Title
An analysis of Syk and the MEK-MAP kinase pathway in B cell antigen receptor signaling

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Publication Date
1998

Peer reviewed|Thesis/dissertation

An Analysis of Syk and the MEK-MAP Kinase Pathway in B Cell Antigen Receptor Signaling

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

Date University Librarian

Degree Conferred: .....

Dedication

I would like to dedicate this thesis to my wife, Rosie, and my parents, Ken and Mary. I truly appreciate the love, encouragement and support they have given me, without which this work would not have been possible. I also dedicate this work to my daughter Sarah, who always seems to have a smile for daddy. Thank you all, and I love you very much.

ACKNOWLEDGMENTS

I first wish to thank my adviser, Tony DeFranco, for many years of support and personal interest in my research. Tony has been optimistic, helpful, and critical, and was instrumental in my development as a scientist. I will always feel fortunate to have been his student. Along those same lines, I also thank Mike Gold and Linda Matsuuchi, my "surrogate" PIs in Vancouver. Mike and Linda were wonderful to work with, and our collaboration was very fruitful. I really enjoyed our time together. I am indebted to several members of the DeFranco lab who have shared their time, expertise and experiences with me. In particular, big thanks to Vivien Chan for always being helpful about pretty much everything. I can't imagine going through graduate school without having Vivien to lean on. Debbie Law and Tracy Stevens were also very helpful to me during my "formative" years, and their presence made the lab a fun place in which to work. I thank Stacey Harmer, Trish Roth, Julie Hambleton, Steve Weinstein, Mary Lee MacKichan, Cheryl Fitzer-Attas and Sasha Finn for their scientific input and especially for their friendship. I also thank Steve Weinstein for taking the plunge into teaching with me--it was fun! I would also like to thank everyone else who has been in the lab during my time here. One of my favorite things about working in the DeFranco lab has been being part of a group of really great people. I also thank my thesis committee, Tony DeFranco, Art Weiss, and Mike Bishop for their time in reviewing this thesis and for their helpful suggestions during my thesis committee meetings. Finally, I thank my wife Rosie and my parents for their continual love, support and encouragement.

Chapter 1 of this thesis is a reprint of a paper originally published in the *Journal of Biological Chemistry*, and is reprinted with the permission of the publishers. The reference is listed below. The work in this chapter was a collaborative effort with Linda Matsuuchi and Mike Gold. Sharon Hourihane, Linda's technician, also contributed. Figures 1.1 and 1.3 were a collaborative effort between Linda, Mike and me. Mike and Linda performed the experiments pictured in figures 1.6-1.8. The other figures and the original isolation and sequencing of the murine *syk* cDNA were completely my work. Tony DeFranco originally had the idea to express Syk in the AtT20 cells to try to reconstitute BCR-mediated signaling events.

Chapter 2 was a collaborative effort with Trish Roth and Alexander Finn. The transfectants expressing Syk and Btk were generated by Trish, and the cells expressing Syk, Btk and the CD19 chimera were generated jointly by Trish and me. Alexander Finn performed the PI 3-kinase activity assay in Figure 2.4. All other work was solely mine.

The work described in Chapter 3 was solely mine.

Tony DeFranco provided advice throughout the work described this thesis.

Chapter 1: 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*, 6458-6466.

An analysis of Syk and the MEK-MAP kinase pathway in B cell antigen receptor signaling

by James David Richards

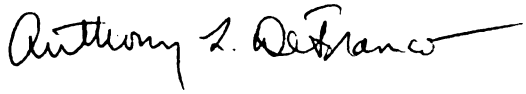
ABSTRACT

B cell antigen receptor (BCR)-induced signaling plays critical roles in B lymphocyte development and activation. The experiments described herein evaluated the roles of the Syk protein tyrosine kinase, and the MEK-MAP kinase cascade in these events. AtT20 pituitary cells, which endogenously expressed the Src-family tyrosine kinase Fyn, were transfected to express the BCR. Anti-IgM stimulation of these cells failed to induce most of the signaling events which occur in B cells. These cells were then transfected to express Syk. Syk expression reconstituted several signaling events upon anti-IgM stimulation, including Syk phosphorylation and association with the BCR, tyrosine phosphorylation of numerous proteins including Shc, and activation of the ERK-MAP kinases. In contrast, Syk expression did not reconstitute anti-IgM-induced inositol phosphate production. Moreover, phosphatidylinositol (PI) 3-kinase was only modestly activated in these cells. Syk therefore plays a key role in BCR signaling and is sufficient to reconstitute some signaling events in a non-lymphoid cellular context.

In an effort to reconstitute additional signaling events, the AtT20 cells were further transfected to express a CD19 chimera, Btk, or both. Syk was required for BCR-induced tyrosine phosphorylation of CD19. However, these proteins were not sufficient to activate the phosphoinositide pathway or PI 3-

kinase, suggesting that additional lymphoid-specific components may be required for activation of these signaling events.

The role of the MEK-MAP kinase cascade in BCR-induced biological responses was evaluated by treating WEHI-231 immature B cells, or mature splenic B cells with the MEK inhibitor PD 98059. Although PD 98059 substantially inhibited BCR-mediated activation of the ERK2 form of MAP kinase, it did not inhibit BCR-induced growth arrest or apoptosis of WEHI-231 cells. In contrast, in mature splenic B cells, PD 98059 suppressed BCR-induced upregulation of Egr-1 and CD44. Moreover, BCR-mediated proliferation, both in the absence and presence of IL-4, was inhibited by PD 98059. Therefore, the MEK-MAP kinase pathway participates in a subset of B cell biological responses to antigen receptor stimulation.



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INTRODUCTION

B cells play a central and unique role in the immune response to infection. Upon recognition of foreign antigen, usually coupled with co-stimulation provided by helper T cells, B cells are prompted to proliferate and differentiate into antibody-secreting plasma cells. Plasma cells secrete large amounts of antibodies which bind to their corresponding antigens on the pathogens, thereby neutralizing the pathogens and marking them for destruction by other components of the immune response.

The B cell antigen receptor (BCR) plays a critical role in this activation of mature B cells. Moreover, the BCR also participates in the elimination of self-reactive B cells during development. In both cases, BCR crosslinking by foreign- or self-antigens induces the activation of a complex network of biochemical signalling events which are crucial for the B cell's physiological response to antigen. It has therefore been of great interest to discover these BCR-induced signalling events, and determine how they act to influence B cell responses to antigen.

BCR-induced Signalling Events

The B cell antigen receptor (BCR) is composed of membrane immunoglobulin (Ig), which recognizes specific antigen, complexed to the Ig-α/Ig-β heterodimer, which mediates transmembrane signalling (DeFranco, 1998). Signal transduction initiated by BCR crosslinking begins with the activation of several tyrosine kinases, leading to the accumulation of numerous cellular tyrosine phosphoproteins (Brunswick et al., 1991; Campbell and Sefton, 1990; Gold et al., 1990; Lane et al., 1990). Tyrosine kinase

activation induces multiple downstream signalling events, including the activation of phospholipase C γ-1 and -2, the activation of phosphatidylinositol 3-kinase (PI 3-kinase), and the activation of the Ras-Mitogen-activated protein kinase (MAP kinase) pathway (Casillas et al., 1991; Fahey and DeFranco, 1987; Gold and Aebersold, 1994; Gold et al., 1992; Gold et al., 1992). B cells which are treated with tyrosine kinase inhibitors (Carter et al., 1991; Lane et al., 1991; Padeh et al., 1991), or which are defective for the expression of the correct tyrosine kinases (Takata and Kurosaki, 1996; Takata et al., 1994) do not activate these downstream signalling events or exhibit biological responses upon stimulation.

Initiation of BCR signalling by Src-family and Syk tyrosine kinases

The earliest signalling event upon BCR crosslinking is the activation of protein tyrosine kinases. Tyrosine phosphorylation is believed to participate in signalling events both by directly increasing or decreasing the enzymatic activity of phosphorylated enzymes, and by promoting the formation of protein-protein signalling complexes by providing binding sites for SH2 and PTB domains (DeFranco, 1997; DeFranco, 1995). Two families of tyrosine kinases participate to initiate signal transduction through the BCR: the Src-family kinases, including Lyn, Fyn, Blk, Lck and Fgr (Burg et al., 1994; Burkhardt et al., 1991; Wechsler and Monroe, 1995; Yamanashi et al., 1991); and the Syk tyrosine kinase, a member of the Syk/ZAP-70 family (Hutchcroft et al., 1992; Hutchcroft et al., 1991; Law et al., 1993).

Among the first proteins to become tyrosine phosphorylated by BCR crosslinking are the BCR proteins Ig-α and Ig-β (Gold et al., 1991). The Ig-α and Ig-β cytoplasmic domains each contain the sequence YxxL/IxxxxxxYxxL/I

(single amino acid code, x= any amino acid), now called the Immunoreceptor Tyrosine-based Activation Motif (ITAM). ITAMs are also present in the cytoplasmic domains of the signalling subunits of the T cell receptor and many Fc receptors (Ravetch, 1994; Reth, 1992), and are sufficient to mediate all the known signalling events that occur upon antigen receptor crosslinking (DeFranco, 1994; Irving and Weiss, 1991; Law et al., 1993; Weiss, 1993). The BCR ITAMs are likely phosphorylated by Src-family tyrosine kinases (Flaswinkel and Reth, 1994; Law et al., 1993). This hypothesis is supported by several observations. First, at least one Src-family kinase, Fyn, associates in low stoichiometry with the BCR prior to stimulation, placing this kinase in the proper location to phosphorylate the ITAMs (Law et al., 1993). Second, the BCR ITAM sequences closely resemble the optimal substrate sequences of several Src-family kinases (Schmitz et al., 1996; Zhou et al., 1995). Third, the Src-family kinases are the first tyrosine kinases to become activated by BCR stimulation, followed quickly by Syk and Btk activation (Saouaf et al., 1994). Finally, Fyn can phosphorylate a GST-Ig-α fusion protein *in vitro* (Flaswinkel and Reth, 1994). The doubly-phosphorylated ITAMs recruit additional Src-family kinases and Syk kinases to the BCR, where they bind to the phosphotyrosines via their SH2 domains and become activated (Chen et al., 1996; Law et al., 1993) (chapter 1). Both Lyn and Syk are activated by binding to phosphorylated ITAMs (Johnson et al., 1995; Rowley et al., 1995; Shiue et al., 1995). Moreover, BCR-associated Syk can be further activated by subsequent autophosphorylation (Rowley et al., 1995; Shiue et al., 1995), or phosphorylation by Src-family kinases (Iwashima et al., 1994; Kurosaki et al., 1994; Watts et al., 1994) (chapter 1).

