure 1.7

Two dimensional (IEF/SDS-PAGE) gel analysis of Lyn and the higher MW forms of Lyn. The membrane fraction of WEHI-231 cells (stimulated with 10 μg/ml of anti-IgM for 1 min) at the 1.0/1.25M interphase of a discontinuous sucrose step gradient was pelleted and lysed in IEF sample buffer. 30 x 10^6 cell equivalents were separated on a pH 3/pH 10 IEF gel. *Left panel*: The lane from the IEF gel was excised and placed horizontally over a 8% thin SDS-polyacrylamide gel for separation in the second dimension. *Right panel*: 30 x 10^6 cell equivalents were boiled in SDS sample buffer and separated on the same SDS-polyacrylamide gel.


p53 p56
p56*
p53*
p56
p53
SDS-PAGE
IEF:
acidic
basic

modification(s) will provide insight into the proximal events of BCR signaling. However, it appears that this task will have to await the purification of sufficient quantities of the modified forms to enable direct chemical identification by mass spectrometry.

MATERIAL AND METHODS

Antibodies

Two different anti-murine Lyn antisera were generated against GST-Lyn fusion proteins. Antiserum #1119 (ab2) was raised against a GST fusion to amino acids 3-67 of $p56^{lyn}$, while antiserum #1274 (ab1) was raised against a GST fusion to amino acids 23-43 of $p56^{lyn}$. Antiserum #1119 (ab2) recognizes both $p53^{lyn}$ and $p56^{lyn}$, while antiserum #1274 (ab1) only recognizes $p56^{lyn}$ since amino acids 23-43 are not present in $p53^{lyn}$ due to alternative splicing (Stanley et al., 1991, Yi et al., 1991). Antisera to Blk (#1127) were similarly raised against a GST fusion to amino acids 3-56 of murine Blk. All the antigenic epitopes lie within the unique regions of the kinases and are not homologous to other members of the Src-family. The GST fusion proteins were purified from bacterial lysates by affinity chromatography with glutathione agarose and injected into rabbits (CalTag Laboratories, South San Francisco, CA). The anti-Lyn and anti-Blk antisera recognize the respective kinases in both immunoprecipitations and immunoblotting. To improve the background for immunoblotting, the antibodies were affinity purified prior to use. In order to avoid purifying for antibodies that recognizes GST and not the kinase unique regions, the affinity columns were prepared by covalently coupling maltose binding protein (MBP) fusions with the same unique regions used for raising the antibodies to cyanogen bromide-activated Sepharose beads.

Anti-Ig-α antisera was a gift from L. Matsuuchi (U. British Columbia). Antibodies against Fyn and the p85 subunit of PI-3 kinase were purchased from UBI (Lake Placid, NY). All immunoblots in this chapter were developed using the ECL detection system (Amersham, IL) using horseradish peroxidase-

conjugated donkey anti-rabbit IgG (Amersham, IL) as a secondary antibody. The goat anti-IgM antibody used for cell stimulation was obtained from Jackson ImmunoResearch Laboratories.

Covalent coupling of antibodies to Sepharose with dimethylpimelimidate

Since the rabbit IgG heavy chain has a molecular weight of 49 kD, its presence causes a distortion of the SDS-polyacrylamide gel in the 50 kD region and prevents the detection of $p53^{lyn}$ and $p56^{lyn}$ on an immunoblot after an anti-Lyn immunoprecipitation. In experiments where this was done (e.g. in Figure 1.5), anti-Lyn antibodies were covalently crosslinked to protein A-Sepharose beads. The anti-Lyn antibodies used for the coupling were either affinity purified over an MBP-Lyn affinity column or partially purified from crude serum by an ammonium sulfate precipitation (200g of $(NH_4)_2SO_4$ per 100 ml serum). 2-10 mg of antibodies were prebound to each ml of protein A-Sepharose at room temperature for at least 1 hour. The beads were washed 3 times with phosphate-buffered saline (PBS), 3 times with reaction buffer (200 mM sodium borate, pH 9.0) and resuspended in 10 times the volume of protein A-Sepharose beads before dimethylpimelimidate (Sigma, D8388) was added to 20 mM. Crosslinking was carried out at room temperature for 30 min on a rocker and terminated by washing the beads 3 times with 200 mM ethanolamine. Beads were further incubated in 200 mM ethanolamine at room temperature, with agitation, for at least 2 hours and subsequently washed with Tris-buffered saline (TBS), followed by 200 mM NaCl in 100 mM glycine, pH 3.0 to remove non-specific binding. Beads were then washed once with TBS, once with 500 mM NaCl, 5% Triton X-100 in 50 mM Tris, pH 7.4, and once again with TBS. Antibody-coupled beads were stored at 4°C in TBS.

Cell culture and cell stimulation

All cell lines were maintained in RPMI 1640 supplemented with 5% FCS (Gibco/BRL), 2 mM pyruvate, 20 mM glutamine and 50 μM 2-mercaptoethanol. The 2PK3 transfectants were grown in the presence of 0.3 mg/ml hygromycin. Prior to stimulation, cells were washed and resuspended to a density of 4×10^6 cells per ml and equilibrated at 37°C for at least 10 minutes. Unless indicated, cells were stimulated with 10 μg/ml goat anti-IgM for 1 minute at 37°C.

Preparation of lysates

Several different lysis methods were used in the experiments described. All lysis buffers contained the following mix of phosphatase and protease inhibitors: 1 mM Na_3VO_4, 1 mM PMSF, 20 μM leupeptin, 0.15 U/ml aprotinin. Brij lysis buffer contained 1% Brij-96 in 10 mM triethanolamine, 150 mM NaCl, 1 mM EDTA (Note that Brij-96 lysates were always used immediately after lysis and never stored at -20°C or -70°C); CHAPS lysis buffer contained 1% CHAPS in 10 mM triethanolamine, 150 mM NaCl, 1 mM EDTA; TX lysis buffer contained 1% Triton X-100 in 20 mM Tris, pH 8, 137 mM NaCl, 2 mM EDTA, 10% glycerol; TX/DC lysis buffer was TX lysis buffer plus 1% deoxycholate. Detergent insoluble material was removed by centrifugation at 4°C at 15,000g.

To isolate membrane proteins, cells were resuspended in 5 μM HEPES, pH 7.4, 1 μM $MgCl_2$, 2 μM EDTA and homogenized with a Dounce homogenizer for 50 strokes on ice. Intact cells and large aggregates were removed by a low speed centrifugation step at 2,500 rpm at 4°C for 2.5 min. Membranes were pelleted by centrifugation in a ti70 rotor for 45 min at 50,000 rpm. Alternatively, cells were permeabilized with 0.2% Triton X-100 in 20 mM Tris, pH 8, 137 mM NaCl, 2 mM

EDTA for 15 minutes on ice and pelleted for 15 minutes in a microcentrifuge to remove most of the cytoplasmic proteins. The pellets were lysed in TX/DC lysis buffer and detergent insoluble material was removed by a second centrifugation step.

To prepare membrane preparations for separation on sucrose step gradients, cells were resuspended in 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 0.25 M sucrose plus inhibitor mix, at 300 x 10^6 cells/ml and homogenized with a Dounce homogenizer for 100 strokes on ice. The homogenates were subjected to centrifugation for 10 min at 4°C at 2,000 rpm to remove intact cells and large aggregates. The supernatants were loaded on top of a 1.58 M sucrose cushion and spun for 15 min at 4°C at 3,000 rpm. The membrane fraction above the sucrose cushion was collected and loaded onto a discontinuous sucrose step gradient (0.5/0.75/1.0/1.25/2.0/2.5 M sucrose in 20 mM Tris, pH 8, 0.5 mM $CaCl_2$ plus phosphatase and protease inhibitor mix as described above) (Bole et al., 1986). The interphases between each step of the sucrose gradient were marked along the outside of centrifuge tube and the samples were subjected to centrifugation at 27,000 rpm for 5 hours at 4°C. The gradients were collected in 500 µl fractions and the molarity of the sucrose was noted for each fraction. These fractions were analyzed by immunoblotting 25 µl of each fraction. The modified forms of Lyn were found to be enriched at the interphase between the 1.0 and 1.25 M layers. The localizations of smooth ER and the plasma membrane to the 1.0/1.25 M interphase and of rough ER and lysosomes to the 1.25/2.0 M interphase were previously determined by marker enzyme analysis (Bole et al., 1986 and P. Bresnahan, personal communications).

Isoelectric focusing

The IEF gel for separation in the first dimension was a thin minigel (5 ml) prepared with ampholytes (Biolyte) purchased from BioRad. The IEF gel contained: 9.2 M urea, 4% acrylamide (acrylamide:bisacrylamide = 17.5:1), 2% Triton X-100, 1% Biolyte 5/7, 1% Biolyte 3/10. Membranes of stimulated WEHI-231 cells were isolated from the 1.0/1.25 M sucrose interphase of a discontinuous sucrose gradient as described above. This membrane fraction was diluted to 0.5 M sucrose and the membranes pelleted by centrifugation at 45,000g for 45 min at 4°C. The pellet was directly resuspended in IEF sample buffer (9.5 M urea, 2% Triton X-100, 5% 2-mercaptoethanol, 1% Biolyte 5/7 and 1% Biolyte 3/10) at 2 x 10^6 cell equivalent per µl. 10 µl was loaded on the first dimension and separated at 300V for 15 min, followed by 500V for 1.25 hour. The lane containing the sample was cut out and equilibrated for 20 min in 1x SDS sample buffer with continuous gentle agitation. The gel slice was then place horizontally over an 8% SDS-polyacrylamide gel. A second 10 µl aliquot of the sample was diluted to 30 µl with 1.5x SDS sample buffer and loaded on the same gel.

***In vitro* kinase assays**

Immunoprecipitates were washed twice in TX lysis buffer, once in kinase assay buffer (20 mM Tris, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.1% Triton X-100) and subsequently incubated in 100 µl of kinase assay buffer containing 10 µCi of ^{32}P-γ-ATP at room temperature for 10 minutes. The reactions were terminated by the addition of 500 µl of kinase wash buffer (10 mM Tris HCl, pH 7.2, 100 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.3% SDS). The immunoprecipitates were washed twice in this buffer and then boiled in SDS

sample buffer and separated on a 10% SDS-polyacrylamide gel. The gel was dried on Whatman paper and directly exposed to film.

In experiments examining kinase association, the kinase assay buffer and wash buffers were similar to those described above except that no detergents were present. After the kinase assays, the immunoprecipitates were washed and the bound proteins were eluted twice by boiling in TX lysis buffer + 0.5% SDS. The supernatants from the two rounds of elutions were pooled, chilled on ice and added to protein A-Sepharose beads plus the appropriate antibody for re-immunoprecipitation.

Calf intestinal phosphatase treatment

Anti-Lyn immunoprecipitates were washed 3 times with TX lysis buffer, twice with CIP buffer (10 mM Tris, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) and resuspended in 50 µl of CIP buffer. Samples were either mock-treated with PBS or treated with 2 µl of CIP (New England Biolabs, 10 U/µl) at 37°C for 1 hour.

Chapter II: The Characterization of the B Lymphocyte Populations in Lyn-Deficient Mice and the Role of Lyn in Signal Initiation and Downregulation

SUMMARY

To analyze the role of Src-family tyrosine kinase Lyn in BCR signaling, mice deficient in Lyn have been generated. These mice had a reduced number of peripheral B cells with a greater proportion of immature cells and a higher than normal turnover rate. Aged *lyn*$^{-/-}$ mice developed splenomegaly, produced autoantibodies and had an expanded population of B lymphoblasts of the B1 lineage. Splenic B cells from young *lyn*$^{-/-}$ mice were capable of initiating early BCR signaling events, although in a delayed fashion. Unexpectedly, *lyn*$^{-/-}$ B cells exhibited a considerable enhancement in MAP kinase activation and an increased proliferative response to BCR engagement. Stimulation of *lyn*$^{-/-}$ B cells with intact and F(ab')$_2$ fragments of anti-IgM revealed at least two defects in mechanisms that negatively regulate BCR signaling, one involving FcγRIIb1. Thus, while other Src-family tyrosine kinases may partially compensate for Lyn in signal initiation, the function of Lyn in the negative regulation of BCR signaling appears to be non-redundant.

INTRODUCTION

A functional immune system has to maintain the ability to recognize a large diversity of foreign antigens while minimizing self-reactivity. Depending on the maturation state of the B lymphocyte and signals supplied by other cells such as helper T cells, antigen binding can lead to the physical or functional elimination of autoreactive B cells, or the proliferation and differentiation of B cells recognizing foreign antigens (Goodnow et al., 1995, Rajewsky, 1996). Antigen binding also appears to participate in the positive selection of B lymphocytes into the peripheral mature B cell pool (Cyster et al., 1996). Therefore, the study of the molecular events triggered by antigen binding is important for understanding how signaling from the same receptor can lead to such diverse consequences.

The BCR consists of membrane immunoglobulin (Ig) complexed with Ig-α/Ig-β dimers, with the former providing antigen recognition, and the latter mediating signaling functions (Reth, 1994). Studies using chimeric transmembrane proteins have shown that conserved sequences, known as the immunoreceptor tyrosine-based activation motif (ITAM), within the cytoplasmic domains of Ig-α and Ig-β are responsible for transducing BCR intracellular signaling events (Sefton and Taddie, 1994). The biochemical events triggered by BCR signaling include increased phosphorylation of cellular proteins, phosphatidylinositol turnover and activation of the Ras pathway. The most rapid response is increased protein tyrosine phosphorylation and this is thought to initiate all of the subsequent signaling events (Gold et al., 1990, Campbell and Sefton, 1990, Lane et al., 1991, Brunswick et al., 1991). Some of the proteins that have been shown to be tyrosine phosphorylated upon BCR engagement include the receptor components Ig-α and Ig-β, the transmembrane proteins CD19 and CD22 and numerous signaling components such as Shc, RasGAP, MAP kinase,

PI-3 kinase, PLCγ1 and γ2, SHP-1 and Vav (reviewed in DeFranco, 1993). Three distinct types of tyrosine kinases have been found to be phosphorylated as well as activated upon BCR signaling: Lyn, Fyn, Lck, Blk and Fgr of the Src-family (Yamanashi et al., 1991, Burkhardt et al., 1991, Wechsler and Monroe, 1995), Syk of the Syk/Zap-70 family (Hutchcroft et al., 1992, Burg et al., 1994) and Btk of the Tec family (Saouaf et al., 1994, Aoki et al., 1994). A multi-step model for BCR signaling has been proposed (DeFranco, 1995), in which the Src-family kinases are the first to be activated upon receptor engagement and they in turn phosphorylate the tyrosine residues in the ITAM sequences within Ig-α and Ig-β. The phosphorylated ITAMs recruit the binding of Syk, which becomes phosphorylated and activated. Both the Src-family kinases and Syk are likely to contribute to the phosphorylation and/or activation of downstream effectors (Takata et al., 1994, Nagai et al., 1995, Richards et al., 1996). Among the subsequent events is the phosphorylation and activation of Btk (Saouaf et al., 1994, Aoki et al., 1994) which is important for the BCR-induced tyrosine phosphorylation of PLCγ2 and the rise in intracellular free calcium (Takata and Kurosaki, 1996)

Among the Src-family kinases, Lyn is widely expressed in hematopoietic cells (Bolen et al., 1992, Law et al., 1992) and has been implicated by a variety of biochemical experiments to be involved in BCR signaling. BCR engagement induces rapid increases in the tyrosine phosphorylation and the kinase activity of Lyn (Burkhardt et al., 1991). In addition, Lyn has been found to associate with the BCR complex as well as with PLCγ2, RasGAP and PI-3 kinase after anti-IgM stimulation (Burkhardt et al., 1991, Yamanashi et al., 1991, Campbell and Sefton, 1992, Leprince et al., 1992, Li et al., 1992, Lin and Justement, 1992, Law et al., 1993, Burg et al., 1994). The predicted optimal substrate recognition sequence for Lyn is also consistent with it being a kinase that can phosphorylate the ITAMs in

the BCR complex (Schmitz et al., 1996). To directly test the involvement of Lyn in BCR signaling, we have generated mice deficient for Lyn by targeted disruption of the active *lyn* gene. Aged *lyn*$^{-/-}$ mice have an activated B cell population which appears to be of the B1 lineage. The conventional B cell population in young *lyn*$^{-/-}$ mice has an increased proportion of less mature B cells and a higher rate of turnover. In contrast to results from a previous study (Nishizumi et al., 1995), we found that early BCR signaling events in *lyn*$^{-/-}$ B cells were delayed but not drastically impaired. Unexpectedly, BCR crosslinking in *lyn*$^{-/-}$ B cells led to a greater activation of MAP kinases compared to wild type B cells and this correlated with an enhanced proliferative response to anti-IgM treatment in *lyn*$^{-/-}$ B cells. These observations suggest that mechanisms that inhibit BCR signaling, such as those mediated by FcγRIIb1, are defective in *lyn*$^{-/-}$ B cells. Thus Lyn, in addition to participating in the initiation of the BCR signal, also stimulates events that negatively regulate certain BCR-induced signaling reactions and the resulting B cell proliferation.