Subsequent to their recruitment to the BCR and their activation, Syk and Src-family kinases phosphorylate numerous downstream proteins,

leading to the activation of many signalling pathways. Btk, PLC-γ, MAP kinase, and PI-3 kinase all depend on Src-family and/or Syk activation. For example, chicken DT40 B cells rendered deficient for Syk and/or Lyn, the only Src-family kinase expressed by these cells, fails to activate any BCR-induced signalling events (Kurosaki et al., 1995; Takata et al., 1994). In addition, reconstitution experiments in a non-lymphoid cell line demonstrate that Syk activation is required for the BCR-induced activation of the MAP kinase pathway (chapter 1). In addition to its role in BCR signalling, Syk is also important developmentally. Mice deficient for Syk exhibit a complete block in B cell development at a time when pre-BCR signals are required (Cheng et al., 1995; Turner et al., 1995).

Activation of downstream signalling events

The Src-family and Syk tyrosine kinases activated by BCR crosslinking set into motion a complex network of biochemical signalling reactions which ultimately alter gene expression and induce the biological responses to antigen. Most of these signalling events are common to many receptors and have been shown to be important for biological responses to many types of stimulation. Four of the more important signalling events regulated by the above-mentioned kinases are the activation of the Btk tyrosine kinase, as well as the PLC, Ras-MAP kinase and PI-3 kinase pathways.

Btk in BCR signalling

Btk is a member of the Tec family of tyrosine kinases (Aoki et al., 1994; de Weers et al., 1994; Saouaf et al., 1994). In contrast to the Src-family kinases

and Syk, Btk does not appear to associate with the BCR. Rather, it appears to be activated by cytoplasmic Src-family kinases (Rawlings et al., 1996). In addition, Btk activation is not required to initiate BCR-induced signalling in the same global manner for which the Src-family kinases and Syk are required, and can therefore be considered a downstream event. For example, DT40 cells that lack Btk expression exhibit near-normal levels of BCR-induced tyrosine phosphorylation (Takata and Kurosaki, 1996). This is not to say, however, that Btk activation is unimportant. DT40 cells lacking Btk expression fail to activate the PLC pathway upon stimulation (Takata and Kurosaki, 1996). In addition, a Btk mutation in mice is responsible for the X-linked immunodefiency (XID) syndrome (Khan et al., 1995). In the XID mice, B cells are produced in substantial numbers but are unable to respond to T cell-independent type 2 (TI-2) antigens, such as many polysaccharides. Moreover, Btk mutations have been linked to the human disease X-linked agammaglobulinemia (XLA) (Tsukada et al., 1993). People with XLA have greatly decreased numbers of B cells and poor antibody responses (Tsukada et al., 1994).

The PLC pathway

BCR crosslinking leads to the tyrosine phosphorylation and activation of PLCγ1 and γ2. Genetic studies in DT40 cells have demonstrated that this phosphorylation and activation requires Syk activity (Takata et al., 1994), although BCR-induced activation of Syk is not sufficient (chapter 1). Btk activation is also required (Takata and Kurosaki, 1996). Although tyrosine phosphorylation increases the enzymatic activation of PLC (Kim et al., 1991),

it must also be translocated to the plasma membrane, where its substrate resides. How this translocation occurs in B cells is not clear.

Activated PLC hydrolyses the cell membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2), yielding the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 binds to receptors in internal membranes, causing the release of intracellular calcium into the cytoplasm and ultimately the opening of calcium channels in the plasma membrane (Cambier and Ransom, 1987). These events result in a rapid and sustained increase in the concentration of free calcium ions in the cell (Gelfand et al., 1989). The released calcium is bound by calmodulin, and promotes the activation of serine/threonine kinases such as calmodulin-dependent kinase II as well as the serine/threonine phosphatase calcineurin (Schreiber and Crabtree, 1992; Valentine et al., 1995). Calcineurin is believed to dephosphorylate and activate the NFAT transcription factor (Rao et al., 1997). In addition, several other transcription factors, such as Ets-1, ATF-2 and NF-κB are activated in a calcium-dependent manner (Dolmetsch et al., 1997; Fisher et al., 1991). The other second messenger generated by PIP_2 hydrolysis, diacylglycerol, activates most protein kinase C (PKC) isotypes. PKC activation also participates in regulating gene expression by activating the CREB (cAMP response element binding protein) transcription factor (Xie and Rothstein, 1995; Xie et al., 1996). Moreover, evidence exists in B cells (Gold et al., 1992) and T cells (Downward et al., 1990; Woodrow et al., 1993) that PKC can participate in the activation of the Ras pathway.

Many labs have demonstrated that activation of the PLC pathway is important for B cell responses to antigen. Treatment of WEHI-231 immature B cells with calcium ionophores and phorbol esters, pharmacologic agents which serve to mimic PLC activation, can partially reproduce BCR-induced

growth arrest in WEHI-231 cells (Page and DeFranco, 1988). In addition, mutants of this cell line which failed to undergo BCR-induced growth arrest and apoptosis were defective in this pathway (Page et al., 1991). Moreover, DT40 chicken B cells rendered deficient for PLCγ2 expression also failed to undergo BCR-mediated apoptosis. Finally, mature B cells treated with calcium ionophores and phorbol esters are induced to proliferate either directly or in combination with IL-4, similarly to what occurs with BCR-stimulated B cells (Monroe and Kass, 1985; Rothstein et al., 1986).

The Ras-MAP kinase pathway

BCR crosslinking also activates Ras, which is an important regulator of cell growth and differentiation in several cell types (Marshall, 1994; Marshall, 1995; Pritchard et al., 1995; Sewing et al., 1997; Woods et al., 1997). Ras is constitutively located at the plasma membrane, and in its inactive state is bound to GDP. BCR crosslinking induces the exchange of this GDP for GTP, thereby activating Ras (Harwood and Cambier, 1993; Kawauchi et al., 1994; Lazarus et al., 1993). The mechanism by which this exchange occurs in B cells is not clear, but it likely involves the adaptor protein Shc. In many cell types, receptor stimulation promotes the tyrosine phosphorylation of the adaptor protein Shc; in B cells, this phosphorylation is likely carried out by Syk (Harmer and DeFranco, 1997; Nagai et al., 1995). Tyrosine phosphorylation of Shc allows it to associate with the adaptor protein Grb-2, which itself binds to the nucleotide exchange factors mSOS1 and mSOS2. Indeed, Shc/Grb-2/mSOS complexes form rapidly in B cells upon BCR crosslinking (Harmer and DeFranco, 1997; Saxton et al., 1994). These complexes become membrane bound, again through an unknown mechanism. Nevertheless, bringing mSOS to the cell membrane is thought to allow it to convert Ras-GDP to Ras-GTP, thereby activating Ras. Ras activity in B cells may also be regulated by the GTP-ase activating protein for Ras, RasGAP. RasGAP increases Ras' slow intrinsic GTPase activity, thereby promoting the conversion of active GTP-bound Ras to inactive GDP-bound Ras. Indeed, RasGAP is also tyrosine phosphorylated upon BCR stimulation (Gold et al., 1993). Also, as mentioned above, PKC may also promote Ras activation.

Ras-GTP triggers activation of the MAP kinase cascade (Marshall, 1994; Marshall, 1996). Activated Ras associates with Raf, delivering Raf to the cell membrane where it becomes phosphorylated and activated. Raf then phosphorylates and activates the MEK1 and MEK2 kinases, which in turn phosphorylate and activate the p44 and p42 classical MAP kinases, ERK1 and ERK2, respectively. The ERK kinases phosphorylate and activate a number of transcription factors, including Elk-1 and SAP-1a, which help enhance transcription of genes whose promoters contain serum response elements (SREs) (Treisman, 1996). As a result, activation of Ras and the MAP kinases participates in BCR-induced changes in expression of genes such as *egr-1* (McMahon and Monroe, 1995)(chapter 3). Until recently, however, little else was known concerning the role of the Ras-MAP kinase pathway in B cell responses to antigen. Experiments addressing this issue will be discussed in chapter 3.

The PI 3-kinase pathway

PI 3-kinase, which consists of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit, adds a phosphate to the 3 position of the inositol ring of phosphoinositides. BCR crosslinking increases both PI 3-kinase tyrosine phosphorylation (Gold et al., 1992; Yamanashi et al., 1992) and activity, as measured by the accumulation its lipid products, including PI 3,4-P_2 and PI 3,4,5-P_3 (Gold and Aebersold, 1994). Another protein that is tyrosine phosphorylated upon BCR crosslinking is an inositol 5-phosphatase called p145/SHIP. Interestingly, PI 3,4,5-P_3 is a substrate for SHIP, which converts it to PI 3,4-P_2. The lipid products of PI 3-kinase and SHIP are binding sites for the plextrin homology (PH) domains of Btk and the serine/threonine kinase

Akt. These lipid products may therefore recruit these kinases to the plasma membrane and participate in their activation (Franke et al., 1997; Klippel et al., 1997; Salim et al., 1996). Importantly, PI 3-kinase activation plays a role in cell cycle regulation in B cells (Beckwith et al., 1996). It is unclear whether SHIP plays a similar role in B cells, but this hypothesis seems likely given the relationship between these two enzymes.

The CD19 co-receptor

CD19 is a transmembrane glycoprotein in B cells. A fraction of CD19 constitutively associates with the BCR (Fearon and Carter, 1995), and CD19 becomes heavily tyrosine phosphorylated upon BCR crosslinking (Chalupny et al., 1993; Uckun et al., 1993). These CD19 phosphotyrosines are thought to provide binding sites for PI 3-kinase (Tuveson et al., 1993) and the protooncogene product Vav (Weng et al., 1994). In human B cells, the level of BCR-induced PI 3-kinase activity is directly proportional to the extent of CD19 tyrosine phosphorylation, and to the amount of PI 3-kinase associated with CD19 (Tuveson et al., 1993). The Src-family kinases Lyn and Fyn also associate with CD19 (Chalupny et al., 1995; van Noesel et al., 1993). Thus, CD19 participates in BCR-induced signalling events by providing docking sites for, and potentially facilitating the activation of several important signalling proteins.

CD19 serves as a potent enhancer of BCR stimulation upon coligation with the BCR. CD19-BCR crosslinking decreases the threshold for BCR-dependent stimulation of proliferation by 100 fold (Carter and Fearon, 1992). This observation is physiologically relevant because CD19 in mature B cells exists in a complex with the complement receptor CD21. Therefore,

complement-coated antigen could co-ligate the BCR with the CD19/CD21 complex, thereby increasing the B cell's sensitivity to antigen. Indeed, antigens expressed as fusion proteins with the C3d complement fragment are much more potent than antigens lacking these fragments (Dempsey et al., 1996). Finally, mice lacking CD19 have decreased responses to T-dependent antigens (Engel et al., 1995; Rickert et al., 1995).

Biological outcomes of BCR signalling

BCR-induced signalling quickly reaches the nucleus, activates transcription factors and alters gene expression (DeFranco, 1998). Although the known signalling events are largely the same in immature and mature B cells, the biological responses to these signals vary greatly depending on the maturation state of the B cell and on the additional signals it receives. Immature B cells that contact self-antigen in the bone marrow can undergo receptor editing in an attempt to change receptor specificity away from self (Radic and Zouali, 1996). Failure to do so, however, results in anergy or apoptosis of the B cell. Similarly, immature B cells in the periphery which contact antigen also become anergized or apoptose (Goodnow, 1992; Klinman, 1996). These events serve to reduce the pool of autoreactive B cells, thereby helping to ensure self-tolerance in the B cell population. In contrast, mature B cells contacting antigen enter G1 phase of the cell cycle and upregulate many proteins involved in adhesion and antigen presentation to helper T cells (Goroff et al., 1986; Maltzman and Monroe, 1996; Maltzman and Monroe, 1996; Mongini et al., 1991; Monroe and Kass, 1985)(chapter 3). Strong BCR stimulation can also induce B cell proliferation and antibody secretion even in the absence of MHC class II-dependent T cell help, although these

responses still require cytokines or other forms of costimulation (Mond et al., 1995). For example, initial antibody responses against the hepatitis B virus, vesticular stomatitis virus and polyoma virus are T cell-independent (Bachmann et al., 1995; Milich and McLachlan, 1986; Szomolanyi-Tsuda and Welsh, 1996). Nevertheless, during infections helper T cells markedly enhance proliferation and antibody secretion by antigen-stimulated B cells, and promote immunoglobulin class switching by providing cell-cell contact signals via CD40 and by releasing cytokines such as IL-4 and IL-5 (Armitage et al., 1992; DeFranco, 1998; Hodgkin et al., 1991; Lederman et al., 1992; Noelle et al., 1992; Oliver et al., 1985; Phillips and Klaus, 1992; Rabin et al., 1985). These events allow the B cell to mount an effective antibody response during infection.

CHAPTER 1: Reconstitution of B Cell Antigen Receptor-Induced Signalling Events in a Non-Lymphoid Cell Line by Expressing the Syk Protein Tyrosine Kinase

Summary

B cell antigen receptor (BCR) crosslinking activates Src-family and Syk tyrosine kinases, results in increased cellular protein tyrosine phosphorylation, and activates several downstream signalling enzymes. To identify lymphoid-specific proteins that are necessary and sufficient to allow BCR-induced signalling to occur, we expressed the BCR in the AtT20 mouse pituitary cell line. These non-lymphoid cells endogenously expressed the Src-family kinase Fyn, but not Syk. Anti-IgM stimulation of these cells failed to induce most of the signalling events which occur in B cells. BCR-expressing AtT20 transfectants were generated that also expressed Syk. Syk expression reconstituted several signalling events upon anti-IgM stimulation, including Syk phosphorylation and association with the BCR, tyrosine phosphorylation of numerous proteins including Shc, and activation of mitogen-activated protein (MAP) kinase. In contrast, Syk expression did not reconstitute anti-IgM-induced inositol phosphate production. A catalytically inactive Syk mutant could associate with the BCR and become tyrosine phosphorylated, but could not reconstitute downstream signalling events. Expression of the Src-family kinase Lck instead of Syk also did not reconstitute signalling. Thus, wild type Syk was required to reconstitute several BCR-induced signalling events, but was not sufficient to couple the BCR to the phosphoinositide signalling pathway.

Introduction

B lymphocytes can recognize a myriad of foreign antigens by virtue of the diversity of their B cell antigen receptors (BCR). The antigen recognition and binding functions of the BCR are performed by membrane immunoglobulin (mIg), whereas the ability to transduce signals across the plasma membrane is fulfilled by the mIg-associated Ig-α/Ig-β heterodimer (DeFranco, 1993). The signalling cascade initiated by Ig-α and Ig-β upon BCR crosslinking can promote different biological outcomes, depending on the differentiation state of the B cell and the nature of additional signals received by the cell. Immature B cells become anergized or undergo apoptosis upon antigen binding, whereas mature B cells enter the cell cycle and can be induced to differentiate into antibody-secreting plasma cells (DeFranco, 1993).

The earliest signalling event initiated by BCR crosslinking is an increase in the tyrosine phosphorylation of numerous proteins (Brunswick et al., 1991; Campbell and Sefton, 1990; Gold et al., 1990; Lane et al., 1990). Upon stimulation, the cytoplasmic tails of Ig-α and Ig-β become tyrosine phosphorylated. This promotes the binding and activation of intracellular tyrosine kinases (DeFranco, 1995). This tyrosine kinase activity is essential for BCR-mediated responses. B cells treated with tyrosine kinase inhibitors (Carter et al., 1991; Lane et al., 1991; Padeh et al., 1991), or which fail to express the correct tyrosine kinases (Takata et al., 1994) do not activate downstream signalling pathways or exhibit biological responses upon stimulation.

Two types of intracellular tyrosine kinases that have been implicated in BCR signalling are Syk, and members of the Src-family of tyrosine kinases. Both Syk and the Src-family kinases p59^{fyn}, p53/56^{lyn}, p55^{blk} and p56^{lck} co-

immunoprecipitate with the BCR and become activated upon BCR crosslinking (DeFranco, 1993; Hutchcroft et al., 1992; Law et al., 1993; Saouaf et al., 1994; Yamada et al., 1993). Evidence for the significance of these two classes of kinases in BCR signalling has been provided by experiments with a chicken B cell line rendered deficient for Lyn or Syk expression by homologous recombination (Takata et al., 1994). Mutant cell lines lacking either Syk or Lyn exhibited markedly decreased anti-Ig-induced protein tyrosine phosphorylation and activation of phospholipase C. In addition, the Lyn^- cells failed to tyrosine phosphorylate and activate Syk as effectively as did the wild type cells, implying that Lyn may act upstream of Syk (Kurosaki et al., 1994).

To identify lymphoid-specific proteins that functionally link the BCR to the activation of downstream signalling pathways, we have attempted to reconstitute BCR signalling in a non-lymphoid cell line. Previously, we transfected the genes encoding the BCR proteins μ heavy chain, λ light chain, Ig-α and Ig-β into the AtT20 mouse pituitary cell line (Matsuuchi et al., 1992). Although the BCR was expressed on the surface of these cells, crosslinking it with anti-Ig antibodies failed to elicit most of the signalling responses normally associated with the BCR, with the exception that the cytoplasmic tails of Ig-α and Ig-β became tyrosine phosphorylated. Notably, a general increase in protein tyrosine phosphorylation did not occur upon anti-Ig treatment, suggesting that one or more tyrosine kinases required for BCR function may have been absent. Of the five tyrosine kinases known to be activated by the BCR, the AtT20 cells expressed only Fyn. Because Syk may be essential for BCR signalling, we isolated a cDNA encoding the murine Syk kinase, transfected it into the BCR^+ AtT20 cells, and selected clones that expressed Syk at levels comparable to Syk expression in B cells. Syk

expression was sufficient to reconstitute in a stimulation-dependent manner several BCR-induced signalling events in the BCR^+ AtT20 cells, including tyrosine phosphorylation of Shc and activation of MAP kinase. Consistent with a requirement for both a Src-family kinase and Syk, BCR signalling in AtT20 cells was not reconstituted by expression of Lck instead of Syk. These results are compatible with a "sequential kinase" model of BCR signalling (Law et al., 1993; Weiss, 1993) in which Src-family kinases are important for the initial tyrosine phosphorylation of Ig-α and Ig-β, whereas subsequent recruitment of Syk to the BCR and Syk activation are required for additional, downstream signalling events to occur. However, Syk expression did not reconstitute activation of the phosphoinositide signalling pathway. Thus, additional lymphoid-specific proteins besides Syk may be required to mediate BCR-induced activation of phospholipase C.