RESULTS

Appearance of autoantibodies and B cell blasts in aged *lyn*$^{-/-}$ mice.

The *lyn*$^{-/-}$ and *lyn*$^{-/+}$ littermates all appeared healthy and were fertile under specific-pathogen-free conditions. However, homozygous *lyn*$^{-/-}$ mice were found to develop autoantibodies and plasma cell accumulation with age, similar to what has been previously reported with independently generated mice (Nishizumi et al., 1995, Hibbs et al., 1995). When wild type and *lyn*$^{-/-}$ mice at various ages were tested for the presence of serum autoantibodies recognizing nuclear antigens or double stranded (ds)-DNA, the *lyn*$^{-/-}$ mice started to become positive around 16 weeks of age (Table II). Both IgM and IgG antibodies to ds-DNA were found among the autoantibodies that developed.

lyn$^{-/-}$ mice older than 14 weeks also developed splenomegaly. Histological sections of these enlarged spleens revealed the presence of large numbers of immature myeloid cells, fully differentiated neutrophils, lymphoblasts and plasma cells (C. A. L., V. W.F. C. and A. L. D., unpublished results). Splenomegaly was often accompanied by an enlargement of the lymph nodes with lymphoblasts and plasma cells. Examination of cells from these enlarged lymph nodes by flow cytometry revealed the presence of an IgM^{+}Mac-1^{+}CD5^{+} population in *lyn*$^{-/-}$ mice (R1 in Figure 2.1A, C) that were neither found in wild type mice of similar age (Figure 2.1A) nor in young *lyn*$^{-/-}$ mice (data not shown). These cells appeared to be activated, as indicated by their high forward light scattering property (an assessment of cell size) (Figure 2.1B). The fact that they stained positively for IgM, Mac-1 and CD5 suggests that these may be B cell blasts derived from cells of the B1 lineage (Hardy et al., 1994). In addition, these

Table II. Presence of autoantibodies in serum.

Antibodies to nuclear antigens †:

Age (weeks)	*lyn*$^{-/-}$ (*n*)	wild type (*n*)
5 - 7	0 (9)	0 (5)
8 - 10	1 (8)	0 (5)
11 - 13	2 (11)	ND
14 - 15	1 (7)	ND
16 - 18	4 (5)	ND
19 - 21	8 (9)	0 (6)
22 - 32	3 (3)	0 (6)

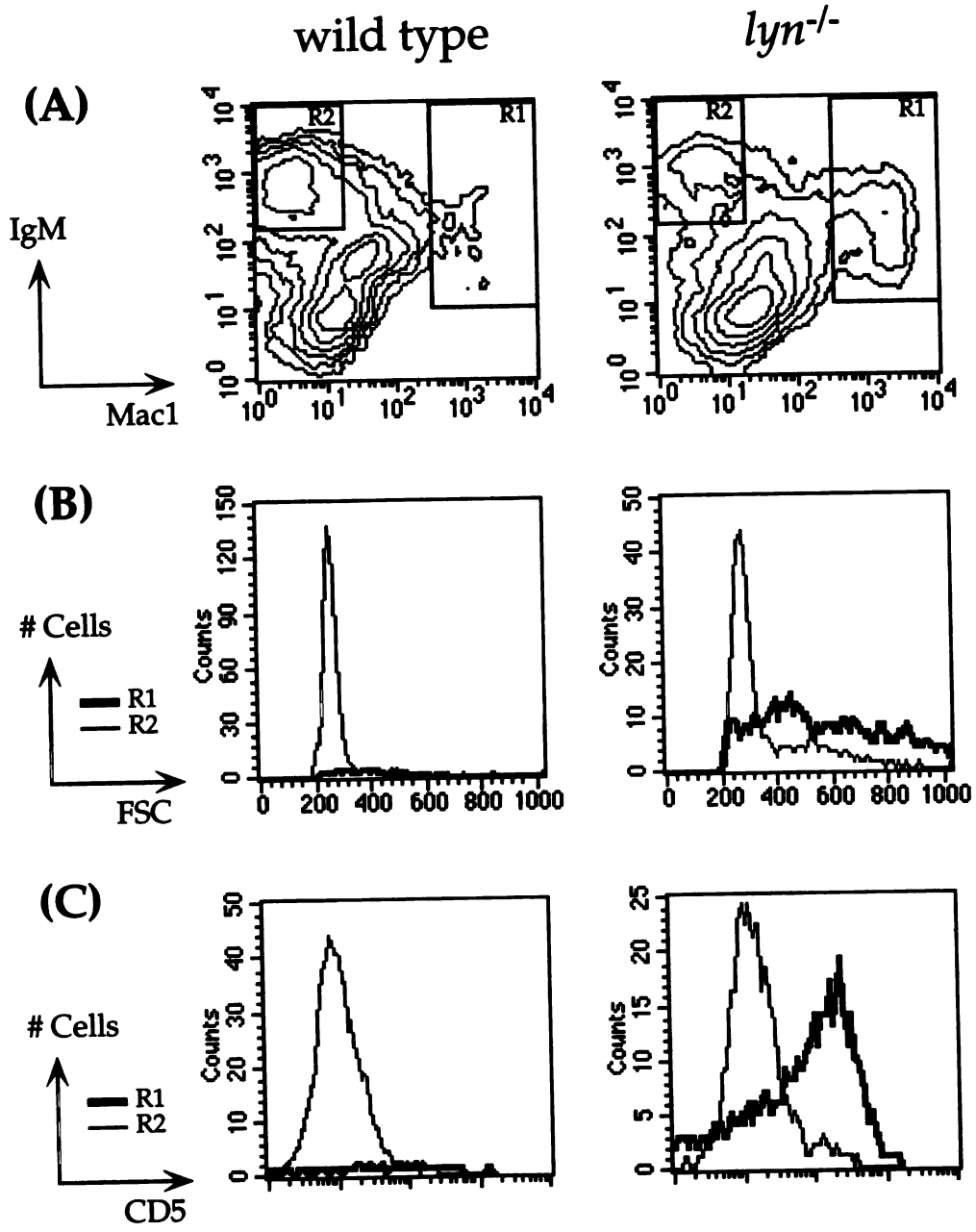
Anti-dsDNA antibodies in *lyn*$^{-/-}$ mice §:

		Antibody isotype	
Age (weeks)	(*n*)	IgM	IgG
6 - 9	(10)	0.224 ± 0.088	0.018 ± 0.015
10 - 13	(10)	0.434 ± 0.161	0.098 ± 0.082
14 - 16	(12)	0.528 ± 0.239	0.513 ± 0.436
17 - 20	(7)	0.859 ± 0.222	0.876 ± 0.478

† Data shown as number of mice whose serum scored positive for antibodies against nuclear antigens, out of a total of (*n*) mice tested.

§ Data shown as the mean ± SE of (*n*) mice per age group . Values represent ELISA readings in A_{450} (triplicate samples per animal) after 30 minutes of color development. Greater variation was seen in IgG isotypes as some animals had very low anti-dsDNA autoantibodies of the IgG isotype. Wild type mice had no detectable anti-dsDNA autoantibodies at any age.

Figure 2.1. Surface phenotype of lymph node cells from 23-week old mice. The *lyn*$^{-/-}$ mice had developed splenomegaly and lymphadenopathy by this age. Cells were triple-stained with antibodies to IgM, Mac-1 and CD5 and analyzed by flow cytometry. (A) The IgM^{+}Mac-1^{+} and IgM^{+}Mac-1^{-} populations are indicated by regions R1 and R2 respectively. Cells in R2 correspond to the conventional B cell population. (B) The forward light scatter (FSC) of the cells within R1 and R2 are represented as histograms. (C) The CD5 staining of cells in R1 and R2. The areas under the two curves within each panel in (B) and (C) are directly proportional to the number of cells present in each population.

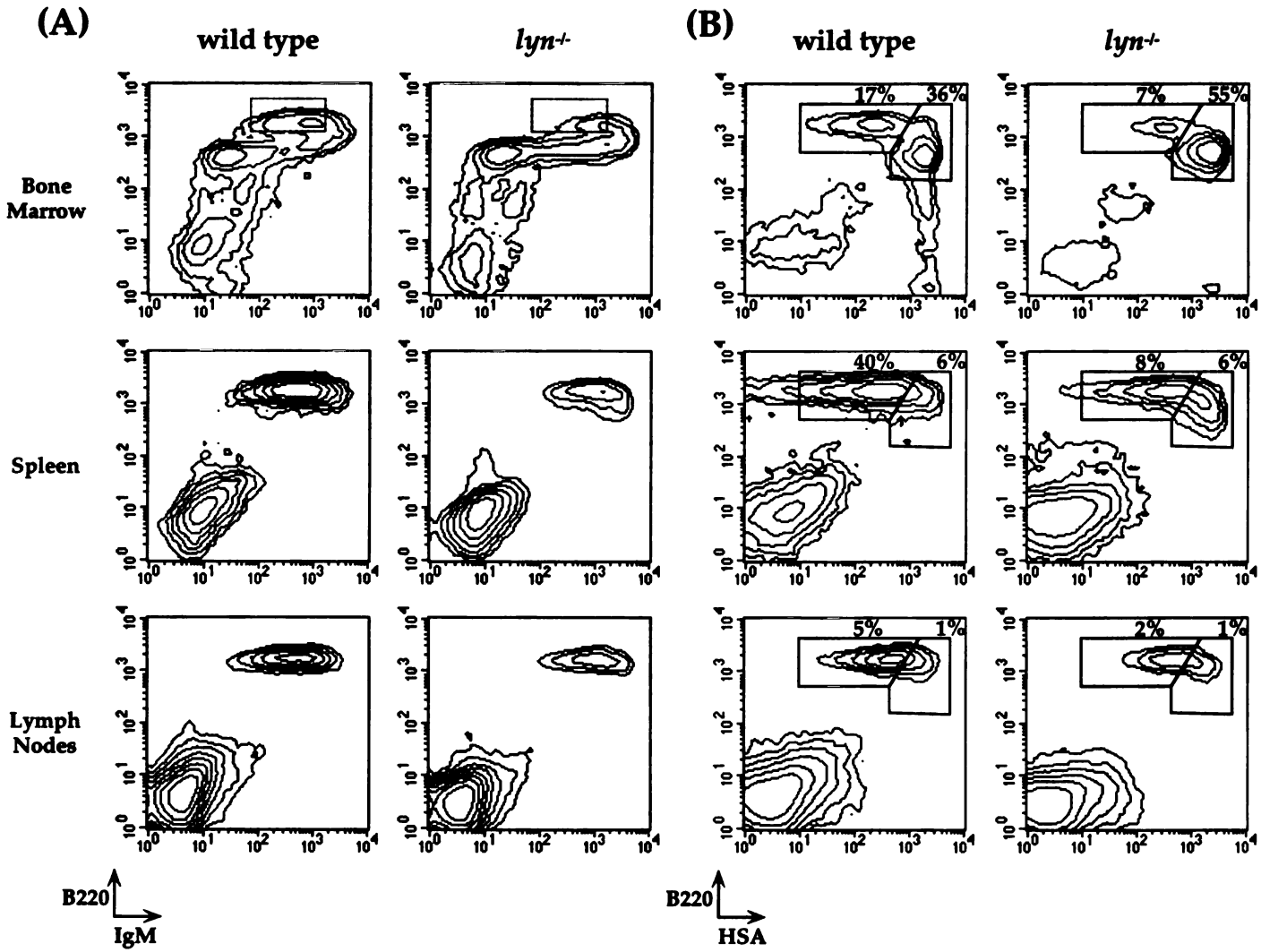

wild type
lyn-/-
(A)
IgM
Mac1
R2
R1
(B)
Cells
R1
R2
Counts
FSC
(C)
Cells
R1
R2
Counts
CD5

cells were also found to be $B220^{lo}IgD^{-}$, consistent with them being B1 cells (data not shown)

Conventional B cells in *lyn*$^{-/-}$ mice have a more immature surface phenotype.

Another observation that could be made from the data in Figure 2.1 is a decrease in the proportion of conventional B cells (R2 in Figure 2.1A) in these aged *lyn*$^{-/-}$ mice. A decreased number of conventional B cells was also observed in *lyn*$^{-/-}$ mice that had not yet developed splenomegaly. Consistent with published data on other *lyn*$^{-/-}$ mice (Nishizumi et al., 1995, Hibbs et al., 1995), our *lyn*$^{-/-}$ mice were found to have a reduced peripheral B cell population (generally 50-70% of wild type levels (n=30)), a significant reduction in the number of recirculating B cells in the bone marrow (Figure 2.2A) and a decrease in the conventional B cell population in the peritoneum (data not shown). These observations led us to postulate that there might be a B cell maturational defect and prompted the examination of the expression level of the heat stable antigen (HSA) on B cells. B cells that are $B220^{lo}HSA^{hi}$ are recent emigrants from the bone marrow that subsequently develop into more mature $B220^{hi}HSA^{lo}$ cells while in the periphery (Carsetti et al., 1995, Allman et al., 1993). As shown in Figure 2.2B, while the percentage of $B220^{lo}HSA^{hi}$ lymphocytes in the bone marrow, spleen and lymph nodes were similar in wild type and *lyn*$^{-/-}$ mice, there was a 2 to 5 fold reduction in the percent of $B220^{hi}HSA^{lo}$ B lymphocytes in these lymphoid compartments in the *lyn*$^{-/-}$ mice. Consistent with the HSA expression data, *lyn*$^{-/-}$ mice also have more $IgM^{hi}IgD^{lo}$ and fewer $IgM^{lo}IgD^{hi}$ cells in the spleen and peripheral blood (data not shown). Examination of the expression of other surface markers such as CD43(S7) confirmed that early B lineage cells are present in comparable numbers in wild type and *lyn*$^{-/-}$ mice (data not shown). Thus, B

Figure 2.2. Surface phenotype of conventional B lymphocytes. Bone marrow, spleen and lymph node cells from 8-week old wild type and *lyn*$^{-/-}$ mice were stained for B220, IgM and HSA simultaneously and analyzed by flow cytometry. (A) B220 x IgM profiles of cells in the lymphocyte gate. The B220^{hi}IgM^{med} population in the bone marrow corresponds to recirculating B lymphocytes and is indicated by the box with dotted lines. (B) Distinct populations of B220^{hi}HSA^{lo} and B220^{lo}HSA^{hi} cells were observed in the bone marrow. The boxes drawn around these two populations in the bone marrow were also used for the analysis of the cells in the spleen and the lymph node. The percentage of lymphocytes that lie within each boxed region is indicated.


(A)
wild type
lyn-/-
(B)
wild type
lyn-/-
Bone Marrow
Spleen
Lymph Nodes
17%
36%
7%
55%
40%
6%
8%
6%
5%
1%
2%
1%
B220
IgM
B220
HSA

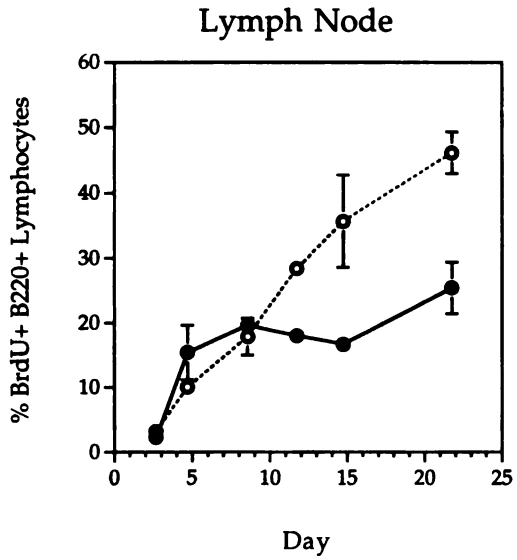
cell production appeared to be normal in the *lyn*$^{-/-}$ mice up until the B220^{lo}HSA^{hi} stage.

B cells in *lyn*$^{-/-}$ mice have a higher turnover rate.

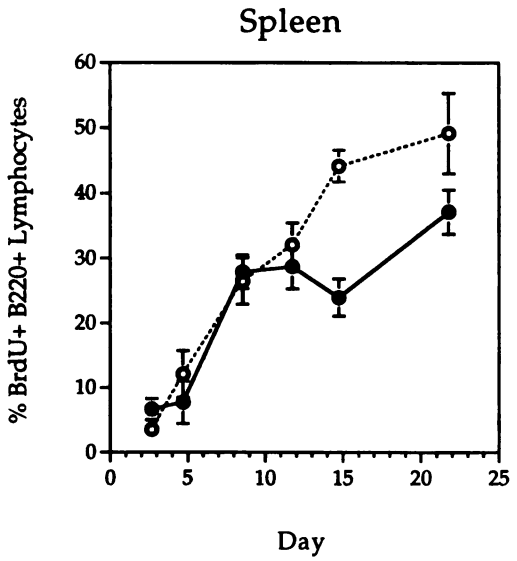
One possible explanation for the decreased percentage of mature B cells in the *lyn*$^{-/-}$ mice would be that a larger portion of peripheral B cells failed to enter the long-lived B cell pool, which would be reflected by an increased rate of B cell turnover. To directly test this hypothesis, we examined the *in vivo* incorporation of 5-bromo-2'-deoxyuridine (BrdU) into splenic and lymph node B cells in 9-week old mice. In the lymph nodes from wild type mice, 20% of B220^{+} lymphocytes were BrdU^{+} by day 8 (Figure 2.3A). The size of this newly synthesized B cell population remained within a range of 17-25% for the rest of the experiment (up to 22 days) indicating that at least 75% of the B220^{+} lymphocytes in wild type lymph nodes were long-lived B cells with a life time of greater than 3 weeks. During the first 8 days of the experiment, B lymphocytes in the lymph nodes from *lyn*$^{-/-}$ mice labeled with BrdU at a rate similar to wild type. However, in contrast to the wild type which appeared to level off beyond 8 days of labeling, the percent of labeled cells in the *lyn*$^{-/-}$ mice continued to increase. Thus, while 20-25% of the B lymphocytes in wild type lymph nodes were short-lived B cells, at least 45% of those in the *lyn*$^{-/-}$ mice were short-lived. The percentage of short-lived splenic B lymphocytes was also higher in *lyn*$^{-/-}$ mice than in wild type mice although the magnitude of the difference was much less than in lymph node (Figure 2.3B). These labeling experiments, together with the phenotypic analysis indicate that *lyn*$^{-/-}$ B cells exhibit a defect either in the maturation to the long-lived mature state or in the survival of mature B cells.

Figure 2.3. Turnover of peripheral B lymphocytes. Continuous administration of BrdU to 9-week old wild type (—●—) and *lyn*$^{-/-}$ (··o··) mice was initiated on day 0. At each indicated time point, 3 mice of each group were sacrificed and the proportion of B220^{+} lymphocytes in the (A) lymph nodes and (B) spleen that was labeled with BrdU (mean ± SEM) was determined by flow cytometric analysis. These data are representative of two independent experiments.

(A)


Lymph Node
% BrdU+ B220+ Lymphocytes
60
50
40
30
20
10
0
0
5
10
15
20
25
Day

(B)

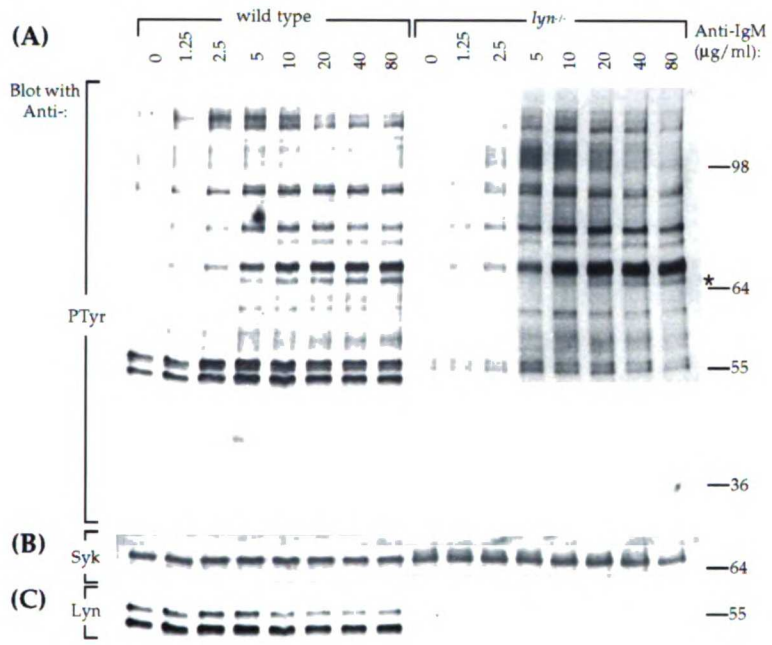

Spleen
% BrdU+ B220+ Lymphocytes
60
50
40
30
20
10
0
0
5
10
15
20
25
Day

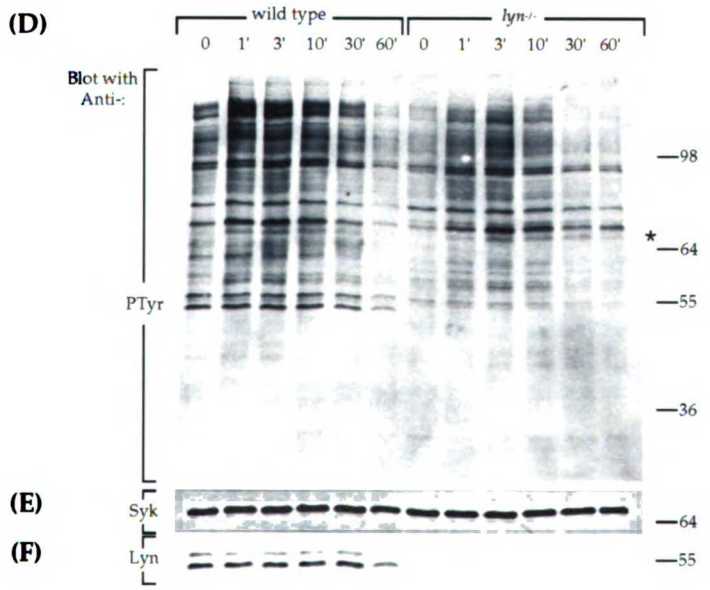
Anti-IgM induced tyrosine phosphorylation in *lyn*$^{-/-}$ B cells.

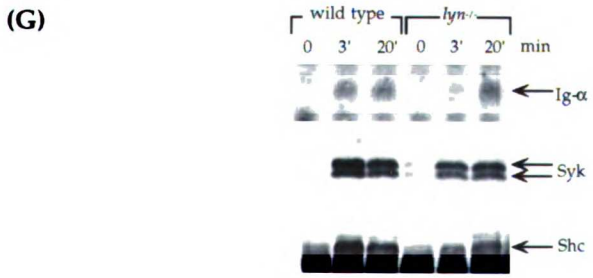
Experiments performed in a chicken B cell line in which the active *lyn* gene was disrupted by gene-targeting demonstrated that anti-IgM-induced tyrosine phosphorylation of many cellular substrates was decreased or abolished in the absence of Lyn (Takata et al., 1994). It was therefore of interest to examine whether the same defect was observed in splenic B cells from the *lyn*$^{-/-}$ mice. As mentioned above, the accumulation of myeloid cells and activated B1 B cells in *lyn*$^{-/-}$ mice older than 14 weeks led to the concern that environmental perturbations such as secreted cytokines could affect the properties of the splenic B cells from these mice. Therefore, all of the experiments below were performed with cells from mice that were 7-11 weeks of age without any signs of splenomegaly.

Anti-IgM stimulation of purified splenic B cells from both wild type and *lyn*$^{-/-}$ mice caused a dose-dependent increase in tyrosine phosphorylation of cellular proteins (Figure 2.4A). Two prominently tyrosine phosphorylated proteins in wild type cells flanking the 55 kD molecular weight marker were distinctly absent in the *lyn*$^{-/-}$ cells. They were confirmed to be the p53 and p56 forms of Lyn by reprobing this region of the same nitrocellulose blot with an anti-Lyn antibody (Figure 2.4C). A stimulation-dependent increase in the tyrosine phosphorylation of Lyn and a 55kD protein can be observed in wild type B cells, most noticeably at a 5 μg/ml dose of goat anti-IgM. A similar increase in the tyrosine phosphorylation of the 55 kD protein can also be observed in *lyn*$^{-/-}$ cells, with maximal response also around 5 μg/ml of goat anti-IgM. This 55 kD protein may correspond to another Src-family tyrosine kinase, such as Blk, which has also been reported to be phosphorylated upon BCR signaling (Burkhardt et al., 1991). The levels and activities of other Src-family kinases present in B cells,

Figure 2.4. Protein tyrosine phosphorylation upon anti-IgM stimulation of purified splenic B cells. (A) Anti-phosphotyrosine immunoblot of whole cell lysates of cells treated with media alone or different concentrations of goat anti-IgM for 3 minutes at 37°C. Regions of the nitrocellulose blot in (A) corresponding to 60-75 kD and 50-60 kD were stripped and reprobed with anti-Syk (B) and anti-Lyn (C) antibodies respectively. The anti-Syk western blot serves as an internal control for equivalent loading between the lanes. (D) Anti-phosphotyrosine immunoblot of whole cell lysates of cells treated with media alone or 20 μg/ml anti-IgM at 37°C for the indicated length of time (in min). Regions of the nitrocellulose blot in (D) corresponding to 60-75 kD and 50-60 kD were stripped and reprobed with anti-Syk (E) and anti-Lyn (F) antibodies respectively. (G) Purified splenic B cells were treated with media alone or goat anti-IgM at 20 μg/ml at 37°C for 3 or 20 min. The indicated proteins were immunoprecipitated from 250 μg of each lysate and analyzed by anti-phosphotyrosine immunoblotting. Equivalent loading between the lanes was verified by stripping and reprobing the immunoblots with the corresponding antibodies (data not shown).


(A)
wild type
lyn-/-
Anti-IgM (μg/ml):
0 1.25 2.5 5 10 20 40 80 0 1.25 2.5 5 10 20 40 80
Blot with Anti-:
PTyr
98
64
55
36
(B)
Syk
64
(C)
Lyn
55


(D)
wild type
lyn-/-
0 1' 3' 10' 30' 60' 0 1' 3' 10' 30' 60'
Blot with Anti-:
PTyr
98
64
55
36
(E)
Syk
64
(F)
Lyn
55


(G)
wild type
lyn-/-
0 3' 20' 0 3' 20' min
Ig-α
Syk
Shc

Blk, Hck, Fgr and Fyn were the same in wild type and *lyn*$^{-/-}$ B cells as determined by immunoblotting as well as by *in vitro* kinase assays (data not shown). A protein (indicated by " * " in Figure 2.4) migrating just above the 64 kD marker in this gel system was less robustly phosphorylated in the anti-IgM stimulated *lyn*$^{-/-}$ B cells compared to wild type B cells. This protein was found to co-migrate with Syk upon reprobing the nitrocellulose blot with an anti-Syk antibody (Figure 2.4B).

To examine whether the absence of Lyn might affect the rate at which the BCR signal is relayed, we stimulated purified splenic B cells with goat anti-IgM for varying lengths of time. While wild type B cells had already attained a maximal level of tyrosine phosphorylation by 1 minute of stimulation, the response in *lyn*$^{-/-}$ B cells did not peak until 3 minutes post-stimulation (Figure 2.4D). A number of proteins, such as Syk and Shc and the mIg accessory molecules, Ig-α and Ig-β, have been reported to have increased tyrosine phosphorylation upon BCR activation (DeFranco, 1995). In order to directly examine the phosphorylation states of these substrates in *lyn*$^{-/-}$ B cells upon anti-IgM stimulation, they were immunoprecipitated from equivalent amounts of lysates and immunoblotted with an anti-phosphotyrosine antibody. Syk immunoprecipitated from stimulated cells often runs as a doublet (Richards et al., 1996) with the lower band co-migrating with Syk from unstimulated cells and the upper band being a small fraction of Syk that is heavily tyrosine phosphorylated. As shown in Figure 2.4G, Ig-α, Syk and Shc immunoprecipitated from wild type lysates exhibited a marked increase in tyrosine phosphorylation after 3 minutes of stimulation and the degree of phosphorylation slightly decreased by 20 min. A 3 minute stimulation of *lyn*$^{-/-}$ B cells resulted in a less dramatic increase in the phosphorylation of Ig-α, Syk and Shc compared to wild type, and the signals never exceeded the wild type levels

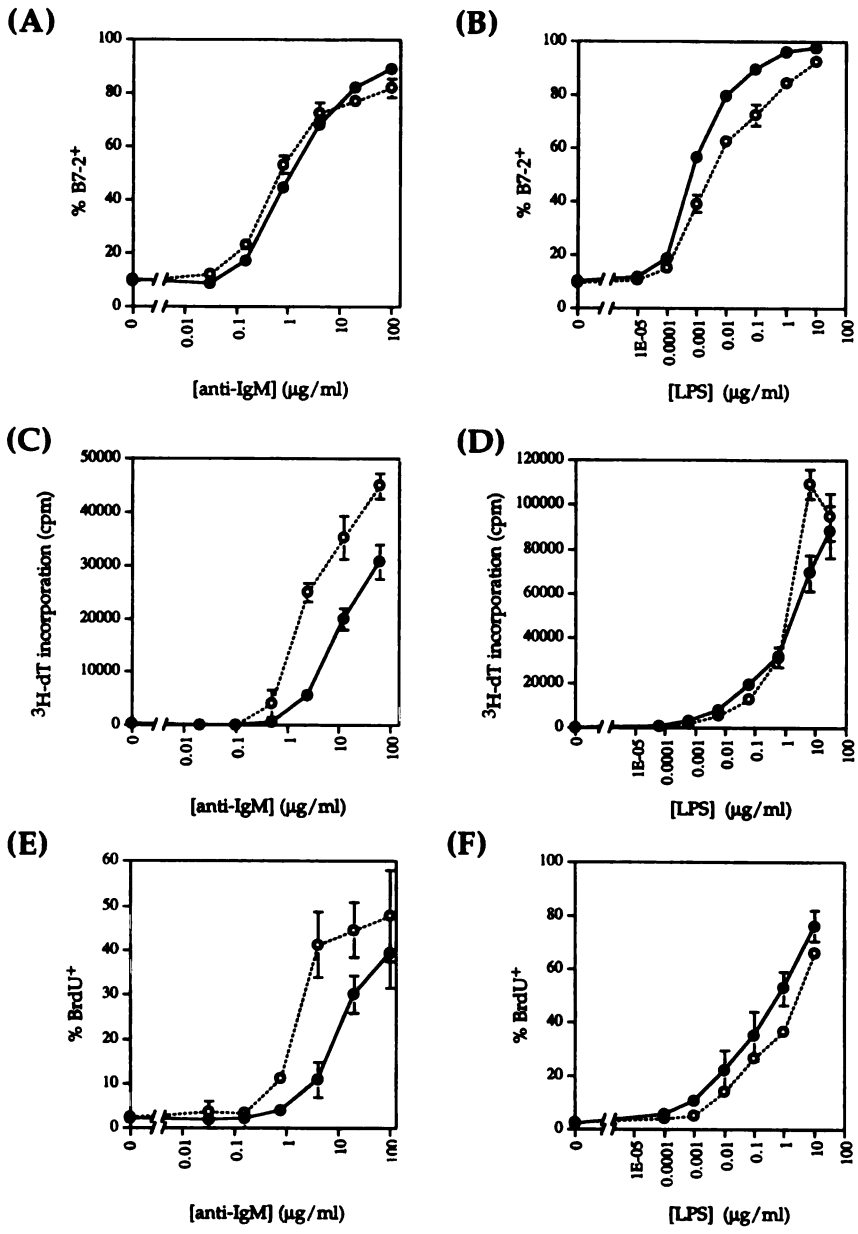
even by 20 minutes of stimulation. These delayed responses observed in the absence of Lyn are consistent with the model that Lyn participates in the initiation of the anti-IgM induced signal and acts upstream of Syk in BCR signaling (Kurosaki et al., 1994, DeFranco, 1995). Although the absence of Lyn resulted in a slightly longer lag time to reach a maximal signal, anti-IgM-induced signaling events can nevertheless be initiated, perhaps by other members of the Src-family tyrosine kinases that are present in the *lyn*$^{-/-}$ B cells.

Induction of B7-2 on *lyn*$^{-/-}$ B cells by LPS and anti-IgM.

Stimulation of B cells via the BCR or by the polyclonal B cell activator, lipopolysaccahride (LPS), has been shown to induce the expression of T cell costimulatory molecules such as B7-1 and B7-2 (Lenschow et al., 1994, Lenschow et al., 1993, Hathcock et al., 1994). Therefore, we tested whether the induced expression of these molecules on B cells is affected by the *lyn*$^{-/-}$ mutation. Non-erythroid splenocytes from wild type and *lyn*$^{-/-}$ mice were cultured *in vitro* in the presence of anti-IgM over a wide concentration range and the expression of B7-2 on B cells was assessed by flow cytometric analysis after 18 hours of stimulation. The percentage of *lyn*$^{-/-}$ B220^{+} cells induced to express B7-2 was similar to that of wild type at all anti-IgM concentrations tested (Figure 2.5A). However, the average intensity of the B7-2 staining (as measured by the mean fluorescence channel) on the B7-2^{+} cells was slightly higher in *lyn*$^{-/-}$ than in wild type cells when stimulated at intermediate doses of goat anti-IgM (0.8 - 4 μg/ml) (data not shown) similar to observations made independently by others (A. Tarakhovsky, personal communication).