Materials and Methods

Cell lines. The mouse endocrine cell line AtT20/D16V-WT#6 (AtT20) (Matsuuchi and Kelly, 1991) was grown at 37°C in DMEM (Stem Cell Technologies, Vancouver, BC) containing 4.5 g/l glucose, 10% fetal calf serum (Canadian Life Technologies, Burlington, Ont.), penicillin/streptomycin and kept in an atmosphere of 10% CO_2. AtT20 cells expressing the $5HT_{1c}$ serotonin receptor, SR1, have been described previously (Matsuuchi et al., 1992). WEHI-231, Bal17, and A20 B cells were grown as previously described (Law et al., 1992) except that media was supplemented with 5% FCS. Jurkat T cells were grown in the same conditions as the B cell lines.

Antibodies. To generate anti-Syk antisera, rabbits were immunized with a synthetic peptide, PYEPTGGPWGPDRGLQREAL (single letter code), corresponding to amino acids 316-335 of murine Syk, coupled to KLH. The peptide was synthesized by Dr. Ian Clark-Lewis (Biomedical Research Centre, Univ. of British Columbia) and the antisera was generated with the assistance of the University of British Columbia Animal Care Facility. In addition, a GST fusion protein containing amino acids 259-333 of murine Syk was produced in *E. coli* and purified by glutathione-agarose affinity chromatography. This fusion protein was used to immunize rabbits at Caltag Laboratory, Inc. (Healdsburg, CA). Rabbit anti-porcine Syk antiserum was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit anti-Lck antiserum was a gift from Dr. Pauline Johnson (Univ. of British Columbia). Rabbit anti-Fyn antiserum was a gift from Dr. Roger Perlmutter (Univ. of Washington). Affinity purified rabbit anti-Blk antibodies and rabbit anti-Lyn antibodies were previously described (Law et al., 1993). Rabbit anti-murine Ig-

α antiserum and mouse anti-phosphotyrosine monoclonal antibody, 4G10, have been described previously (Gold et al., 1991). An additional rabbit anti-Ig-α antiserum was a gift from Drs. Jason Cyster and Chris Goodnow (Stanford Univ.). Rabbit anti-λ light chain antibodies were purchased from ICN Biomedicals Canada, Ltd. (St. Laurent, Quebec). Rabbit anti-Shc antibody was purchased from Upstate Biotechnology Inc. Anti-MAP kinase antibody anti-ERK2 (sc-154, Santa Cruz Biotechnology, Santa Cruz, CA) was used for p42 MAP kinase activity assays. For immunoblotting MAP kinase in electrophoretic mobility shift assays, antibody sc-94 was used (Santa Cruz Biotechnology). Affinity purified Goat anti-IgM, μ chain specific antibodies used in cell stimulations were purchased from Jackson ImmunoResearch (West Grove, Pa) or Bio-Can (Mississauga, Ontario, Canada).

Library Construction and Screening and Sequence Analysis. WEHI-231 cells were grown to log phase, and total RNA was purified (Bothwell et al., 1990). PolyA RNA was isolated as described (Ausubel et al., 1994). cDNA was synthesized with reverse transcriptase as recommended by Promega (Madison, WI), except that for first strand synthesis 1000 U M-MLV RNase H^- RT (Promega) and 31 U AMV RT XL (Life Sciences, St. Petersburg, FL) were used. cDNA ends were made blunt by incubating at 37° C for 30 minutes with dNTPs and 10 U T4 DNA Polymerase (NEB, Beverly, MA). Reaction products were extracted with phenol:chloroform and precipitated with ethanol. The cDNAs were ligated to adaptors and fragments less than 1 kb were removed following electrophoretic separation. The cDNAs were then ligated into the λZAPII vector (Stratagene, La Jolla, CA) in the *Xho* I site. The ligation was then packaged with Gigapack II Gold Packaging Extract (Stratagene).

Approximately 220,000 phage were plated on the XL-1 Blue bacterial strain. Phage plaques were transferred in duplicate to Hybond-N filters

(Amersham), and the DNA was crosslinked onto the filters by UV irradiation with a Stratalinker 1800 (Stratagene). The filters were screened with either of two ^{32}P-labelled DNA probes. The first was a murine *syk* DNA probe which was generated by PCR using the 5' oligonucleotide primer 5'-GACTACCTGGTCCAGGGGGGC-3' and the 3' primer 5'-GTCTGCCTGCTCAAGAACCCT-3', which were chosen based on the porcine *syk* sequence. The second probe was a 1.1 kb fragment of the porcine *syk* gene (gift of K. Chu and D.R. Littman, UCSF). Filters were hybridized overnight at 42° C. Hybridization and washing conditions were as described (Mittelstadt and DeFranco, 1993), except that the filters probed with porcine *syk* were hybridized with 30% formamide and washed at 37° C. Four plaques that hybridized with both probes were picked and purified by two additional rounds of screening, and excised into pBluescript SK$^-$. The longest cDNA, U4.1, was sequenced in both directions using the dideoxy chain termination procedure with Sequenase (United States Biochemical Corp.), and found to contain an open reading frame encoding the full length Syk kinase. The other three clones were partially sequenced, and found to be fragments of the same gene. Sequence alignments were performed by the GAP program of the Wisconsin Package (Program Manual for the Wisconsin Package, Version 8, Sept. 1994, Genetics Computer Group, Madison, WI), which also yielded the % identity scores.

Site-Directed Mutagenesis and Subcloning. To generate the catalytically inactive mutated form of Syk, the lysine at position 396 was mutated to an alanine by site-directed mutagenesis with the oligonucleotide 5'-CTTCAGGATTGCCACAGCCACG-3', using standard techniques (Ausubel et al., 1994). The open reading frames encoding both wild type and catalytically

inactive Syk were subcloned into the *Hind* III and *Xba* I sites of the pRc/CMV expression vector (InVitrogen, San Diego, CA).

DNA transfection and preparation of AtT20 cell lines. A murine B cell antigen receptor-expressing AtT20 cell line was made by co-transfecting cells using the calcium phosphate technique (Matsuuchi and Kelly, 1991) with 20μg of a plasmid containing the hygromycin resistance gene expressed from a TK promoter, pHS-53, and 25μg each of the following plasmids: pRSV μmembrane cDNA, pRSV λ1 genomic clone, pCMV mb-1 and pLpA B29. These plasmids encoding the four chains of the BCR have been previously described (Matsuuchi et al., 1992). Transfected cells were selected in complete growth media containing 175 μg/ml hygromycin (Calbiochem, La Jolla, CA and Boehringer Mannheim Biochemicals Canada, Laval, Quebec), and drug resistant clones were recovered and screened by immunofluorescence for the expression of surface μ chain using goat anti-mouse μ-FITC (ICN Biochemicals, Mississaugua, Ont.). All four BCR chains must be expressed in order for the BCR to be expressed at the cell surface (Matsuuchi et al., 1992). The original clone with the highest level of surface μ chain was subcloned three successive times, yielding the recipient cell line for future transfections, 100-33. The 100-33 cell line expresses high levels of μ chain on its surface and is homogeneous. In addition to the immunofluorescence experiments, surface expression of λ chain, μ chain and Ig-α was confirmed by surface biotinylation of 100-33 cells, selective immunoprecipitation with anti-μ, anti-λ and anti-Ig-α antibodies, analysis on SDS-PAGE, transfer to nitrocellulose and identification of biotinylated proteins using streptavidin-HRP and enhanced chemiluminescence detection (ECL, Amersham Canada Ltd., Oakville, Ontario).

The 100-33 cell line was transfected as described above, with 20μg of pSV2neo and 100μg per transfection of the pRc/CMV expression vector containing either the *syk* or *lck* cDNA. A cDNA clone encoding Lck was obtained from Dr. Jamey Marth, Biomedical Research Center, Univ. of British Columbia. Alternatively, 100μg of the expression vector encoding Syk or catalytically inactive Syk was introducted into 100-33 cells alone in the pRc/CMV vector. Transfected cells were selected in growth medium containing 0.4mg/ml Geneticin (GIBCO/BRL, Burlington, Ontario), with individual clones recovered and screened for expression of Lck or Syk protein using specific antibodies.

Cell stimulation and preparation of cell lysates. Cells grown in 10 cm dishes were washed three times with PBS containing 1mg/ml glucose and the medium was replaced with buffer A (25 mM NaHepes, pH 7.2, 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1 mg/ml glucose, 2 mM glutamine, 1 mM Na pyruvate, 50 μM 2-mercaptoethanol, 1 mg/ml BSA). After 15 min at 37°C, the cells were washed three times with PBS/glucose and fresh 37°C buffer A was added back. Cells were stimulated for 5 min with 20 μg/ml goat anti-mouse IgM. Reactions were terminated by aspirating the buffer and washing the cells three times with ice-cold PBS/glucose containing 1 mM Na_3VO_4. Cells were lysed by adding 1 ml buffer B (20 mM Tris-HCl, pH 8, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin) to each dish. For MAP kinase enzyme assays, the cells were lysed in 1 ml of buffer C (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 1 mM DTT, 1 mM Na_2MoO_4, 0.2 mM Na_3VO_4, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 0.23 mM PMSF, 10 μg/ml soybean trypsin inhibitor). After rocking 20 min in the cold, detergent-

insoluble material was removed by centrifugation and the lysates were stored at -80°C until assayed. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

Immunoprecipitations. Unless otherwise indicated, immunoprecipitations were performed as follows: Cell lysates were pre-cleared for 1 hr with protein A-sepharose (Sigma, St. Louis, MO), with or without precoating with normal rabbit serum (Gold et al., 1994). The appropriate antisera was added to the precleared lystates with new protein A-sepharose and incubated with rocking at 4° C for 1 to 3 hr. Beads were then washed once with Buffer B plus 0.5M NaCl, and twice with Buffer B, except anti-Shc immunopreciptates were washed 3 times with Buffer B and 1 time with 20mM Tris, pH 7.5 / 50 μM Na_4VO_4. Beads were then pelleted and bound proteins were eluted with SDS-PAGE sample buffer containing 100μM dithiothreitol and separated by SDS-PAGE gels.