LPS has been reported to stimulate Lyn activity in macrophages (Stefanova et al., 1993) but not in B cells (Burkhardt et al., 1991). To assess whether the

Figure 2.5. Biological responses of wild type and *lyn*$^{-/-}$ B cells to LPS and anti-IgM. Unpurified splenocytes (A-B, E-F) or purified splenic B cells (C-D) from wild type (—●—) and *lyn*$^{-/-}$ (··o··) mice were cultured with various concentrations of goat anti-IgM or LPS. In (A-B), splenocytes were incubated with media or with stimuli for 18 h prior to staining and flow cytometric analysis. The data presented (mean ± SE) are the proportion of viable cells (as determined by forward light scatter and propidium iodide exclusion) that were B220^{+} and positively stained for B7-2. In (C-D), purified splenic B cells were incubated with media or with stimuli for 44 h and subsequently pulsed with ^{3}H-dT for 4 h. In (E-F), splenocytes were incubated with media or with stimuli for 24 h and then labeled with 1 μM BrdU for the following 24 h. The proportion of B220^{+} cells that incorporated BrdU (mean ± SE) was determined by flow cytometric analysis. The data shown are representative of two or more independent experiments each involving at least 2 mice of each genotype.


(A)
% B7-2+
[anti-IgM] (μg/ml)
(B)
% B7-2+
[LPS] (μg/ml)
(C)
3H-dT incorporation (cpm)
[anti-IgM] (μg/ml)
(D)
3H-dT incorporation (cpm)
[LPS] (μg/ml)
(E)
% BrdU+
[anti-IgM] (μg/ml)
(F)
% BrdU+
[LPS] (μg/ml)

responses of B cells to LPS was affected by the *lyn*$^{-/-}$ mutation, B7-2 expression induced by LPS was also examined (Figure 2.5B). When stimulated with LPS, a slightly lower percentage of *lyn*$^{-/-}$ B cells expressed B7-2 as compared to wild type, but the difference was never greater than 18%. In a parallel experiment, it was found that B7-1 expression on both wild type and *lyn*$^{-/-}$ B cells was not significantly increased after an 18 hour stimulation with anti-IgM or LPS stimulation (data not shown) consistent with what has previously been reported for normal B cells (Lenschow et al., 1994).

lyn$^{-/-}$ B cells display a hypersensitive growth response to anti-IgM.

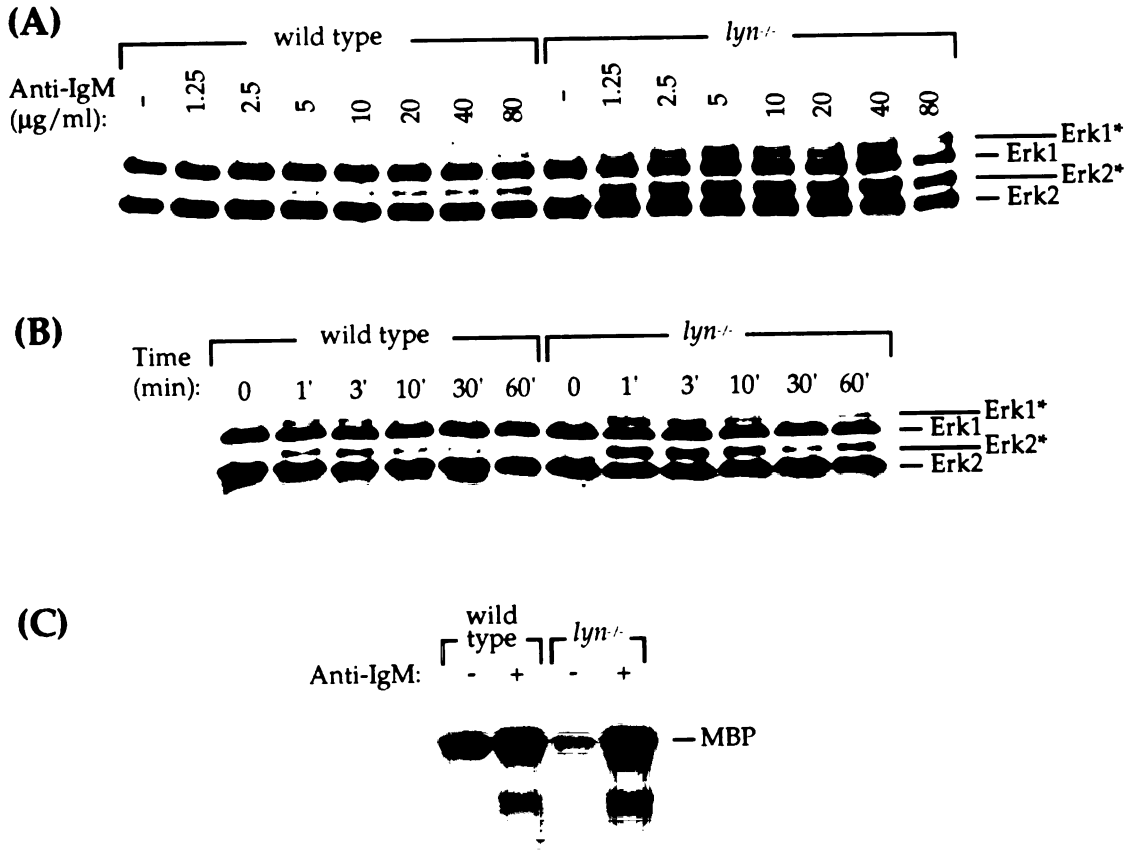
To further examine the effects of the *lyn*$^{-/-}$ mutation on biological responses of B cells, we assessed the proliferation of purified splenic B cells following LPS or anti-IgM stimulation. As shown in Figure 2.5D, wild type and *lyn*$^{-/-}$ B cells exhibited similar proliferative responses to LPS. In contrast, *lyn*$^{-/-}$ B cells were significantly more sensitive to anti-IgM than wild type B cells as indicated by a shift in the anti-IgM dose response curve (Figure 2.5C). The maximal difference was observed at 2.5 μg/ml anti-IgM, where the level of ^{3}H-dT incorporation in *lyn*$^{-/-}$ B cells was 4.5 times that of wild type B cells. To confirm that the increased proliferation observed in *lyn*$^{-/-}$ cells was attributable to the B cells and not to the small percentage of contaminating non-B cells in our splenic B cell preparations, we used flow cytometric analysis to assess the *in vitro* proliferation of B220^{+} cells by their ability to incorporate BrdU. While the wild type and *lyn*$^{-/-}$ B cells exhibited equal proliferative responses to various concentrations of LPS (Figure 2.5F), the *lyn*$^{-/-}$ B cells again proliferated at lower concentrations of anti-IgM than did the wild type B cells (Figure 2.5E). The maximal difference in this assay was observed at 4 μg/ml anti-IgM, where the percent of *lyn*$^{-/-}$ B cells that

incorporated BrdU was 3.8 times that of wild type. The hypersensitivity of *lyn*$^{-/-}$ B cells to anti-IgM was not due to a defect in receptor downregulation as the rate of internalization of surface IgM upon receptor crosslinking was the same for both wild type and *lyn*$^{-/-}$ B cells (data not shown).

Anti-IgM causes enhanced stimulation of MAP kinases in *lyn*$^{-/-}$ B cells.

The enhanced proliferative response of the *lyn*$^{-/-}$ B cells to anti-IgM stimulation was unexpected in light of the finding that most early BCR signaling events were slightly decreased (Figure 2.4D, G). Since the duration of MAP kinase activation has been shown to be important for the proliferative/ differentiation responses of cells in other systems (reviewed in Marshall, 1995), we tested whether the BCR-induced activation of MAP kinases in B cells was altered in the absence of Lyn. BCR signaling has previously been shown to activate a fraction of the total cellular pool of the Erk2 form of MAP kinase in B lymphoma lines (Gold et al., 1992b) as well as in purified splenic B cells (Cyster et al., 1996). This activation is accompanied by an increase in tyrosine phosphorylation of Erk2 and a decrease in its mobility on an SDS-polyacrylamide gel (Gold et al., 1992b). Using an antibody that recognizes both the Erk2 and Erk1 forms of MAP kinase, the appearance of slower-migrating forms of both MAP kinases was detected after anti-IgM treatment of purified splenic B cells (Figures 2.6A-B). As shown in Figure 2.6A, a greater fraction of Erk1 and Erk2 shifted to a slower mobility in *lyn*$^{-/-}$ B cells than in wild type cells following anti-IgM treatment. It is interesting to note that at low doses of anti-IgM (1.25 to 5 μg/ml), there was little or no slower-migrating forms of Erk1 and Erk2 in wild type cells, while there was a substantial amount induced in *lyn*$^{-/-}$ cells. This is a

Figure 2.6. Enhanced activation of the MAP kinases in *lyn*$^{-/-}$ B cells by anti-IgM stimulation. Purified splenic B cells were stimulated as described in Figure 2.4. Panels (A) and (B) correspond to the 35-50 kD regions of the immunoblots shown in Figure 2.4(A) and (D) respectively, which were stripped and reprobed with an antibody that recognizes both the Erk1 and Erk2 forms of MAP kinase. The slower migrating forms of Erk1 and Erk2 are indicated on the right by Erk1* and Erk2* respectively. (Equivalent loading of proteins in all lanes was verified by anti-Syk immunoblot shown in Figures 2.4B and E). (C) Purified splenic B cells from wild type and *lyn*$^{-/-}$ mice were treated with media alone or 10 μg/ml of goat anti-IgM for 1 minute at 37°C. Erk2 was immunoprecipitated from 100 μg of each lysate and subjected to an in vitro kinase assay using MBP as a substrate. Phosphorylation of MBP was visualized by autoradiography after SDS-PAGE.


(A)
wild type
lyn-/-
Anti-IgM (μg/ml):
-
1.25
2.5
5
10
20
40
80
Erk1*
Erk1
Erk2*
Erk2
(B)
Time (min):
0
1'
3'
10'
30'
60'
(C)
wild type
Anti-IgM:
MBP

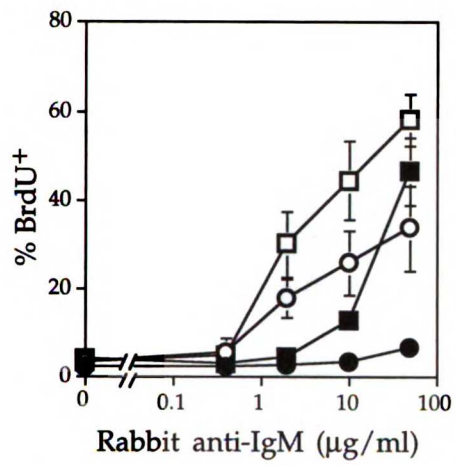
striking parallel to the proliferation results where the maximal difference was also observed in this concentration range of anti-IgM (Figures 2.5C, E).

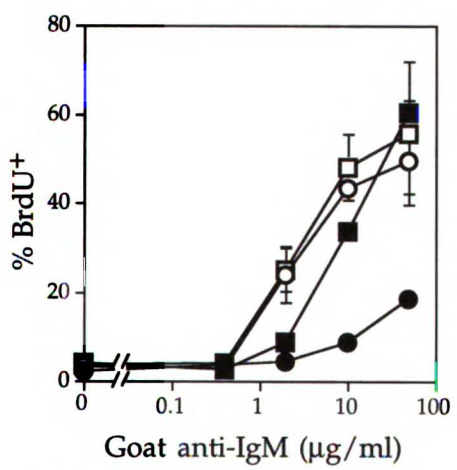
To examine whether the kinetics of the formation of the slower-migrating forms of Erk1 and Erk2 is different between *lyn*$^{-/-}$ and wild type B cells, we stimulated purified splenic B cells with 20 μg/ml of anti-IgM for various lengths of time. As shown in Figure 2.6B, although a greater fraction of Erk1 and Erk2 in *lyn*$^{-/-}$ B cells shifted to slower mobility upon anti-IgM stimulation as compared to wild type, the peak response was observed between 1 to 3 minutes of anti-IgM stimulation in both wild type and *lyn*$^{-/-}$ B cells. However, the slower-migrating forms of MAP kinases appeared to persist for a longer period of time in *lyn*$^{-/-}$ B cells as compared to wild type (Figure 2.6B). In other experiments in which longer stimulation times were examined (up to 36 hours), the amount of slower-migrating forms of Erk1 and Erk2 observed was always higher in *lyn*$^{-/-}$ B cells than in wild type (data not shown).

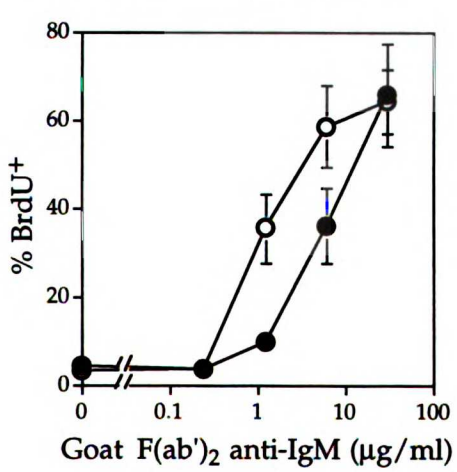
To confirm that the appearance of the slower-migrating forms of Erk1 and Erk2 seen upon BCR engagement corresponded to increases in MAP kinase activities, we used an antibody that preferentially recognizes Erk2 to immunoprecipitate this protein from control and stimulated B cell lysates and then measured its kinase activity *in vitro* using myelin basic protein (MBP) as a substrate. As shown in Figure 2.6C, anti-IgM stimulation resulted in a greater increase in Erk2 kinase activity in *lyn*$^{-/-}$ B cells than in wild type B cells. By quantitating the major MBP band using a phosphorimager, anti-IgM stimulation was found to result in a 4.6 fold increase in the Erk2 kinase activity in *lyn*$^{-/-}$ B cells as compared to a 1.6 fold increase in wild type B cells. Thus, anti-IgM caused a greater activation of the MAP kinases in *lyn*$^{-/-}$ B cells, both in the magnitude and in the duration of the response.

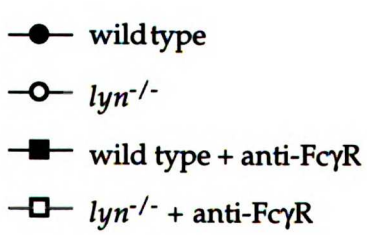
Figure 2.7. Enhanced responses of *lyn*$^{-/-}$ B cells to BCR signaling in the presence and in the absence of FcγR-mediated inhibition. RBC-depleted splenocytes (A) or purified splenic B cells (B-C) were preincubated with media alone, or with media containing the anti-FcγR monoclonal antibody (2.4G2) for 30 minutes. (A) The splenocytes were plated out at 0.5×10^6 per ml and cultured with rabbit anti-IgM, goat anti-IgM or goat $F(ab')_2$ anti-IgM at the indicated concentrations. Proportion of $B220^+$ cells that incorporated BrdU (mean ± SE) during 24-48 hour post-stimulation was determined by flow cytometric analysis. (B-C) Anti-Erk1/2 and anti-Syk immunoblots of whole cell lysates of purified splenic B cells treated with media, goat anti-IgM (B) or goat $F(ab')_2$ anti-IgM (C) for 3 minutes at 37°C. Note that the goat anti-IgM antibodies used in the experiments in this figure belonged to a batch different from that used in the prior figures. The affinity of this batch of antibodies appeared to be lower and resulted in a decreased level of stimulation as compared to prior experiments at comparable doses. Data shown are representative of two or three experiments with the same batch of antibodies.