Immunoblotting. Cell lysates were separated by SDS-PAGE and transferred to BA83 nitrocellulose (Schleicher and Schuell) or Immobilon (Amersham) membranes. For MAP kinase electrophoretic mobility shift assays, whole cell lysates were separated on 12.5% low bis acrylamide (12.36% acrylamide, 0.1% bis-acrylamide final concentrations) SDS-PAGE gels. Filters were blocked for 1 hr to overnight with 5% (w/v) BSA or non-fat dried milk in TBS (10 mM Tris, pH 7.5, 150 mM NaCl). The filters were washed for 10 min in TBS/0.05% Tween-20 (TBST) or TBST/0.5% NP40 (TBST-NP40) and then incubated with the primary antibody for 2-3 hr at room temperature at the indicated dilution in TBST or TBST-NP40. Antibodies used were the 4G10 anti-P-Tyr mAb (60 ng/ml), rabbit anti-murine Syk antiserum (diluted 1:2000), rabbit anti-Ig-α (diluted 1:1000) or affinity-purified rabbit anti-ERK1/ERK2 antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA).

The 4G10 mAb was detected with HRP-conjugated sheep anti-mouse IgG (1:10,000; Amersham, Oakville, Ontario, Canada) while rabbit antibodies were detected with HRP-conjugated goat anti-rabbit IgG (1:20,000; Bio-Rad, Mississauga, Ontario, Canada) or protein A-HRP (1:10,000; Amersham) . The filters were washed extensively with TBS/0.1% Tween-20 or TBST-NP40 and immunoreactive bands were visualized by enhanced chemiluminescence detection (ECL, Amersham). Blots which were stripped of bound antibody in order to be reprobed were incubated at 50° C for 30 minutes in 100mM 2-mercaptoethanol / 2% SDS / 62.5 mM Tris-HCl pH 6.7.

To examine the association between Syk and the BCR, lysates were immunoprecipitated with anti-Ig-α antisera as described above. The beads were split into 2 fractions and the immunoprecipitates were separated on 7.5% gels by SDS-PAGE and transferred to nitrocellulose. Membranes were immunoblotted with 4G10 or anti-Syk antisera as described above. Membranes were then stripped as above and reprobed with anti-Ig-α antisera to ensure equal loading of lanes.

***In vitro* kinase assay.** Lysates were pre-cleared with protein A sepharose beads that had been coated with normal rabbit serum. Lysates were then immunoprecipitated with the appropriate antisera and fresh protein A-sepharose. Beads were then washed twice with Buffer B with 0.5M LiCl, and once with kinase assay buffer. Autophosphorylation reactions were performed as described (Gold et al., 1994), and reaction products were separated by SDS-PAGE and visualized by autoradiography. The *in vitro* kinase activity of Syk was determined as above, except lysates were pre-cleared with protein A-sepharose alone, beads were washed in Buffer B plus 0.5M NaCl instead of LiCl, and reaction products were separated on 8% gels.

p42 MAP kinase (ERK2) activity. Cell lysates (0.5 mg protein) in buffer C were incubated for 1.5 hr at 4°C with 15 µl agarose beads to which affinity-purified rabbit anti-ERK 2 antibodies had previously been covalently coupled. The beads were washed three times with buffer C and once with MAP kinase assay buffer (20 mM Na Hepes, pH 7.2, 5 mM MgCl2, 1 mM EGTA, 5 mM 2-mercaptoethanol, 2 mM Na3VO4, 10 µg/ml aprotinin, 1 mM PMSF). Reactions were initiated by adding 30 µl of kinase assay buffer containing 1 mg/ml myelin basic protein (MBP; Sigma) and 5 µCi ^{32}P-ATP. Reactions were carried out for 15 min at 30°C and terminated by addition of 35 µl 2X SDS-PAGE sample buffer. After boiling, portions of the sample (15-20 µl) were separated on 15% SDS-PAGE gels and then transferred to nitrocellulose. The MBP bands were detected by Ponceau S staining of the filter. After autoradiography, the stained MBP bands were excised and ^{32}P incorporation determined by liquid scintillation counting. The upper portion of the blot was analyzed by anti-MAP kinase immunoblotting to confirm that equal amounts of p42 MAP kinase had been precipitated in all lanes.

Phosphoinositide Breakdown. Inositol phosphate production was measured essentially as described (Matsuuchi et al., 1992). Cells were grown overnight in 10 µCi of ^{3}H-inositol, and washed and stimulated as described above, except that they were stimulated for 30 minutes to allow for the accumulation of inositol phosphates. Inositol phosphate production is expressed as % inositol phosphate release, which was calculated as the amount of ^{3}H-labelled inositol phosphates divided by the sum of ^{3}H-inositol phosphates plus the ^{3}H-inositol-labelled phospholipids present in the TCA-insoluble fraction. The TCA-insoluble fraction was washed once in ice cold 10% TCA before it was sonicated, resuspended in methanol and scintillation counted.

Measurement of Intracellular Free Calcium. Cells were grown in 2 well coverglass chambers (Nunc, Naperville, IL), and incubated with 2-4 μM indo-1 AM (Molecular Probes, Inc., Eugene, OR) for 90 minutes at 37° C. Cells were washed 3 times with buffer D (buffer A minus glutamine, Na pyruvate and 2-mercaptoethanol), and then stimulated with buffer D alone, or buffer D plus either 20 μg/ml goat anti-mouse IgM, 10% dialyzed FCS, or 1μM serotonin (5-hydroxytryptamine, Sigma). Fluorescence intensity of intracellular indo-1 was monitored by image analysis on a laser-based fluorescence image cytometer (ACAS 470, Meridian Instruments, Inc.) as described (Szöllösi et al., 1991).

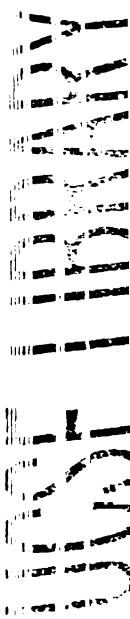
Results

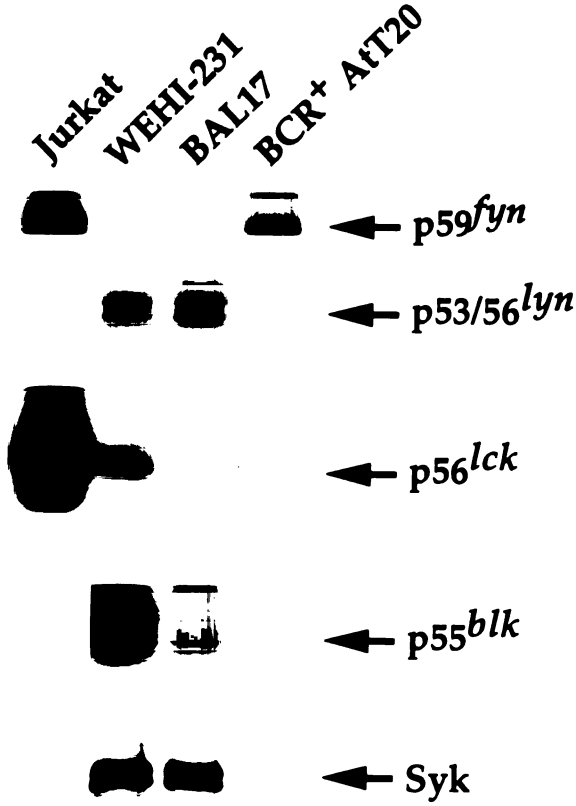
Tyrosine Kinase Expression in AtT20 Cells. *In vitro* kinase assays were performed to determine which of the tyrosine kinases that associate with the BCR in B cells were expressed in the BCR^+ AtT20 cells. AtT20 lysates were incubated with antibodies specific to these kinases, and the immunoprecipitates were incubated with γ-^{32}P-ATP and allowed to autophosphorylate *in vitro* (Figure 1.1). Lysates from the Jurkat T lymphoma cell line and the B lymphoma cell lines WEHI-231 and Bal17 also were used as controls. In the anti-Fyn immunoprecipitates from both Jurkat and AtT20 lysates, a band of approximately 59 kDa became labeled. No labeled band corresponding to Fyn was observed after anti-Fyn immunoprecipitation from WEHI-231 cell lysates, in agreement with a previous report that WEHI-231 cells have little or no *fyn* mRNA (Law et al., 1992). Although they expressed Fyn, AtT20 cells did not express Lyn, Blk or Lck. Moreover, the AtT20 cells did not express Syk. In the anti-Syk immunoprecipitates from WEHI-231 and Bal17 lysates, but not from AtT20 lysates, a ^{32}P-labeled doublet of approximately 72 kDa was observed. Both bands in the doublet are Syk; purified baculovirus-expressed Syk also autophosphorylates *in vitro* to form two or more bands (Stacey Harmer and A.L.D., unpublished observations). Subsequent immunoblotting experiments (see below) confirmed that Syk protein was not expressed in AtT20 cells. Thus, AtT20 cells expressed at least one Src-family tyrosine kinase implicated in BCR signalling, but they did not express Syk.

Isolation of a cDNA Clone Encoding the Murine Syk Protein Tyrosine Kinase. In order to clone the murine *syk* cDNA, we constructed a murine B cell cDNA library and screened it with both a PCR-generated fragment of

Figure 1.1 Tyrosine Kinase Expression in BCR$^+$ AtT20 Transfectants

The indicated cell lines were lysed in 1% Triton-X 100 lysis buffer. The indicated protein tyrosine kinases were immunoprecipitated separately from cell lysates (for Src-family kinases: 150 μg protein for Jurkat, WEHI-231 and BAL17 lanes, 300 μg for AtT20 lane; for Syk: 250 μg protein for each lane) with antisera specific for p59*fyn*, p53/56*lyn*, p56*lck*, p55*blk*, or Syk. Kinase expression and activity were assessed by autophosphorylation as described in experimental procedures, and reaction products were visualised by autoradiography. The positions of these kinases are indicated.




Jurkat
WEHI-231
BAL17
BCR+ AtT20
p59fyn
p53/56lyn
p56lck
p55blk
Syk

murine *syk* and a fragment of the porcine *syk* cDNA. Four plaques that hybridized positively with each probe were obtained. DNA sequencing of the longest cDNA clone revealed a full length open reading frame encoding the entire murine Syk kinase, as determined by its homolgy with porcine Syk (Taniguchi et al., 1991). Partial sequencing of the other cDNAs revealed that they were fragments of the same gene. The cDNA nucleotide sequence and deduced amino acid sequence of the full length clone are shown in Figure 1.2. An open reading frame is present beginning at nucleotide 220 and terminating at nucleotide 2106. It codes for a 629 amino acid polypeptide with a deduced molecular weight of 71,371 daltons. At the amino acid level, murine Syk is 91.5% and 92.9% identical to porcine Syk (Taniguchi et al., 1991) and human Syk (Law et al., 1994), respectively. It is 56.3% identical to murine ZAP-70 (Gauen et al., 1994), and 55.2% identical to human ZAP-70 (Chan et al., 1992).

Syk Expression and Activity in AtT20 Transfectants. The BCR^+ AtT20 clonal cell line 100-33 was generated, as described in experimental procedures. In turn, 100-33 cells were used to generate BCR^+ AtT20 transfectants that expressed either wild type Syk, catalytically inactive mutated Syk, or wild type Lck. Individual AtT20 clonal transfectants and the B cell lines WEHI-231, Bal17, and A20 were screened by immunoblotting with anti-Syk antisera (Figure 1.3A). The 100-33 and lck10 cell lines were both negative for Syk. Syk was expressed in the syk13, syk38 and syk41 clonal transfectants, and the mutated form of Syk was expressed in the kd17, kd16 and kd21 clonal transfectants. Syk expression in the syk38, syk41 and kd17 clones was similar to that in the WEHI-231 B cell line, whereas it was higher in the syk13 clone, and lower in the kd16 and kd21 clones. Lck was expressed in lck10 cells (data not shown).