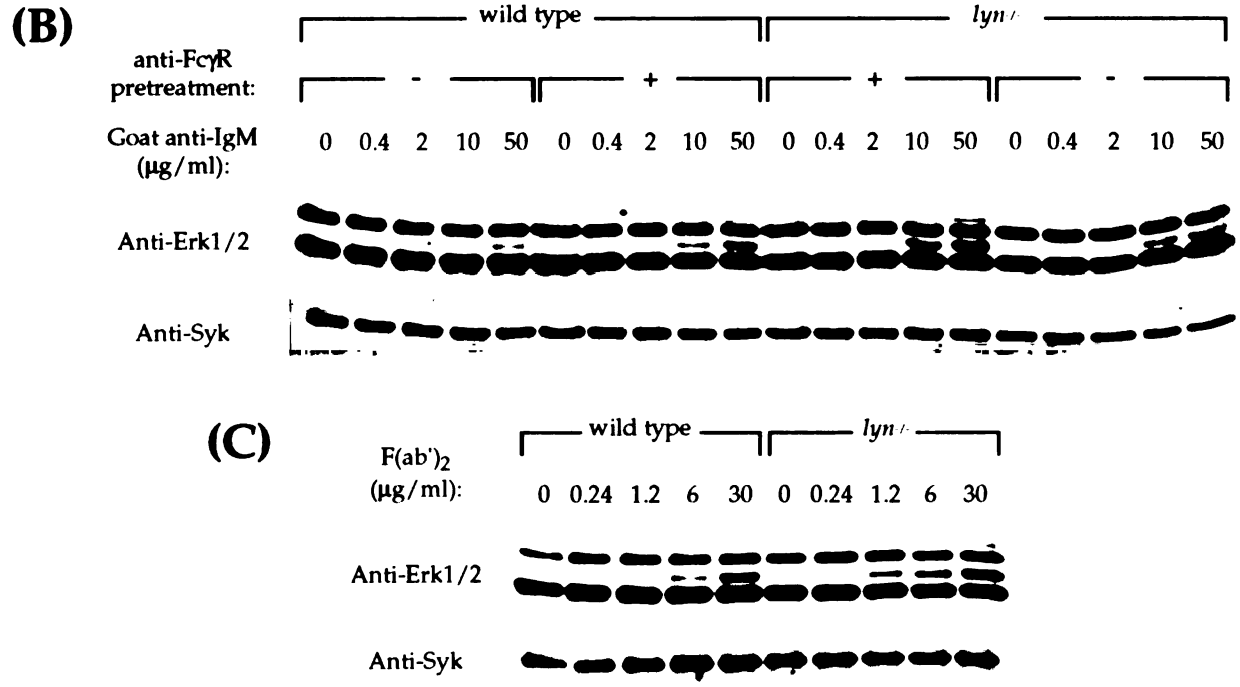
(A)


% BrdU+
80
60
40
20
0
0
0.1
1
10
100
Rabbit anti-IgM (µg/ml)


% BrdU+
80
60
40
20
0
0
0.1
1
10
100
Goat anti-IgM (µg/ml)


% BrdU+
80
60
40
20
0
0
0.1
1
10
100
Goat F(ab')2 anti-IgM (µg/ml)


wild type
lyn-/-
wild type + anti-FcγR
lyn-/- + anti-FcγR


(B)
wild type
lyn-/-
anti-FcγR pretreatment:
-
+
+
-
Goat anti-IgM (μg/ml):
0 0.4 2 10 50 0 0.4 2 10 50 0 0.4 2 10 50 0 0.4 2 10 50
Anti-Erk1/2
Anti-Syk
(C)
wild type
lyn-/-
F(ab')2 (μg/ml):
0 0.24 1.2 6 30 0 0.24 1.2 6 30
Anti-Erk1/2
Anti-Syk

Enhanced responses of *lyn*$^{-/-}$ B cells to anti-IgM in the presence and absence of Fcγ-receptor co-ligation.

The enhanced MAP kinase activation and proliferative response to anti-IgM suggested a defect in signal downregulation in *lyn*$^{-/-}$ B cells. One of the mechanisms by which the BCR signal is negatively regulated involves the receptor for the Fc region of IgG, FcγRIIb1 on B cells. When FcγRIIb1 is co-ligated with the BCR using intact rabbit anti-Ig antibodies, B cell proliferation (Phillips and Parker, 1983), phosphatidylinositol turnover (Bijsterbosch and Klaus, 1985) and transmembrane calcium influx (Choquet et al., 1993) are inhibited. To test whether the FcγRIIb1-mediated inhibition is defective in *lyn*$^{-/-}$ B cells, the proliferative responses of wild type and *lyn*$^{-/-}$ B cells to intact rabbit anti-IgM was examined (Figure 2.7A, top panel). Rabbit anti-IgM at doses above 1 μg/ml was clearly mitogenic to *lyn*$^{-/-}$ B cells (Figure 2.7A, open circles) while wild type B cells (solid circles) exhibited only a very weak proliferative response, indicating that Fcγ receptor-mediated inhibition is defective in *lyn*$^{-/-}$ B cells. To further examine this issue, cells were pretreated with a blocking monoclonal antibody against Fcγ receptors, 2.4G2, which prevents the co-ligation of these receptors when the B cells were stimulated with intact rabbit anti-IgM antibodies. As expected, wild type B cells exhibited a strong proliferative response to rabbit anti-IgM when the Fcγ receptor negative signal was blocked. *lyn*$^{-/-}$ B cells that were pretreated with the anti-Fcγ receptor antibody (open squares) proliferated better than untreated *lyn*$^{-/-}$ B cells (open circles), indicating that some negative signaling could still be mediated by the Fcγ receptor in *lyn*$^{-/-}$ B cells. Interestingly, rabbit anti-IgM induced proliferation remained higher in *lyn*$^{-/-}$ B cells than in wild type B cells, even when both cell types were pretreated with anti-Fcγ receptor antibody (open and solid squares respectively). This suggests

that inhibitory mechanisms other than that mediated by Fcγ receptors might also be defective in *lyn*$^{-/-}$ B cells.

A similar experiment was also carried out with intact goat anti-IgM antibodies (Figure 2.7A, middle panel). Goat antibodies interact with murine Fcγ receptors less well than do rabbit antibodies, but some Fcγ receptor inhibition of B cell proliferation was evident. Again, the proliferative response of *lyn*$^{-/-}$ B cells was greater than the response of wild type B cells even in the presence of anti-Fcγ receptor antibody. To rule out the possibility that this difference was due to an incomplete blockage of the Fcγ receptor by the antibody treatment, goat F(ab')$_2$ fragments of anti-IgM antibodies were used (Figure 2.7A, bottom panel). *lyn*$^{-/-}$ B cells were found to proliferate better than wild type B cells, even in the absence of Fcγ receptor involvement, indicating that other negative regulation mechanisms might be defective in *lyn*$^{-/-}$ B cells.

To test whether the enhanced proliferation observed upon Fcγ receptor blockage is accompanied by a corresponding increase in MAP kinase activation, purified splenic B cells were pretreated with the anti-Fcγ receptor antibody and stimulated with various concentrations of intact goat anti-IgM (Figure 2.7B). Anti-MAP kinase immunoblot revealed that anti-Fcγ receptor antibody pretreatment resulted in the appearance of greater amounts of slower-migrating Erk1 and Erk2 in both wild type and *lyn*$^{-/-}$ B cells. Thus, the activation of MAP kinases also appear to be negatively regulated by Fcγ receptor signaling. In agreement with our previous results (Figure 2.6), the amounts of the slower-migrating forms of MAP kinases were consistently higher in *lyn*$^{-/-}$ B cells as compared to wild type B cells when stimulated under identical conditions. In parallel with the enhanced proliferation of *lyn*$^{-/-}$ B cells in response to F(ab')$_2$ fragments of anti-IgM antibodies (Figure 2.7A, bottom panel), greater amounts of the slower-migrating forms of MAP kinases were also observed in *lyn*$^{-/-}$ B cells as

compared to wild type B cells. Taken together, the results in Figure 2.7 suggest that in addition to the partial defect in the inhibitory signals mediated by the Fcγ receptors, other negative regulation mechanisms may also be impaired by the *lyn*$^{-/-}$ mutation.

DISCUSSION

We and others have recently generated mice deficient in the Src-family tyrosine kinase Lyn. These mice exhibit defects in several different lineages of hematopoietic cells (Hibbs et al., 1995b and C.A.L., V.W.F.C, A.L.D. unpublished observations). In the work described here, we have focused on the characterization of the B cell population in *lyn*$^{-/-}$ mice. We and others have found that these mice have a reduced peripheral B cell population, but at the same time exhibit an accumulation of B cell blasts and serum autoantibodies. We have extended previous observations by demonstrating that the B cell blasts seen in older mice express CD5 and Mac-1 and therefore appear to be of the B1 lineage. Moreover, the conventional B cell population in *lyn*$^{-/-}$ mice has an increased proportion of less mature B cells and exhibits a higher turnover rate. To try to understand the possible causes of these *in vivo* B cell abnormalities, we have examined the signaling and biological responses to BCR stimulation of resting splenic conventional B cells. Our findings not only support the previously proposed role of Lyn in the initiation of BCR signaling reactions but also reveal an unexpected role of Lyn in signal downregulation.

Positive and negative roles of Lyn in BCR signaling

We observed a delayed time course for the BCR-induced tyrosine phosphorylation of some proteins in whole cell lysates, including Ig-α and Syk (Figure 2.4G). As the phosphorylation of Ig-α and Syk are postulated to be initial steps in BCR signaling, their delayed phosphorylation is consistent with the model that Lyn participates in the initiation of the BCR signaling cascade (DeFranco, 1995). Lyn appears to be the most highly expressed of the Src-family

tyrosine kinases in splenic B cells, so it is not surprising that its absence noticeably affects this step in BCR signaling. Although the induction of tyrosine phosphorylation of some proteins require a longer lag time in the absence of Lyn, the BCR signal can nevertheless be initiated, probably by other Src-family kinases that are still present in the *lyn*$^{-/-}$ cells. On the other hand, the observations that the activation of MAP kinases was enhanced (Figure 2.6) and that *lyn*$^{-/-}$ B cells exhibited a hypersensitive proliferative response to intact and F(ab')$_2$ fragments of anti-IgM antibodies suggested that Lyn might also participate in the downregulation of the BCR signal as a feedback mechanism. Thus, while other Src-family kinases might be able to compensate for Lyn in signal initiation, the role of Lyn in signal downregulation appears to be non-redundant.

The striking observation that co-ligation of FcγRIIb1 with the BCR by rabbit anti-IgM only slightly inhibited the proliferation of *lyn*$^{-/-}$ B cells indicates that the inhibitory signal through FcγRIIb1 is defective in the absence of Lyn (Figure 2.7A). However, since rabbit anti-IgM-induced proliferation of *lyn*$^{-/-}$ B cells was enhanced when Fcγ receptors were blocked, some negative signaling from FcγRIIb1 remains, although its effect is much less than in wild type cells. Moreover, when the involvement of FcγRIIb1 was avoided by stimulating the B cells with F(ab')$_2$ fragments of anti-IgM, *lyn*$^{-/-}$ B cells still responded better than wild type B cells, suggesting that another inhibitory pathway is also likely to be defective in *lyn*$^{-/-}$ B cells. One candidate for this additional inhibitory mechanism is CD22 since the prevention of CD22 co-aggregation with the BCR lowers the threshold for BCR-induced proliferation (Pezzutto et al., 1987, Doody et al., 1995) and B cells from CD22-deficient mice are hyperresponsive to BCR signaling (O'Keefe et al., 1996).

Proteins involved in BCR signal downregulation

The ability of coligated FcγRIIb1 to downregulate BCR signaling involves the phosphorylation of a tyrosine in the cytoplasmic domain of FcγRIIb1 (Muta et al., 1994). One attractive possibility is that Lyn may be important for carrying out this phosphorylation (Bewarder et al., 1996). The phosphorylated tyrosine of FcγRIIb1 serves as a binding site for the recently renamed SH2-containing protein tyrosine phosphatase, SHP-1 (D'Ambrosio et al., 1995, Adachi et al., 1996)]. Presumably SHP-1 then acts to dephosphorylate one or more key signaling components participating in BCR signaling. SHP-1 also binds to CD22 in an inducible fashion (Doody et al., 1995), which is consistent with the possibility that CD22 mediates the additional negative regulatory event that is absent in *lyn*$^{-/-}$ B cells.

The negative role of SHP-1 in regulating antigen receptor responses of B cells has been indicated by studies of B cells in motheaten (*me*) and viable motheaten (*me*^{v}) mice (Pani et al., 1995, Cyster and Goodnow, 1995b, D'Ambrosio et al., 1995), which have mutations in the gene encoding SHP-1. However, it should be noted that SHP-1 is not the only protein that has been implicated in the negative regulation by FcγRIIb1. Recent work indicates that the co-ligation of FcγRIIb1 by intact anti-Ig treatment of B cells resulted in the tyrosine phosphorylation of the phosphatidylinositol 5-phosphatase, p145^{ship} (Chacko et al., 1996) and its association with FcγRIIb1 (Ono et al., 1996). Tyrosine phosphorylated p145^{ship} also associates with Shc upon a wide variety of stimuli in many hematopoietic cell types (see references in Lioubin et al., 1996), including B cells stimulated through the BCR (Crowley et al., 1996). In anti-Ig-stimulated DT40, a chicken B cell line, Shc also coprecipitated with a 140 kD tyrosine phosphorylated protein (likely to be p145^{ship}), but this induced association was absent in *lyn*$^{-/-}$ DT40 cells

(Nagai et al., 1995). These results are intriguing since p145^{ship} has been implicated in negatively regulating cell growth when expressed in myeloid cells (Lioubin et al., 1996). Thus, it is possible that p145^{ship} is also a negative regulator of BCR-induced B cell proliferation and that this pathway is regulated by Lyn.

Enhanced activation of MAP kinase by anti-IgM in *lyn*$^{-/-}$ B cells

BCR signaling induces the tyrosine phosphorylation of Shc, and the subsequent formation of the Shc/Grb2/Sos complex is thought to mediate the activation of Ras and MAP kinase (Saxton et al., 1994, Kumar et al., 1995). In light of the delayed induction of tyrosine phosphorylation on Shc in *lyn*$^{-/-}$ cells (Figure 2.4G), it was surprising to find an enhanced activation of the MAP kinases (Figure 2.6). The reason for this discrepancy is not known. One possible explanation would be that the Shc/Grb2/Sos pathway may not be the sole mediator of BCR-induced MAP kinase activation in B cells. Indeed, the PKC agonist PMA has been shown in a human B cell line to activate the Raf-1/MEK-1 pathway without the induction of Shc tyrosine phosphorylation or Shc/Grb2 association (Kumar et al., 1995). Whether there is an enhanced activation of the PKC-dependent pathway leading to MAP kinase activation by anti-IgM in the *lyn*$^{-/-}$ B cells has yet to be determined.

Enhanced proliferation to anti-IgM by *lyn*$^{-/-}$ B cells

By measuring cell proliferation using two independent methods, we have demonstrated that *lyn*$^{-/-}$ B cells proliferate at lower doses of anti-IgM than wild type B cells (Figures 2.5 C, E). Doses of goat anti-IgM that resulted in the most dramatic differences in proliferation corresponded to anti-IgM doses at which

wild type B cells exhibited much lower MAP kinase activation than *lyn*$^{-/-}$ B cells (Figure 2.6A). In addition to the magnitude of MAP kinase activation, the duration of this response was also found to be greater in *lyn*$^{-/-}$ B cells (Figure 2.6B and data not shown). The duration of MAP kinase activation has been shown in other cell systems to be critical for cell fate decisions (Traverse et al., 1994). Therefore, it is tempting to speculate that the enhanced activation of MAP kinase in the *lyn*$^{-/-}$ B cells contributes to the hyperproliferative response to anti-IgM. It is also worth noting that the increased proliferation of *lyn*$^{-/-}$ B cells appears to be specific for BCR-signaling since the LPS dose response curves for wild type and *lyn*$^{-/-}$ B cell proliferation were very similar (Figure 2.5D, F).

In contrast to our findings presented here, others initially reported an impaired proliferative response of *lyn*$^{-/-}$ B cells to both anti-IgM and LPS treatment (Nishizumi et al., 1995, Hibbs et al., 1995), as well as a reduction in many early biochemical signaling responses to anti-IgM (Nishizumi et al., 1995). The methodologies by which we used to prepare cells (purified B cells or unpurified splenocytes) for our experiments were similar to those described by the other two groups. One exception is that when measuring proliferation by BrdU incorporation *in vitro*, we corrected for the difference in B cell numbers in the unpurified splenocyte populations from wild type and *lyn*$^{-/-}$ mice by using three-color flow cytometric analysis. The discrepancy between the initial published reports and our findings may be attributable to the fact that *lyn*$^{-/-}$ mice develop splenomegaly. We found that the accumulations of plasma cells and neutrophils, as well as a substantial number of hematopoietic progenitors in the enlarged spleens of older *lyn*$^{-/-}$ mice made it difficult to purify B cells without substantial numbers of contaminating B220^{-} cells. The extramedullary hematopoiesis and autoantibody production occurring in these mice might also be accompanied by environmental changes that could affect the responses of

their splenic B cells. Therefore, all our biochemical and proliferative experiments were performed with B cells from mice that were between 7 - 11 weeks old without any signs of splenomegaly. More recently, Wang et al. (Wang et al., 1996) reported that B cells purified from 4-8 week old *lyn*$^{-/-}$ mice have an enhanced proliferative response to anti-Ig, similar to what we observed. They also found that the proliferation of *lyn*$^{-/-}$ B cells is not inhibited by co-ligation of the FcγRIIb1 with the BCR, in agreement with our results.

Phenotypic similarities between motheaten mice and *lyn*$^{-/-}$ mice

The hyperproliferative response to anti-IgM and the impaired FcγRIIb1-mediated inhibition observed in *lyn*$^{-/-}$ B cells are reminiscent of properties of the B cells from *me* and *me*^{v} mice (Pani et al., 1995, D'Ambrosio et al., 1995). Some of the other phenotypes of the *me* and *me*^{v} mice, namely, the decrease in peripheral B cell numbers, the development of splenomegaly and neutrophilia, the appearance of autoantibodies and the accumulation of large numbers of plasma cells (Davidson et al., 1979, Shultz and Green, 1976, Green and Shultz, 1975) were also mirrored in *lyn*$^{-/-}$ mice (Table II; Hibbs et al., 1995b and C.A.L., V.W.F.C and A.L.D. unpublished results). Although the progression of the *lyn*$^{-/-}$ mice to the diseased stage is much slower than that seen in *me* or *me*^{v} mice, the striking phenotypic similarities and the signaling properties of their B cells suggest that Lyn and SHP-1 might lie along a common signaling pathway for signal downregulation.