Figure 1.2 Nucleotide and Deduced Amino Acid Sequence of Murine *syk*

The two SH2 domains of Syk are underlined with solid lines. The kinase domain is underlined with a dashed line. The lysine which was mutated to alanine to create mutant Syk is boxed. @ represents the stop codon. These sequence data are available from EMBL/GenBank/DDBJ under accession number U36776.

```
   1  CCACTTCCCTAGTTACAGACACCACTTAACAGCAGGAAACCTCCACTTGCTCTCCTCTGC
  61  CTTCTCTCCATTGCAGCCATTCCCATTTTCAAGACTGCCAGTCTGGTCCTTTCAACGTTC
 121  CATGCTGCCTGGTAGCCCGGGAGCCCAGGCCTTCTGTGACTCCAGGACAGGAAGGTACTT
 181  CTCCATAGCGACTTCCCAGAACTCTGAAGGGGTGCAGACATGGCGGGAAGTGCTGTGGAC
                                          M  A  G  S  A  V  D      7
 241  AGCGCCAACCACCTGACCTACTTTTTTGGCAACATCACCCGGGAAGAGGCTGAAGACTAC
       S  A  N  H  L  T  Y  F  F  G  N  I  T  R  E  E  A  E  D  Y   27
 301  CTGGTCCAGGGAGGCATGACCGATGGGCTCTACCTGCTACGCCAGAGCCGCAATTACCTG
       L  V  Q  G  G  M  T  D  G  L  Y  L  L  R  Q  S  R  N  Y  L   47
 361  GGTGGTTTTGCTTTGTCGGTGGCTCACAACAGGAAGGCACACCACTACACCATCGAGAGG
       G  G  F  A  L  S  V  A  H  N  R  K  A  H  H  Y  T  I  E  R   67
 421  GAACTTAATGGCACCTACGCCATCTCCGGGGGCAGGGCCCATGCCAGCCCAGCAGACCTC
       E  L  N  G  T  Y  A  I  S  G  G  R  A  H  A  S  P  A  D  L   87
 481  TGCCATTACCACTCCCAGGAACCTGATGGCCTTATCTGCCTCCTTAAGAAGCCCTTCAAC
       C  H  Y  H  S  Q  E  P  D  G  L  I  C  L  L  K  K  P  F  N  107
 541  CGGCCCCCGGGAGTACAGCCCAAGACCGGACCCTTTGAGGACCTGAAGGAGAACCTCATC
       R  P  P  G  V  Q  P  K  T  G  P  F  E  D  L  K  E  N  L  I  127
 601  AGGGAATATGTGAAACAGACCTGGAACCTTCAGGGCCAGGCTCTGGAGCAAGCCATCATC
       R  E  Y  V  K  Q  T  W  N  L  Q  G  Q  A  L  E  Q  A  I  I  147
 661  AGCCAGAAGCCCCAGCTGGAGAAGCTGATCGCCACCACGGCCCATGAGAAGATGCCCTGG
       S  Q  K  P  Q  L  E  K  L  I  A  T  T  A  H  E  K  M  P  W  167
 721  TTCCATGGCAACATCTCCAGAGATGAATCAGAGCAGACGGTCCTCATAGGGTCAAAGACC
       F  H  G  N  I  S  R  D  E  S  E  Q  T  V  L  I  G  S  K  T  187
 781  AATGGAAAATTCCTGATCAGGGCCAGAGACAACAGCGGCTCCTATGCTCTGTGCCTGCTG
       N  G  K  F  L  I  R  A  R  D  N  S  G  S  Y  A  L  C  L  L  207
 841  CACGAAGGGAAAGTATTGCACTACCGCATTGACAGGGACAAGACCGGGAAGCTCTCCATT
       H  E  G  K  V  L  H  Y  R  I  D  R  D  K  T  G  K  L  S  I  227
 901  CCTGAGGGGAAGAAGTTTGACACCCTCTGGCAGCTAGTGGAACATTACTCTTACAAGCCA
       P  E  G  K  K  F  D  T  L  W  Q  L  V  E  H  Y  S  Y  K  P  247
 961  GATGGGCTACTAAGAGTCCTCACGGTACCATGCCAAAAGATTGGTGCACAGATGGGCCAC
       D  G  L  L  R  V  L  T  V  P  C  Q  K  I  G  A  Q  M  G  H  267
1021  CCAGGAAGCCCAAATGCCCATCCCGTGACTTGGTCACCGGGTGGAATAATCTCAAGGATC
       P  G  S  P  N  A  H  P  V  T  W  S  P  G  G  I  I  S  R  I  287
1081  AAATCCTACTCCTTCCCAAAGCCTGGCCACAAAAAGCCTGCCCCACCCCAAGGGAGCCGT
       K  S  Y  S  F  P  K  P  G  H  K  K  P  A  P  P  Q  G  S  R  307
1141  CCAGAGAGCACTGTGTCCTTCAACCCCTATGAGCCAACGGGAGGGCCCTGGGGCCCAGAC
       P  E  S  T  V  S  F  N  P  Y  E  P  T  G  G  P  W  G  P  D  327
1201  AGAGGCCTTCAGAGAGAAGCCCTGCCCATGGACACAGAGGTGTACGAGAGCCCTTATGCT
       R  G  L  Q  R  E  A  L  P  M  D  T  E  V  Y  E  S  P  Y  A  347
1261  GACCCTGAAGAGATCCGGCCCAAAGAGGTCTACCTGGACAGGAGCCTGCTGACCCTGGAG
       D  P  E  E  I  R  P  K  E  V  Y  L  D  R  S  L  L  T  L  E  367
1321  GACAATGAACTGGGCTCCGGTAACTTCGGGACTGTGAAAAAGGGATACTACCAAATGAAA
       D  N  E  L  G  S  G  N  F  G  T  V  K  K  G  Y  Y  Q  M  K  387
1381  AAAGTTGTGAAAACCGTGGCTGTGAAAATCCTGAAGAACGAGGCCAACGACCCGGCTTTG
       K  V  V  K  T  V  A  V  K  I  L  K  N  E  A  N  D  P  A  L  407
1441  AAGGACGAGCTGCTGGCAGAGGCGAACGTCATGCAGCAGCTGGACAACCCCTACATTGTG
       K  D  E  L  L  A  E  A  N  V  M  Q  Q  L  D  N  P  Y  I  V  427
1501  CGCATGATCGGAATCTGCGAGGCGGAGTCCTGGATGCTGGTGATGGAGATGGCGGAGCTG
       R  M  I  G  I  C  E  A  E  S  W  M  L  V  M  E  M  A  E  L  447
1561  GGGCCGCTCAACAAGTACCTGCAGCAGAACAGGCACATTAAGGATAAGAACATCATAGAG
       G  P  L  N  K  Y  L  Q  Q  N  R  H  I  K  D  K  N  I  I  E  467
1621  CTGGTTCACCAGGTTTCCATGGGGATGAAGTATTTGGAAGAGAGCAACTTTGTGCACAGA
       L  V  H  Q  V  S  M  G  M  K  Y  L  E  E  S  N  F  V  H  R  487
1681  GATCTGGCTGCGCGGAACGTGCTTCTGGTCACACAGCACTATGCCAAGATCAGCGATTTC
       D  L  A  A  R  N  V  L  L  V  T  Q  H  Y  A  K  I  S  D  F  507
1741  GGTCTTTCCAAAGCCCTGCGTGCTGATGAAAACTACTACAAGGCCCAGACCCACGGGAAG
       G  L  S  K  A  L  R  A  D  E  N  Y  Y  K  A  Q  T  H  G  K  527
1801  TGGCCCGTGAAGTGGTACGCCCCCGAATGCATCAACTACTACAAGTTCTCCAGTAAGAGT
       W  P  V  K  W  Y  A  P  E  C  I  N  Y  Y  K  F  S  S  K  S  547
1861  GACGTCTGGAGCTTCGGAGTCCTGATGTGGGAAGCGTTCTCCTATGGGCAGAAGCCCTAC
       D  V  W  S  F  G  V  L  M  W  E  A  F  S  Y  G  Q  K  P  Y  567
1921  AGAGGGATGAAAGGGAGCGAAGTGACCGCCATGCTGGAGAAAGGAGAGCGGATGGGGTGC
       R  G  M  K  G  S  E  V  T  A  M  L  E  K  G  E  R  M  G  C  587
1981  CCTGCAGGATGCCCGAGAGAGATGTACGACCTGATGAACCTGTGCTGGACTTACGATGTG
       P  A  G  C  P  R  E  M  Y  D  L  M  N  L  C  W  T  Y  D  V  607
2041  GAGAACAGGCCAGGATTCACAGCTGTGGAACTGAGGCTTCGCAATTACTACTACGACGTG
       E  N  R  P  G  F  T  A  V  E  L  R  L  R  N  Y  Y  Y  D  V  627
2101  GTTAACTAACAGCTGGCGCCGCCTGTCGTGCACACCACGGATTCCCAAGCGATCACAGGA
       V  N  @
2161  AATTCATTCAGATGAACTGGCTCTCAGAGTTTCATCTCCCTCTGCCCGGAGTGAGAGCTA
2221  AGCTAACATAGGACTCACCCTCACAACAGGTCTGGTGCCCAGAGACAGACAAGCAGCAAG
2281  ACCTTGGGGC
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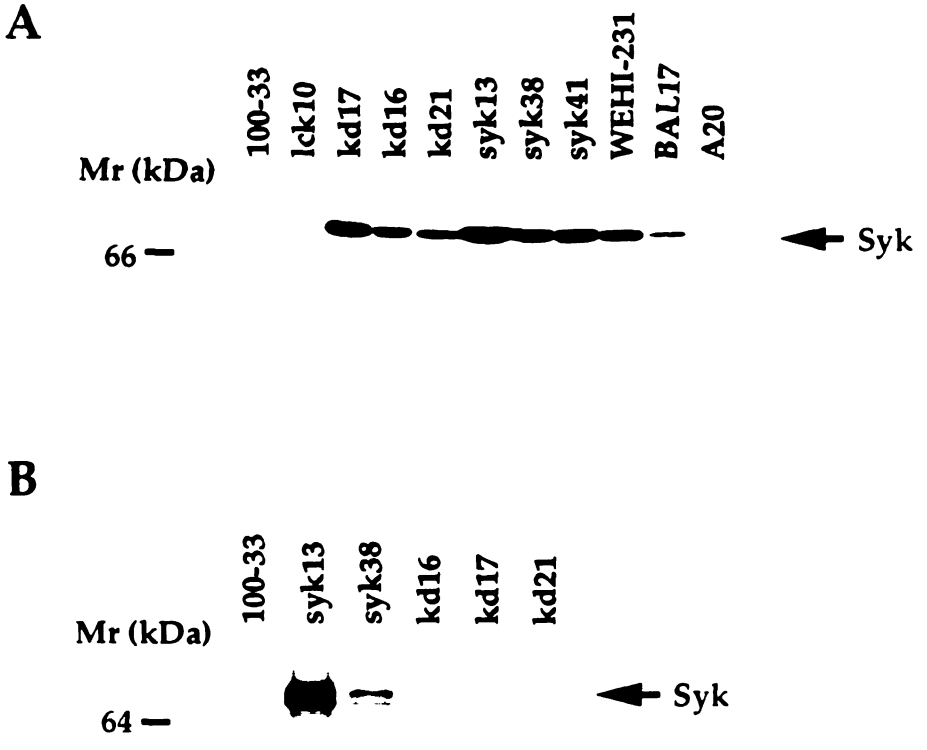
Figure 1.3 Syk Expression and Activity in AtT20 Transfectants and B Cell Lines

(A) Relative levels of Syk expression were determined from the 100-33 parental BCR-expressing AtT20 cells, and from 100-33 cells transfected with *lck* (lck10), with wild type *syk* (syk13, syk38 and syk41), or with mutated *syk* (kd17, kd16 and kd21), as well as from the B cell lines WEHI-231, Bal17, and A20. Cell lysates (15 μg protein per lane) were separated on an 8% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with anti-Syk antisera, as described in Experimental Procedures. The position of Syk is indicated. (B) Syk activity was determined from the indicated cells. Cell lysates (250 μg) were immunoprecipitated with anti-Syk antisera, and the ability of Syk to autophosphorylate was determined, as in Figure 1.1. The position of the labelled Syk doublet is indicated. The positions of molecular weight markers are indicated on both panels.

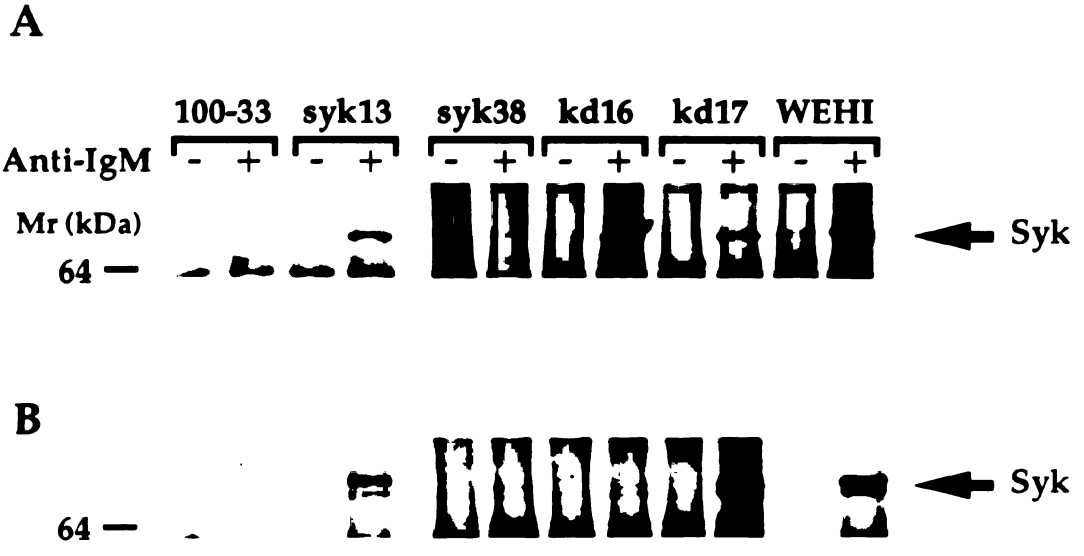

A
100-33
lck10
kd17
kd16
kd21
syk13
syk38
syk41
WEHI-231
BAL17
A20
Mr (kDa)
66
Syk
B
100-33
syk13
syk38
kd16
kd17
kd21
Mr (kDa)
64
Syk

The catalytic activity of Syk was analyzed in several AtT20 clones expressing either wild type or mutated Syk. Syk was immunoprecipitated and *in vitro* autophosphorylation reactions were performed. The syk13 and syk38 transfectants expressed catalytically active Syk, as shown by the labelled doublet at 72 kDa (Figure 1.3B). In contrast, no Syk activity was immunoprecipitated from kd16, kd17, or kd21, even though the mutated Syk protein was expressed (Figure 1.3A). These results confirmed that the mutated Syk was catalytically inactive.