From signaling properties to phenotype

In older *lyn*$^{-/-}$ mice, there is a marked expansion of activated B1 cells and

considerable production of autoantibodies. The B1 population in normal mice is generated from B cell precursors early in life and is maintained by self-renewal. This population exhibits an increase in the proportion of cells with autospecificity as animals age, possibly due to antigen-based selection (Hardy et al., 1994). Mutations in SHP-1, which has been implicated in negatively regulating BCR signaling, favors the development of large B1 cells in the *me* mice (Cyster and Goodnow, 1995b, Sidman et al., 1986). On the other hand, mutation of genes encoding signaling components involved in positively regulating the BCR signal, such as Syk, Btk, CD19 and Vav, leads to a loss of the B1 population (Turner et al., 1995, Cheng et al., 1995, Khan et al., 1995, Engel et al., 1995, Rickert et al., 1995, Zhang et al., 1995, Tarakhovsky et al., 1995). These observations suggest that certain BCR signaling events are required for the maturation or maintenance of B1 cells. Thus, one attractive hypothesis is that BCR signaling in the B1 cells of *lyn*$^{-/-}$ mice also causes enhanced MAP kinase activation and proliferation, similar to that observed for the splenic conventional B cells, leading to the activation and expansion of B1 cells in *lyn*$^{-/-}$ mice.

Although the early development of conventional B cells in *lyn*$^{-/-}$ mice occurs normally, subsequent maturation of immature B cells to enter the long-lived mature B cell population is adversely affected by the *lyn*$^{-/-}$ mutation (Figure 2.2, 2.3). Studies of the maturation of CD45$^{-/-}$ B cells carrying the anti-lysozyme Ig transgenes suggest that a low level of BCR signaling provides a positive signal that promotes B cell maturation (Cyster et al., 1996). As Lyn has been shown to be involved in many early BCR signaling events, it is attractive to postulate that the poor maturation of B cells in *lyn*$^{-/-}$ mice may reflect an impairment of a signal normally required for positive selection of B cells. However, in light of the enhanced MAP kinase signaling seen in *lyn*$^{-/-}$ B cells, it is also possible that a greater fraction of B cells are deleted or anergized in *lyn*$^{-/-}$ mice due to their

signaling above a certain threshold when they come into contact with normally non-tolerizing self antigens (Goodnow, 1996, Cyster and Goodnow, 1995a). Since anergic B cells have shorter half lives in the presence of competing B cells, this may account for the increased B cell turnover rate and the increased proportion of immature B cells observed in *lyn*$^{-/-}$ mice. Introduction of various Ig transgenes into the *lyn*$^{-/-}$ genetic background will enable the testing of these possibilities. In any case, it is already apparent that studies of *lyn*$^{-/-}$ mice will provide a number of insights into not only Lyn function, but also into the roles of BCR signaling in B cell maturation and activation.

Acknowledgments

I am grateful to Cliff Lowell for providing the *lyn*$^{-/-}$ mice used in this study and for performing the analyses of serum autoantibodies, as well as Fanying Meng for animal husbandry. I am also thankful to Tony Capobianco, Stacey Harmer, Jim Richards, Trish Roth and Steve Weinstein for critical reading of the manuscript and Jason Cyster and Julie Hambleton for helpful discussions.

MATERIALS AND METHODS

Antibodies and other reagents

Affinity purified anti-Lyn antibody that recognizes the N-terminal unique regions of both the p53 and p56 forms of Lyn has been described (Law et al., 1993). Anti-Shc antibody was affinity purified from rabbits immunized with human Shc expressed as a GST fusion protein (S. L. Harmer, M. T. Crowley and A. L. D., manuscript in preparation). This antibody crossreacts with murine Shc in immunoblots as well as immunoprecipitations. Affinity purified goat-anti-mouse IgM (whole IgG and $F(ab')_2$) and rabbit-anti-mouse IgM (whole IgG) were obtained from Jackson Immunological Research (West Grove, PA). LPS from *Salmonella minnesota* R595 was purchased from List Biological (Campbell, CA). Cultured supernatants from HO13.4 (anti-Thy1), 53.6.172 (anti-CD8), GK1.5 (anti-CD4), 2.4G2 (anti-FcγRII) were generated from hybridomas obtained from ATCC. Antibodies obtained from Pharmingen (San Diego, CA) included: biotinylated M1/69 (anti-HSA), PE-conjugated RA3-6B2 (anti-B220), PE-conjugated GL-1 (anti-B7-2) and PE-conjugated B7 (anti-B7-1). FITC-conjugated anti-BrdU was obtained from Becton-Dickinson. Avidin-TriColor and biotinylated anti-CD5 were from CalTag (South San Francisco, CA). Both the antibody used for immunoprecipitation of Erk2 (C-14) for MBP kinase assays and the antibody used for the detection of Erk1 and Erk2 (K-23) in western blots were from Santa Cruz Biotechnology (Santa Cruz, CA). BrdU was from Sigma Chemical. Tissue culture medium used in all experiments was RPMI 1640 supplemented with 5% FCS (Gibco/BRL), 2 mM pyruvate, 20 mM glutamine and 50 μM 2-mercaptoethanol.

Flow Cytometric Analysis

Analysis was performed on a FACScan® (Becton Dickinson) and the data acquired to a Macintosh Quadra running the CellQuest v2.1 software.

BrdU-labeling

In vivo labeling: BrdU was administered in the drinking water to 9-week old wild type and *lyn*$^{-/-}$ mice. The drinking water, which was protected from light, contained 0.25 mg/ml BrdU and 2.5% glucose and was changed every 3-4 days. Single cell suspensions from the bone marrow, spleen, axillary and mesenteric lymph nodes were depleted of RBCs and stained for HSA, B220 and BrdU as previously described (Hartley et al., 1993). The stained cells were analyzed on a FACScan®. Data collection for each sample was terminated when 20,000 events within the B220$^+$ gate were acquired. The acquired data were analyzed by gating on small lymphocytes by forward and side scatter as well as their positive staining for B220. These B220$^+$ small lymphocytes consist of two distinct populations of BrdU$^+$ and BrdU$^-$ cells. The data is presented as the percent of B220$^+$ lymphocytes that is BrdU$^+$.

In vitro labeling: splenocytes depleted of RBCs were plated in tissue culture medium at 0.5 x 10^6 cells per ml in the presence of various concentrations of anti-IgM or LPS. Cells were labeled with BrdU (10 μM) from 24-48 h and stained for B220 and BrdU at the end of the labeling period, following the same procedure as above. Data acquisition was terminated for each sample when 20,000 B220$^+$ cells were collected. Two distinct populations, one BrdU$^+$ and one BrdU$^-$, were observed among the B220$^+$ cells. The data is presented as the percent of B220$^+$ cells that is BrdU$^+$.

Purification of Splenic B Lymphocytes

Splenic B lymphocytes from 8-11 week old mice were prepared as described (DeFranco et al., 1982) except that anti-Thy1, anti-CD8 and anti-CD4 were used in combination for the complement lysis of T cells. Cells that were collected from the 50%/70% percoll interphase were washed and subsequently rested in tissue culture medium for 3 hours in 100 mm diameter tissue culture dishes (Corning 25020) prior to all stimulations. The purity of each of the splenic B cell preparations was verified by flow cytometric analysis, and was typically ≥84% $B220^+$ from *lyn*$^{-/-}$ mice and ≥93% from wild type mice.

In vitro ^{3}H-dT incorporation.

Purified splenic B lymphocytes (2.0×10^5 per 200 µl) were cultured in triplicates or quadruplicates in the presence of anti-IgM or LPS at various concentrations in tissue culture medium. The cells were pulsed with ^{3}H-dT (1 µCi/well) from 44-48 h, harvested onto glass fiber filters (Wallac, Gaithersburg, MD) and the amount of ^{3}H-dT incorporated was determined using the Betaplate™ reader (Wallac).

Surface expression of B7-2

RBC-depleted splenocytes (2×10^6) from 7-week old wild type and *lyn*-/- mice were cultured at 0.5×10^6 cells per ml in the presence of various concentrations of LPS or anti-IgM for 18 h. Cells were co-stained with B220 and B7-2 and analyzed by flow cytometric analysis. The B7-2-negative gate was defined by cells that were stained by a control antibody.

Immunoprecipitations and immunoblotting.

Purified splenic B lymphocytes were resuspended to 8 x 10^6 cells per ml of medium at 37°C. Control and stimulated cells were washed with ice cold PBS containing 1 mM Na_3VO_4, and lysed in ice cold TX/DC-lysis buffer (1% Triton X-100, 1% deoxycholate in 20 mM Tris (pH 8), 137 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin, 0.15 U/ml aprotinin) for 10-30 minutes. Cell lysates were cleared of detergent insoluble material by centrifugation at 15,000xg at 4°C for 15 minutes. The amount of soluble protein in whole cells lysates was quantitated by BCA assays (Pierce, Rockford, IL). For immunoblotting of whole cell lysates, equal amounts of lysates, as quantitated by BCA assays, were separated by 10% SDS-PAGE gels containing an acrylamide:bis-acrylamide ratio of 120:1 (Figure 2.4 A-F, 2.6A-B, 2.7B-C), transferred to nitrocellulose membranes and equivalent loading was further verified by Ponceau S staining of the membranes. For immunoprecipitations: equivalent amounts of cell lysates, as determined by BCA assays, were each added to 20 μl of packed protein A Sepharose for a 30 minute preclearing step at 4°C. The precleared lysates were added to a fresh aliquot of protein A Sepharose plus the immunoprecipitating antibody and incubated at 4°C for 1-2 hours. The protein A Sepharose beads were washed twice with TX/DC-lysis buffer, boiled in SDS sample buffer and proteins were separated on a 10% SDS-polyacrylamide (acrylamide:bisacrylamide = 37.5:1) (Figure 2.4G, 2.6C) and transferred to a nitrocellulose membrane. After blocking the membranes with 2% BSA/TBS, they were first probed with the monoclonal anti-phosphotyrosine antibody 4G10, washed with TBS + 0.05% Tween-20, incubated with HRP-conjugated sheep anti-mouse IgG, and developed using the Amersham ECL system. To verify equivalent loading, or to detect specific proteins, the nitrocellulose membranes

were stripped with 100 mM 2-mercaptoethanol/2% SDS/62.5 mM Tris pH 6.7 for 15 minutes at room temperature and blocked with 5% milk before the addition of specific primary antibodies. The nitrocellulose membranes were washed, probed with HRP-conjugated secondary antibodies and developed with ECL as above.

MAP kinase assay

Erk2 was immunoprecipitated from control or stimulated (1 min stimulation at 10 μg/ml anti-IgM, 37°C) splenic B cells using the C-14 antibody from Santa Cruz Biotechnology following procedures similar to that described in the preceding section. The immunoprecipitates were washed twice with TX/DC-lysis buffer and then once with kinase assay buffer (30 mM β-glycerol phosphate, 20 mM HEPES, pH 7.2, 20 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT and 1 mM Na_3VO_4). The kinase reaction was initiated by adding 25 μl of kinase assay buffer containing 25 μg of MBP as substrate with 50 μM ^{32}P-ATP and incubated at 30°C for 15 minutes. The reaction was terminated by boiling in SDS sample buffer and the proteins were resolved on 12% SDS-PAGE (acrylamide:bisacrylamide = 37.5:1), dried on Whatman paper and exposed to film. Quantitation was achieved by directly exposing the dried gel to a phosphor screen and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Autoantibodies

Autoantibodies directed against anti-nuclear antigens (ANA) or anti-double stranded DNA (anti-dsDNA) were detected in the serum of animals by immunofluorescence or ELISA assay, respectively. For ANA titers, serum was

diluted 1/500 in PBS and incubated with slides containing fixed HEp-2 cells (Sanofi Diagnostics) for 30 min at room temperature. Slides were washed 3 times in PBS and autoantibodies were detected by incubation with FITC-labeled rabbit anti-mouse Ig. Nuclear staining was considered positive only if it was present in a diffuse/homogenous pattern (with or without rim staining). Speckled nuclear staining was scored negative. Anti-dsDNA autoantibodies were detected by ELISA assay using the Bindazyme kit (Binding Site) as described by the manufacturer. Mouse sera was diluted 1/1000 in PBS, incubated with the dsDNA coated wells and binding was quantitated using HRP-labeled anti-mouse IgM or IgG. All ELISA's were done in triplicate. Data is reported as A_{450} after 30 min of color development. Wild type mice showed no anti-dsDNA autoantibodies at any age.

CONCLUDING REMARKS

The experiments described here suggest two ways in which Lyn participates in BCR signaling. The ability of Lyn to phosphorylate Ig-α *in vitro*, the enhanced association of Lyn with the BCR complex upon stimulation and the slight delay in the induction of protein tyrosine phosphorylations in Lyn-deficient B cells are all consistent with the conclusion that Lyn participates in the initiation of the BCR signal upon receptor crosslinking. Based on the biochemical characterizations of the B cells from *lyn*$^{-/-}$ mice, it also appears that the absence of Lyn causes defects in at least two mechanisms leading to the downregulation of the BCR signal, one of which involves FcγRIIb1. Moreover, many of the phenotypes observed in the Lyn-deficient mice resemble those of CD22-deficient mice as well as that of SHP-1 deficient mice (O'Keefe et al., 1996, Davidson et al., 1979, Shultz and Green, 1976, Green and Shultz, 1975, Sidman et al., 1986). These similarities are consistent with Lyn participating in the negative regulation of the BCR signal (See Table I on page 12). An attractive hypothesis is that proteins such as FcγRIIb1, CD22 and SHP-1 possess or bind to regulatory tyrosine residues that requires the phosphorylation by Lyn in order for them to exert their inhibitory effects on BCR signaling. Experiments are currently underway to directly test this hypothesis.

The BCR signaling pathway may not be the only pathway that is affected by the absence of Lyn in B cells. Although *lyn*$^{-/-}$ B cells responded normally to LPS, they do not appear to respond to crosslinking of the recently described B cell-specific protein, RP-105. RP-105 is a transmembrane protein that was identified by virtue of the ability of anti-RP-105 monoclonal antibodies to rescue B cells from radiation-induced apoptosis (Miyake et al., 1994). Unirradiated splenic B cells exhibit a proliferative response to anti-RP-105 (Miyake et al., 1994,

Yamashita et al., 1996) and this was not observed in B cells from *lyn*$^{-/-}$ mice (A. Tarakhovsky, personal communication). Our preliminary results indicate that anti-RP-105 treatment triggers the activation of MAP kinase and JNK in wild type B cells. Current efforts are focused on testing whether the activation of these kinases might be defective in *lyn*$^{-/-}$ B cells.

The effects of the *lyn*$^{-/-}$ mutation on mice are not limited to B lineage cells. The spleens of aged *lyn*$^{-/-}$ mice contain increased numbers of mature neutrophils as well as myeloid and erythroid precursors. These observations suggest that the development of those cell types is also affected in the absence of Lyn. By performing colony-forming assays, substantial numbers of myeloid and erythroid precursors were also found in the peripheral blood from *lyn*$^{-/-}$ mice, suggesting that there might be an adhesion or homing defect of these precursor cells (C. Lowell, unpublished results).

Lyn is also highly expressed in macrophages. The macrophages from *lyn*$^{-/-}$ mice appear to be hyper-responsive to stimuli such as LPS and cytokines (F. Meng, unpublished results), reminiscent of the hyper-proliferative response of *lyn*$^{-/-}$ B cells to anti-Ig. The molecular mechanism by which Lyn might mediate an inhibitory effect on these signaling pathways in the macrophages is less well understood, but it is intriguing to note that macrophages from mice lacking all three of the Src-family kinases present in macrophages, Lyn, Hck and Fgr, exhibit an even more dramatic hyper-responsiveness to LPS and cytokines (F. Meng, unpublished results). Mutations in Hck and Fgr, alone or in combination, did not cause such hyper-responsiveness (Lowell et al., 1994). Taken together, these results suggest that the absence of Lyn is primarily responsible for the hyper-responsiveness of the *lyn*$^{-/-}$*hck*$^{-/-}$*fgr*$^{-/-}$ macrophages, although the additional deficiencies in Hck and Fgr exacerbated the effect of the *lyn*$^{-/-}$ mutation.

Experiments described in Chapter II revealed that the absence of Lyn leads to hyper-responsiveness to anti-Ig treatment in B cells. Similar effects were not observed when the other Src-family tyrosine kinases in B cells, Blk, Fyn and Fgr, were mutated (S. Tarakhovsky, personal communication, Sillman and Monroe, 1994, Lowell et al., 1994) . It is likely that mutations of Blk, Fyn or Fgr in combination with the *lyn*$^{-/-}$ mutation will have more dramatic effects than the *lyn*$^{-/-}$ mutation alone. Therefore, it will be interesting to perform experiments similar to those described here with B cells from the double and triple mutants once they become available.

Experiments in this thesis demonstrated that Lyn has both positive and negative roles in BCR signaling, an aspect that was previously unappreciated of the Src-family tyrosine kinases. Part of the current challenge is to understand how the B cell abnormalities in the Lyn-deficient mice arise, based on what we have learnt about the signaling properties of their B cells. One approach would be to utilize the transgenic model systems for tolerance, which have been used to elucidate the positive and negative roles of tyrosine phosphatases in B cell signaling (Cyster et al., 1996, Cyster and Goodnow, 1995b). Future experiments will likely lead to further insights into the role of Src-family tyrosine kinases in B cell development and function.

REFERENCES

Adachi, M., Fischer, E. H., Ihle, J., Imai, K., Jirik, F., Neel, B., Pawson, T., Shen, S.-H., Thomas, M., Ullrich, A., and Zhao, Z. (1996). Mammalian SH2-containing protein tyrosine phosphatases. Cell *85*, 15.

Allman, D. M., Ferguson, S. E., Lentz, V. M., and Cancro, M. P. (1993). Peripheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immune developmental intermediate in the production of long-lived marrow-derived B cells. J. Immunol. *151*, 4431-44.

Aoki, Y., Isselbacher, K. J., and Pillai, S. (1994). Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligated B cells. Proc. Natl. Acad. Sci. USA *91*, 10606-9.

Appleby, M. W., Kerner, J. D., Chien, S., Maliszewski, C. R., Bondadaa, S., and Perlmutter, R. M. (1995). Involvement of $p59^{fynT}$ in interleukin-5 receptor signaling. J. Exp. Med. *182*, 811-20.

Beckwith, M., Fenton, R. G., Katona, I. M., and Longo, D. L. (1996). Phosphatidylinositol-3-kinase activity is required for the anti-ig-mediated growth inhibition of a human B-lymphoma cell line. Blood *87*, 202-10.

Bewarder, N., Weinrich, V., Budde, P., Hartmann, D., Flaswinkel, H., Reth, M., and Frey, J. (1996). In vivo and in vitro specificity of protein tyrosine kinases for immunoglobulin G receptor (FcγRII) phosphorylation. Mol. Cell. Biol. *16*, 4735-43.

Bijsterbosch, M. K., and Klaus, G. G. (1985). Crosslinking of surface immunoglobulin and Fc receptors on B lymphocytes inhibits stimulation of inositol phospholipid breakdown via the antigen receptors. J. Exp. Med. *162*, 1825-36.

Blum, J. H., Stevens, T. L., and DeFranco, A. L. (1993). Role of the mu immunoglobulin heavy chain transmembrane and cytoplasmic domains in B

cell antigen receptor expression and signal transduction. J. Biol. Chem. *268*, 27236-45.

Bole, D. G., Hendershot, L. M., and Kearney, J. F. (1986). Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. *102*, 1558-66.

Bolen, J. B., Rowley, R. B., Spana, C., and Tsygankov, A. Y. (1992). The Src family of tyrosine protein kinases in hemopoietic signal transduction. Faseb J. *6*, 3403-9.

Bradbury, L., Kansas, G. S., Levy, S., Evans, R. L., and Tedder, T. F. (1992). CD19 is a component of a signal transducing complex on the surface of B cells that includes CD21, TAPA-1 and Leu-13. J. Immunol. *149*, 2841-50.

Brunswick, M., Samelson, L. E., and Mond, J. J. (1991). Surface immunoglobulin crosslinking activates a tyrosine kinase pathway in B cells that is independent of protein kinase C. Proc. Natl. Acad. Sci. USA *88*, 1311-4.

Burg, D. L., Furlong, M. T., Harrison, M. L., and Geahlen, R. L. (1994). Interactions of Lyn with the antigen receptor during B cell activation. J. Biol. Chem. *269*, 28136-42.

Burkhardt, A. L., Brunswick, M., Bolen, J. B., and Mond, J. J. (1991). Anti-immunoglobulin stimulation of B lympocytes activates src-related protein-tyrosine kinases. Proc. Natl. Acad. Sci. USA *88*, 7410-4.

Bustelo, X. R., and Barbacid, M. (1992). Tyrosine phosphorylation of the vav proto-oncogene product in activated B cells. Science *256*, 1196-9.

Byth, K. F., Conroy, L. A., Howlett, S., Smith, A. J. H., May, J., Alexander, D. R., and Holmes, N. (1996). CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of $CD4^+CD8^+$ thymocytes, and in B cell maturation. J. Exp. Med. *183*, 1707-18.

Campbell, M.-A., and Sefton, B. M. (1990). Protein tyrosine phosphorylation is induced in murine B lymphocytes in response to stimulation with anti-immunoglobulin. EMBO J. *9*, 2125-31.

Campbell, M.-A., and Sefton, B. M. (1992). Association between B-lymphocyte membrane immunoglobulin and multiple members of the Src family of protein tyrosine kinases. Mol. Cell. Biol. *12*, 2315-21.

Carsetti, R., Kohler, G., and Lamers, M. C. (1995). Transitional B cells are the target of negative selection in the B cell compartment. J. Exp. Med. *181*, 2129-40.

Carter, R. H., and Fearon, D. T. (1992). CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. Science *256*, 105-7.

Carter, R. H., Park, D. J., Rhee, S. G., and Fearon, D. T. (1991). Tyrosine phosphorylation of phospholipase C induced by membrane immunoglobulin in B lymphocytes. Proc. Natl. Acad. Sci. USA *88*, 2745-9.

Chacko, G. W., Tridandapani, S., Damen, J. E., Liu, L., Krystal, G., and Coggeshall, K. M. (1996). Negative signaling in B lymphocytes induces tyrosine phosphorylation of the 145-kDa inositol polyphosphate 5-phosphatase, SHIP. J. Immunol. *157*, 2234-8.

Chalupny, N. J., Aruffo, A., Esselstyn, J. M., Chan, P. Y., Bajorath, J., Blake, J., Gilliland, L. K., Ledbetter, J. A., and Tepper, M. A. (1995). Specific binding of Fyn and phosphatidylinositol 3-kinase to the B cell surface glycoprotein CD19 through their src homology 2 domains. Eur. J. Immunol. *25*, 2978-84.

Chalupny, N. J., Kanner, S. B., Schieven, G. L., Wee, S. F., Gilliland, L. K., Aruffo, A., and Ledbetter, J. A. (1993). Tyrosine phosphorylation of CD19 in pre-B and mature B cells. EMBO J. *12*, 2691-6.

Cheng, A. M., Rowley, B., Pao, W., Hayday, A., Bolen, J. B., and Pawson, T. (1995). Syk tyrosine kinase required for mouse viability and B-cell development. Nature *378*, 303-6.

Choquet, D., Partiseti, M., Amigorena, S., Bonnerot, C., Fridman, W. H., and Korn, H. (1993). Cross-linking of IgG receptors inhibits membrane immunoglobulin-stimulated calcium influx in B lymphocytes. J. Cell Biol. *121*, 355-63.

Clark, M. R., Johnson, S. A., and Cambier, J. C. (1994). Analysis of Ig-alpha-tyrosine kinase interaction reveals two levels of binding specificity and tyrosine phosphorylated Ig-alpha stimulation of Fyn activity. EMBO J. *13*, 1911-9.

Coggeshall, K. M., McHugh, J. C., and Altman, A. (1992). Predominant expression and activation-induced tyrosine phosphorylation of phospholipase C-γ2 in B lymphocytes. Proc. Natl. Acad. Sci. USA *89*, 5660-4.

Crowley, M. T., Harmer, S. L., and DeFranco, A. L. (1996). Activation-induced association of a 145-kDa tyrosine-phosphorylated protein with Shc and Syk in B lymphocytes and macrophages. J. Biol. Chem. *271*, 1145-52.

Cyster, J. G., and Goodnow, C. C. (1995a). Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. Immunity *3*, 691-701.

Cyster, J. G., and Goodnow, C. C. (1995b). Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. Immunity *2*, 13-24.

Cyster, J. G., Healy, J. I., Kishihara, K., Mak, T. W., Thomas, M. L., and Goodnow, C. G. (1996). Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. Nature *381*, 325-8.

D'Ambrosio, D., Hippen, K. H., Minskoff, S. A., Mellman, I., Pani, G., Siminovitch, K. A., and Cambier, J. C. (1995). Recruitment and activation of PTP1C in negative regulation of antigen receptor signaling by FcγRIIB1. Science *268*, 293-6.

Davidson, W. F., Morse III, H. C., Sharrow, S. O., and Chused, T. M. (1979). Phenotypic and functional effects of the motheaten gene on murine B and T lymphocytes. J. Immunol. *122*, 884-91.

de Weers, M., Brouns, G. S., Hinshelwood, S., Kinnon, C., Schuurman, R. K., Hendriks, R. W., and Borst, J. (1994). B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. J. Biol. Chem. *269*, 23857-60.

DeFranco, A. (1995). Transmembrane signaling by antigen receptors of B and T lymphocytes. Curr. Opin. Cell Biol. *7*, 163-75.

DeFranco, A. L. (1993). Signaling pathways activated by protein tyrosine phosphorylation in lymphocytes. Curr. Opin. Immunol. *6*, 364-71.

DeFranco, A. L., Raveche, E. S., Asofsky, R., and Paul, W. E. (1982). Frequency of B lymphocytes responsive to anti-immunoglobulin. J. Exp. Med. *155*, 1523-36.

Dempsey, P. W., Allison, M. E. D., Akkaraju, S., Goodnow, C. C., and Fearon, D. T. (1996). C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science *271*, 348-50.

Doody, G. M., Justement, L. B., Delibrias, C. C., Matthews, R. J., Lin, J., Thomas, M. L., and Fearon, D. T. (1995). A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. Science *269*, 242-4.

Engel, P., Zhou, L. J., Ord, D. C., Sato, S., Koller, B., and Tedder, T. F. (1995). Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. Immunity *3*, 39-50.

Fahey, K. A., and DeFranco, A. L. (1987). Cross-linking membrane IgM induces production of inositol trisphosphate and inositol tetrakisphosphate in WEHI-231 B lymphoma cells. J. Immunol. *138*, 3935-42.

Flaswinkel, H., and Reth, M. (1994). Dual role of the tyrosine activation motif of the Ig-alpha protein during signal transduction via the B cell antigen receptor. EMBO J. *13*, 83-9.

Gold, M. R., and Aebersold, R. (1994). Both phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase products are increased by antigen receptor signaling in B cells. J. Immunol. *152*, 42-50.

Gold, M. R., Chan, V. W. F., Turck, C. W., and DeFranco, A. L. (1992a). Membrane Ig cross-linking regulates phosphatidylinositol 3-kinase in B lymphocytes. J. Immunol. *148*, 2012-22.

Gold, M. R., Crowley, M. T., Martin, G. A., McCormick, F., and DeFranco, A. L. (1993). Targets of B lymphocyte antigen receptor signal transduction include the $p21^{ras}$ GTPase-activating protein (GAP) and two GAP-associated proteins. J. Immunol. *150*, 377-86.

Gold, M. R., Law, D. A., and DeFranco, A. L. (1990). Stimulation of protein tyrosine phosphorylation by the B-lymphocyte antigen receptor. Nature *345*, 810-3.

Gold, M. R., Matsuuchi, L., Kelly, R. B., and DeFranco, A. L. (1991). Tyrosine phosphorylation of components of the B cell antigen receptors following receptor crosslinking. Proc. Natl. Acad. Sci. USA *88*, 3436-40.

Gold, M. R., Sanghera, J. S., Stewart, J., and Pelech, S. L. (1992b). Selective activation of p42 MAP kinase in murine B lymphoma cell lines by membrane immunoglobulin crosslinking. Evidence for protein kinase C-independent and -dependent mechanisms of activation. Biochem. J. *286*, 269-76.

Goodnow, C. C. (1996). Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. Proc. Natl. Acad. Sci. USA *93*, 2264-71.

Goodnow, C. C., Cyster, J. G., Hartley, S. B., Bell, S. E., Cooke, M. P., Healy, J. I., Akkaraju, S., Rathmell, J. C., Progue, S. L., and Shokat, K. P. (1995). Self-

tolerance checkpoints in B lymphocyte development. Adv. Immunol. *59*, 279-368.

Gordon, J. A. (1991). Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. Meth. Enzymol. *201*, 477-82.

Green, M. C., and Shultz, L. D. (1975). Motheaten, an immunodeficient mutant of the mouse. I. Genetics and pathology. J. Hered. *66*, 250-8.

Hardy, R. R., Carmack, C. E., Li, Y. S., and Hayakawa, K. (1994). Distinctive developmental origins and specificities of murine $CD5^+$ B cells. Immunol. Rev. *137*, 91-118.

Hartley, S. B., Cooke, M. P., Fulcher, D. A., Harris, A. W., Cory, S., Basten, A., and Goodnow, C. C. (1993). Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. Cell *72*, 325-35.

Harwood, A. E., and Cambier, J. C. (1993). B cell antigen receptor cross-linking triggers rapid protein kinase C independent activation of $p21^{ras1}$. J. Immunol. *151*, 4513-22.

Hathcock, K. S., Laszlo, G., Pucillo, C., P, P. L., and Hodes, R. J. (1994). Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. J. Exp. Med. *180*, 631-40.

Hempel, W. M., Schatzman, R. C., and DeFranco, A. L. (1992). Tyrosine phosphorylation of phospholipase C-γ2 upon cross-linking of membrane Ig on murine B lymphocytes. J. Immunol. *148*, 3021-7.

Hibbs, M. L., Tarlinton, D. M., Armes, J., Grail, D., Hodgson, G., Maglitto, R., Stacker, S. A., and Dunn, A. R. (1995). Multiple defects in the immune system of Lyn-deficient mice culminating in autoimmune disease. Cell *83*, 301-11.

Hutchcroft, J. E., Harrison, M. L., and Geahlen, R. L. (1992). Association of the 72-kDa protein-tyrosine kinase PTK72 with the B cell antigen receptor. J. Biol. Chem. *267*, 8613-9.

Jackson, S. P., Schoenwaelder, S. M., Matzaris, M., Brown, S., and Mitchell, C. A. (1995). Phosphatidylinositol 3,4,5-trisphosphate is a substrate for the 75 kDa inositol polyphosphate 5-phosphatase and a novel 5-phosphatase which forms a complex with the p85/p110 form of phosphoinositide 3-kinase. EMBO J. *14*, 4490-500.

Johnson, S. A., Pleiman, C. M., Pao, L., Schneringer, J., Hippen, K., and Cambier, J. C. (1995). Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. J. Immunol. *155*, 4596-603.

Justement, L. B., Campbell, K. S., Chien, N. C., and Cambier, J. C. (1991). Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. Science *252*, 1839-42.

Kawauchi, K., Lazarus, A. H., Rapoport, M. J., Harwood, A., Cambier, J. C., and Delovitch, T. L. (1994). Tyrosine kinase and CD45 tyrosine phosphatase activity mediate $p21^{ras}$ activation in B cells stimulated through the antigen receptor. J. Immunol. *152*, 3306-16.

Khan, W. N., Alt, F. W., Gerstein, R. M., Malynn, B. A., Larsson, I., Rathbun, G., Davidson, L., Muller, S., Kantor, A. B., Herzenberg, L. A., Rosen, F. S., and Sideras, P. (1995). Defective B cell development and function in Btk-deficient mice. Immunity *3*, 283-99.

Kim, K. M., Alber, G., Weiser, P., and Reth, M. (1993). Differential signaling through the Ig-alpha and Ig-beta components of the B cell antigen receptor. Eur. J. Immunol. *23*, 911-6.

Kumar, G., Wang, S., Gupta, S., and Nel, A. (1995). The membrane immunoglobulin receptor utilizes a Shc/Grb2/hSOS complex for activation of the mitogen-activated protein kinase cascade in a B-cell line. Biochem. J. *307*, 215-23.

Kurosaki, T., Johnson, S. A., Pao, L., Sada, K., Yamamura, H., and Cambier, J. C. (1995). Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. J. Exp. Med. *182*, 1815-23.

Kurosaki, T., Takata, M., Yamanashi, Y., Inazu, T., Taniguchi, T., Yamamoto, Y., and Yamamura, H. (1994). Syk activation by the Src-family tyrosine kinase in the B cell receptor signaling. J. Exp. Med. *179*, 1725-9.

LaBaer, J., Tsien, R. Y., Fahey, K. A., and DeFranco, A. L. (1986). Stimulation of the antigen receptor on WEHI-231 B lymphoma cells results in a voltage-independent increase in cytoplasmic calcium. J. Immunol. *137*, 1836-44.

Lane, P. J. L., Ledbetter, J. A., McConnell, F. M., Draves, K., Deans, J., Schieven, G. L., and Clark, E. A. (1991). The role of tyrosine phosphorylation in signal transduction through surface Ig in human B cells. J. Immunol. *146*, 715-22.

Law, C. L., Sidorenko, S. P., Chandran, K. A., Zhao, Z., Shen, S. H., Fischer, E. H., and Clark, E. A. (1996). CD22 associates with protein tyrosine phosphatase 1C, Syk, and phospholipase C-γ1 upon B cell activation. J. Exp. Med. *183*, 547-60.

Law, D. A., Chan, V. W. F., Datta, S. K., and DeFranco, A. L. (1993). B-cell antigen receptor motifs have redundant signalling capabilities and bind the tyrosine kinases PTK72, Lyn and Fyn. Curr. Biol. *3*, 645-57.

Law, D. A., Gold, M. R., and DeFranco, A. L. (1992). Examination of B-lympoid cell lines for membrane immunoglobulin-stimulated tyrosine phosphorylation and src-family tyrosine kinase messanger RNA expression. Mol. Immunol. *29*, 917-26.

Lazarus, A. H., Kawauchi, K., Rapoport, M. J., and Delovitch, T. L. (1993). Antigen-induced B lymphocyte activation involves the $p21^{ras}$ and RasGAP signaling pathway. J. Exp. Med. *178*, 1765-9.

Lenschow, D. J., Sperling, A. I., Cooke, M. P., Freeman, G., Rhee, L., Decker, D. C., Gray, G., Nadler, L. M., Goodnow, C. C., and Bluestone, J. A. (1994).

Differential up-regulation of the B7-1 and B7-2 costimulatory molecules after Ig receptor engagement by antigen. J. Immunol. *153*, 1990-7.

Lenschow, D. J., Su, G. H., Zuckerman, L. A., Nabavi, N., Jellis, C. L., Gray, G. S., Miller, J., and Bluestone, J. A. (1993). Expression and functional significance of an additional ligand for CTLA-4. Proc. Natl. Acad. Sci. USA *90*, 11054-8.

Leprince, C., Draves, K. E., Geahlen, R. L., Ledbetter, J. A., and Clark, E. A. (1993). CD22 associates with the human surface IgM-B-cell antigen receptor complex. Proc. Natl. Acad. Sci. USA *90*, 3236-40.

Leprince, C., Draves, K. E., Ledbetter, J. A., Torres, R. M., and Clark, E. A. (1992). Characterization of the molecular components associated with surface immunoglobulin M in human B lymphocytes: presence of tyrosine and serine/threonine protein kinases. Eur. J. Immunol. *22*, 2093-9.

Li, Z.-H., Mahajan, S., Prendergast, M. M., Fargnoli, J., Zhu, Z., Klages, S., Adam, D., Schieven, G. L., Blake, J., Bolen, J. B., and Burkhardt, A. L. (1992). Cross-linking of surface immunoglobulin activates src-related tyrosine kinases in WEHI 231 cells. Biochem. Biophys. Res. Comm. *187*, 1536-44.

Lin, J., and Justement, L. B. (1992). The MB-1/B29 heterodimer couples the B cell antigen receptor to multiple src family protein tyrosine kinases. J. Immunol. *149*, 1548-55.

Lioubin, M. N., Algate, P. A., Tsai, S., Carlberg, K., Aebersold, A., and Rohrschneider, L. R. (1996). p150^{ship}, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. Genes Dev. *10*, 1084-95.

Lowell, C. A., Soriano, P., and Varmus, H. E. (1994). Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. Genes Dev. *8*, 387-98.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179-85.

Maruta, H., and Burgess, A. W. (1994). Regulation of the Ras signalling network. Bioessays *16*, 489-96.

Matsuuchi, L., Gold, M. R., Travis, A., Grosschedl, R., DeFranco, A. L., and Kelly, R. B. (1992). The membrane IgM-associated proteins MB-1 and Ig-beta are sufficient to promote surface expression of a partially functional B-cell antigen receptor in a nonlymphoid cell line. Proc. Natl. Acad. Sci. USA *89*, 3404-8.

Miyake, K., Yamashita, Y., Hitoshi, Y., Takatsu, K., and Kimoto, M. (1994). Murine B cell proliferation and protection from apoptosis with an antibody against a 105-kD molecule: unresponsiveness of X-linked immunodeficient B cells. J. Exp. Med. *180*, 1217-24.

Mizuguchi, J., Yamanashi, Y., Ehara, K., Tamura, T., Nariuchi, H., Gyotoku, Y., Fukazawa, H., Uehara, Y., and Yamamoto, T. (1992). Tyrosine protein kinase is involved in anti-IgM-mediated signaling in BAL17 B lymphoma cells. J. Immunol. *148*, 689-94.

Mustelin, T., and Burn, P. (1993). Regulation of src family tyrosine kinases in lymphocytes. Trends Biochem. Sci. *18*, 215-20.

Muta, T., Kurosaki, T., Misulovin, Z., Sanchez, M., Nussenzweig, M. C., and Ravetch, J. V. (1994). A 13-amino-acid motif in the cytoplasmic domain of FcγRIIB modulates B-cell receptor signalling. Nature *368*, 70-3.

Nagai, K., Takata, M., Yamamura, H., and Kurosaki, T. (1995). Tyrosine phophorylation of Shc is mediated through Lyn and Syk in B cell receptor signaling. J. Biol. Chem. *270*, 6824-9.

Nishizumi, H., Taniuchi, I., Yamanashi, Y., Kitamura, D., Ilic, D., Mori, S., Watanabe, T., and Yamamoto, T. (1995). Impaired proliferation of peripheral B cells and indication of autoimmune disease in *lyn*-deficient mice. Immunity *3*, 549-60.

O'Keefe, T. L., Williams, G. T., Davies, S. L., and Neuberger, M. S. (1996). Hyperresponsive B Cells in CD22-deficient mice. Science *274*, 798-801.

Ono, M., Bolland, S., Tempst, P., and Ravetch, J. V. (1996). Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor FcγRIIB. Nature *383*, 263-6.

Page, D. M., and DeFranco, A. L. (1988). Role of phosphoinositide-derived second messengers in mediating anti-IgM-induced growth arrest of WEHI-231 B lymphoma cells. J. Immunol. *140*, 3717-26.

Page, D. M., Gold, M. R., Fahey, K. A., Matsuuchi, L., and DeFranco, A. L. (1991). Mutational analysis of antigen receptor regulation of B lymphocyte growth. Evidence for involvement of the phosphoinositide signaling pathway. J. Biol. Chem. *266*, 5563-74.

Pani, G., Kozlowski, M., Cambier, J. C., Mills, G. B., and Siminovitch, K. A. (1995). Identification of the tyrosine phosphatase PTP1C as a B cell antigen receptor-associated protein involved in the regulation of B cell signaling. J. Exp. Med. *181*, 2077-84.

Peaker, C. J., and Neuberger, M. S. (1993). Association of CD22 with the B cell antigen receptor. Eur. J. Immunol. *23*, 1358-63.

Pesando, J. M., Bouchard, L. S., and McMaster, B. E. (1989). CD19 is functionally and physically associated with surface immunoglobulin. J. Exp. Med. *170*, 2159-64.

Pezzutto, A., Dorken, B., Moldenhauer, G., and Clark, E. A. (1987). Amplification of human B cell activation by a monoclonal antibody to the B cell-specific antigen CD22, Bp 130/140. J. Immunol. *138*, 98-103.

Phillips, N. E., and Parker, D. C. (1983). Fc-dependent inhibition of mouse B cell activation by whole anti-mu antibodies. J. Immunol. *130*, 602-6.

Pleiman, C. M., Abrams, C., Gauen, L. T., Bedzyk, W., Jongstra, J., Shaw, A. S., and Cambier, J. C. (1994a). Distinct p53/56^{lyn} and p59^{fyn} domains associate with nonphosphorylated and phosphorylated Ig-alpha. Proc. Natl. Acad. Sci. USA *91*, 4268-72.

Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994b). Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. Science *263*, 1609-12.

Rajewsky, K. (1996). Clonal selection and learning in the antibody system. Nature *381*, 751-8.

Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S., Kato, R. M., Fluckiger, A. C., Witte, O. N., and Kinet, J. P. (1996). Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. Science *271*, 822-5.

Reth, M. (1992). Antigen receptors on B lymphocytes. Ann. Rev. Immunol. *10*, 97-121.

Reth, M. (1994). B cell antigen receptors. Curr. Opin. Immunol. *6*, 3-8.

Richards, J. D., Gold, M. R., Hourihane, S. L., DeFranco, A. L., and Matsuuchi, L. (1996). Reconstitution of B cell antigen receptor-induced signaling events in a nonlymphoid cell line by expressing the Syk protein-tyrosine kinase. J. Biol. Chem. *271*, 6458-66.

Rickert, R. C., Rajewsky, K., and Roes, J. (1995). Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. Nature *376*, 352-5.

Rowley, R. B., Burkhardt, A. L., Chao, H. G., Matsueda, G. R., and Bolen, J. B. (1995). Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Ig alpha/Ig beta immunoreceptor tyrosine activation motif binding and autophosphorylation. J. Biol. Chem. *270*, 11590-4.

Sanchez, M., Misulovin, Z., Burkhardt, A. L., Mahajan, S., Cost, T., Franke, R., Bolen, J. B., and Nussenzweig, M. (1993). Signal transduction by immunoglobulin is mediated through Ig alpha and Ig beta. J. Exp. Med. *178*, 1049-55.

Saouaf, S. J., Kut, S. A., Fargnoli, J., Rowley, R. B., Bolen, J. B., and Mahajan, S. (1995). Reconstitution of the B cell antigen receptor signaling components in COS cells. J. Biol. Chem. *270*, 27072-8.

Saouaf, S. J., Mahajan, S., Rowley, R. B., Kut, S. A., Fargnoli, J., Burkhardt, A. L., Tsukada, S., Witte, O. N., and Bolen, J. B. (1994). Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. Proc. Natl. Acad. Sci. USA *91*, 9524-8.

Saxton, T. M., van Oostveen, I., Bowtell, D., Aebersold, R., and Gold, M. R. (1994). B cell antigen receptor cross-linking induces phosphorylation of the p21^{ras} oncoprotein activators SHC and mSOS1 as well as assembly of complexes containing SHC, GRB-2, mSOS1 and a 145-kDa tyrsoine-phosphorylated protein. J. Immunol. *153*, 623-36.

Schmitz, R., Baumann, G., and Gram, H. (1996). Catalytic specificity of phosphotyrosine kinases Blk, Lyn, c-Src and Syk as assessed by phage display. J. Mol. Biol. *260*, 664-77.

Schulte, R. J., Campbell, M.-A., Fischer, W. H., and Sefton, B. M. (1992). Tyrosine phosphorylation of CD22 during B cell activation. Science *258*, 1001-4.

Sefton, B. M., and Taddie, J. A. (1994). Role of tyrosine kinases in lymphocyte activation. Curr. Opin. Immunol. *6*, 372-9.

Shultz, L. D., and Green, M. C. (1976). Motheaten, an immunodeficient mutant of the mouse. II. Depressed immune competence and elevated serum immunoglobulins. J. Immunol. *116*, 936-43.

Shultz, L. D., Schweitzer, P. A., Rajan, T. V., Yi, T., Ihle, J. N., Matthews, R. J., Thomas, M. L., and Beier, D. R. (1993). Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. Cell *73*, 1445-54.

Sidman, C. L., Shultz, L. D., Hardy, R. R., Hayakawa, K., and Herzenberg, L. A. (1986). Production of immunoglobulin isotypes by Ly-1$^+$ B cells in viable motheaten and normal mice. Science *232*, 1423-5.

Sillman, A. L., and Monroe, J. G. (1994). Surface IgM-stimulated proliferation, inositol phospholipid hydrolysis, Ca^{2+} flux, and tyrosine phosphorylation are not altered in B cells from p59$^{fyn-/-}$ mice. J. Leuk. Biol. *56*, 812-6.

Stanley, E., Ralph, S., McEwen, S., Boulet, I., Holtzman, D. A., Lock, P., and Dunn, A. R. (1991). Alternatively spliced murine *lyn* mRNAs encode distinct proteins. Mol. Cell. Biol. *11*, 3399-406.

Stefanova, I., Corcoran, M. L., Horak, E. M., Wahl, L. M., Bolen, J. B., and Horak, I. D. (1993). Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56^{lyn}. J. Biol. Chem. *268*, 20725-8.

Takai, T., Ono, M., Hikida, M., Ohmori, H., and Ravetch, J. V. (1996). Augmented humoral and anaphylactic responses in FcγRII-deficient mice. Nature *379*, 346-9.

Takata, M., and Kurosaki, T. (1996). A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-γ2. J. Exp. Med. *184*, 31-40.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., and Kurosaki, T. (1994). Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca^{2+} mobilization through distinct pathways. EMBO J. *13*, 1341-9.

Taniuchi, I., Kitamura, D., Maekawa, Y., Fukuda, T., Kishi, H., and Watanabe, T. (1995). Antigen-receptor induced clonal expansion and deletion of lymphocytes are impaired in mice lacking HS1 protein, a substrate of the antigen-receptor-coupled tyrosine kinases. EMBO J. *14*, 3664-78.

Tarakhovsky, A., Turner, M., Schaal, S., Mee, P. J., Duddy, L. P., Rajewsky, K., and Tybulewicz, V. L. (1995). Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. Nature *374*, 467-70.

Thomas, M. L. (1994). The regulation of B- and T-lymphocyte activation by the transmembrane protein tyrosine phosphatase CD45. Curr. Opin. Cell Biol. *6*, 247-52.

Townley, R., Shen, S. H., Banville, D., and Ramachandran, C. (1993). Inhibition of the activity of protein tyrosine phosphate 1C by its SH2 domains. Biochemistry *32*, 13414-8.

Traverse, S., Seedorf, K., Paterson, H., Marshall, C. J., Cohen, P., and Ullrich, A. (1994). EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. Curr. Biol. *4*, 694-701.

Tsukada, S., Rawlings, D. J., and Witte, O. N. (1994). Role of Bruton's tyrosine kinase in immunodeficiency. Curr. Opin. Immunol. *6*, 623-30.

Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature *378*, 298-302.

van der Geer, P., and Hunter, T. (1993). Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. EMBO J. *12*, 5161-72.

van Noesel, C. J., Lankester, A. C., van Schijndel, G. M. W., and van Lier, R. A. W. (1993). The CR2/CD19 complex on human B cells contains the src-family kinase Lyn. Int. Immunol. *5*, 699-705.

Venkitaraman, A. R., Williams, G. T., Dariavach, P., and Neuberger, M. S. (1991). The B-cell antigen receptor of the five immunoglobulin classes. Nature *352*, 777-81.

Wang, J., Koizumi, T., and Watanabe, T. (1996). Altered antigen receptor signaling and impaired Fas-mediated apoptosis of B cells in Lyn-deficient mice. J. Exp. Med. *184*, 831-38.

Wechsler, R. J., and Monroe, J. G. (1995). Src-family tyrosine kinase p55^{fgr} is expressed in murine splenic B cells and is activated in response to antigen receptor cross-linking. J. Immunol. *154*, 3234-44.

Weng, W. K., Jarvis, L., and LeBien, T. W. (1994). Signaling through CD19 activates Vav/mitogen-activated protein kinase pathway and induces formation of a CD19/Vav/phosphatidylinositol 3-kinase complex in human B cell precursors. J. Biol. Chem. *269*, 32514-21.

Yamanashi, Y., Fukui, Y., Wongsasant, B., Kinoshita, Y., Ichimori, Y., Toyoshima, K., and Yamamoto, T. (1992). Activation of Src-like protein-tyrosine kinase Lyn and its association with phosphatidylinositol 3-kinase upon B-cell antigen receptor-mediated signaling. Proc. Natl. Acad. Sci. USA *89*, 1118-22.

Yamanashi, Y., Kakiuchi, T., Mizuguchi, J., Yamamoto, T., and Toyoshima, K. (1991). Association of B cell antigen receptor with protein tyrosine kinase lyn. Science *251*, 192-4.

Yamanashi, Y., Okada, M., Semba, T., Yamori, T., Umemori, H., Tsunasawa, S., Toyoshima, K., Kitamura, D., Watanabe, T., and Yamamoto, T. (1993). Identification of HS1 protein as a major substrate of protein-tyrosine kinase(s) upon B-cell antigen receptor-mediated signaling. Proc. Natl. Acad. Sci. USA *90*, 3631-5.

Yamashita, Y., Miyake, K., Miura, Y., Kaneko, Y., Yagita, H., Suda, T., Nagata, S., Nomura, J., Sakaguchi, N., and Kimoto, M. (1996). Activation mediated by RP105 but not CD40 makes normal B cells susceptible to anti-IgM-induced apoptosis: a role for Fc receptor coligation. J. Exp. Med. *184*, 113-20.

Yao, X. R., Flaswinkel, H., Reth, M., and Scott, D. W. (1995). Immunoreceptor tyrosine-based activation motif is required to signal pathways of receptor-mediated growth arrest and apoptosis in murine B lymphoma cells. J. Immunol. *155*, 652-61.

Yi, T. L., Bolen, J. B., and Ihle, J. N. (1991). Hematopoietic cells express two forms of lyn kinase differing by 21 amino acids in the amino terminus. Mol. Cell. Biol. *11*, 2391-8.

Zhang, R., Alt, F. W., Davidson, L., Orkin, S. H., and Swat, W. (1995). Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. Nature *374*, 470-3.

Zhou, S., Carraway III, K. L., Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Ponder, B. A. J., Mayer, B. J., and Cantley, L. C. (1995). Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. Nature *373*, 536-9.