Association of Both Wild Type and Kinase Inactive Syk with the BCR. Syk associates with the BCR in B cells, and upon BCR stimulation the extent of this association increases and Syk becomes tyrosine phosphorylated (Hutchcroft et al., 1992; Law et al., 1993; Yamada et al., 1993). We determined whether Syk was regulated similarly in the AtT20 transfectants. Cell lysates from unstimulated and anti-IgM stimulated cells were immunoprecipitated with an anti-Ig-α antiserum. The immunoprecipitates were split, and examined by immunoblotting with either an anti-Syk antiserum or with the anti-phosphotyrosine monoclonal antibody 4G10 (Figures 1.4A and 1.4B, respectively). In the Syk-expressing AtT20 transfectants, as well as in WEHI-231 cells, there was often a low basal level of association between Ig-α/Ig-β and Syk (Figure 1.4A). However, the amount of BCR-associated Syk clearly increased upon anti-IgM stimulation. The syk38 transfectant was atypical in that it generally had only a very modest level of Syk associated with the BCR. Anti-phosphotyrosine immunoblotting revealed that both wild type and kinase inactive BCR-associated Syk from stimulated AtT20 cells were tyrosine phosphorylated, and that there was no consistent difference in the extent of tyrosine phosphorylation between these two populations of Syk. As with autophosphorylated Syk (Figures 1.1 and 1.3B), both bands in the doublet are

Figure 1.4 Anti-IgM-Induced Syk/BCR Association and Tyrosine Phosphorylation of BCR-Associated Syk

The indicated cells were incubated for 5 minutes with or without 20 μg/ml anti-IgM and lysed in 1% Triton X-100 lysis buffer. Lysates (2.5 mg for AtT20 transfectants, 1.5 mg for WEHI-231 cells) were immunoprecipitated with an anti-Ig-α antiserum. The immunoprecipitates were split into two fractions, each of which was separated on a 7.5% gel by SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes and immunoblotted with either anti-Syk antisera (A) or the anti-phosphotyrosine mAb 4G10 (B). The WEHI-231 lanes in (B) were exposed for substantially less time than the rest of the lanes on that membrane (25 seconds versus 15 minutes). The membranes were then stripped of bound antibody and reprobed with anti-Ig-α antisera to verify equal loading had occurred (not shown).

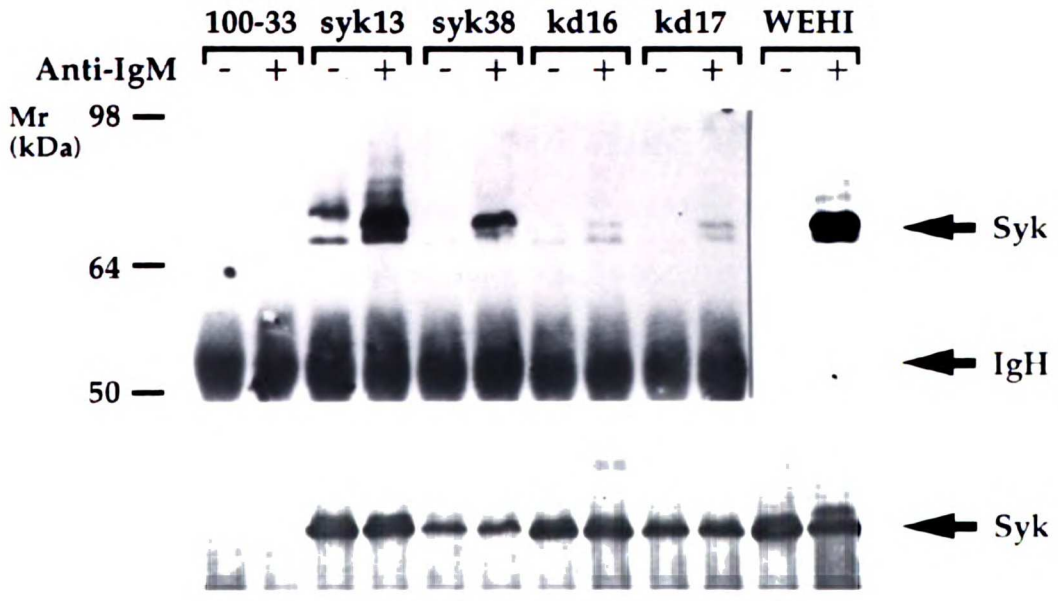

A
100-33
syk13
syk38
kd16
kd17
WEHI
Anti-IgM
- + - + - + - + - + - +
Mr (kDa)
64
Syk
B
64
Syk

Syk; the upper band is a more highly phosphorylated form of Syk, and is recognized by our anti-Syk antisera, although inefficiently (See Figure 1.5, especially the WEHI-231 lanes) Whereas WEHI-231 cells appeared to have a similar level of Syk/BCR association as that seen in the AtT20 transfectants, the WEHI-231 BCR-associated Syk was tyrosine phosphorylated to a much greater degree. The reasons for this difference are not known. However, these experiments demonstrate that Syk/BCR association and Syk tyrosine phosphorylation were regulated in AtT20 cells as they are in B cells. The observation that kinase inactive Syk also associated with Ig-α/Ig-β and became tyrosine phosphorylated upon stimulation demonstrates that Syk kinase activity was not required for association with the BCR, and that Syk must have been phosphorylated by a separate tyrosine kinase.

Differential Tyrosine Phosphorylation of Wild Type and Kinase Inactive Syk Upon BCR Crosslinking. Only a small fraction of total cellular Syk could be immunoprecipitated with Ig-α/Ig-β. In order to examine the effect of BCR-crosslinking on the tyrosine phosphorylation of total cellular Syk, Syk was immunoprecipitated directly from lysates of unstimulated or anti-IgM stimulated cells. Its tyrosine phosphorylation state was then examined by anti-phosphotyrosine immunoblotting (Figure 1.5). The syk13 and syk38 clones exhibited a strong induction of Syk tyrosine phosphorylation upon BCR crosslinking, although this phosphorylation was less robust than in WEHI-231 cells. The kd16 and kd17 transfectants also showed a BCR-induced tyrosine phosphorylation of kinase inactive Syk, but consistently to a lesser extent than seen with wild type Syk. This difference was not due to a smaller amount of catalytically inactive Syk being immunoprecipitated, as shown by reprobing this blot with anti-Syk antisera (Figure 1.5). Thus, BCR crosslinking induced a more extensive tyrosine phosphorylation of wild type

Figure 1.5 BCR-Induced Tyrosine Phosphorylation of Total Cellular Syk

The indicated cells were incubated for 5 minutes with or without 20 μg/ml anti-IgM and lysed in 1% Triton X-100 lysis buffer. For each lane, 1 mg of lysate was immunoprecipitated with an anti-Syk antiserum. Immunoprecipitates were separated by SDS-PAGE (8% gel) and immunoblotted with the anti-phosphotyrosine mAB 4G10 (upper panel). The lack of visible IgH in the WEHI-231 lanes reflects a shorter exposure time for these lanes than for the AtT20 lanes (1 minute versus 20 minutes). The membrane was then stripped of bound antibody and reprobed with anti-Syk antisera, and all lanes were exposed for the same amount of time (lower panel).

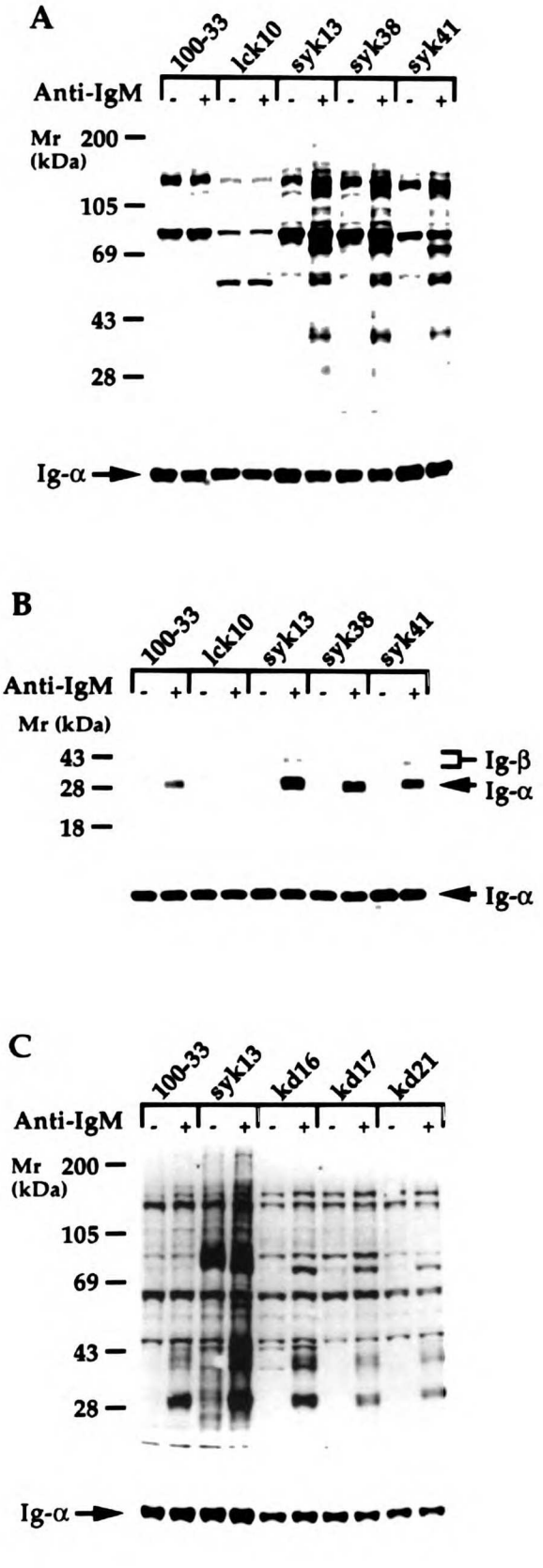

100-33
syk13
syk38
kd16
kd17
WEHI
Anti-IgM
- + - + - + - + - + - +
Mr (kDa)
98
64
50
Syk
IgH
Syk

Syk than catalytically inactive Syk, although this difference was not seen in the population of Syk associated with the BCR (Figure 1.4B). One possible explanation for these results is that in addition to being phosphorylated by a separate tyrosine kinase, wild type Syk was able to autophosphorylate and dissociate from the BCR.

Syk Reconstitution of BCR-Induced Tyrosine Phosphorylation of Cellular Proteins. A direct consequence of the rapid activation of tyrosine kinases upon BCR stimulation in B cells is the tyrosine phosphorylation of a variety of cellular proteins (Brunswick et al., 1991; Campbell and Sefton, 1990; Gold et al., 1990; Lane et al., 1990). These effects were largely absent in the BCR-expressing AtT20 cells which lacked Syk (Matsuuchi et al., 1992). In order to test whether Syk expression would reconstitute these events, we examined lysates from unstimulated and anti-IgM stimulated AtT20 transfectants by immunoblotting with the anti-phosphotyrosine mAb, 4G10. In the 100-33 cells, BCR crosslinking weakly induced the tyrosine phosphorylation of 30-40 kDa proteins which included Ig-α and Ig-β (Figure 1.6A), as previously reported (Matsuuchi et al., 1992). However, a general increase in the number and intensity of tyrosine phosphorylated bands was not seen. In contrast, the wild type Syk-expressing clones exhibited a dramatic increase in both the number and intensity of tyrosine phosphorylated bands upon anti-IgM stimulation. The pattern of induced bands was similar in the different clones, and included the same 30-40 kDa bands as were seen in the 100-33 cells. The anti-IgM-induced bands also included a prominent band just above the 69 kDa marker, which was probably Syk (see above). Unlike the Syk-expressing transfectants, the Lck-expressing transfectant lck10 did not exhibit a BCR-induced increase in tyrosine phosphorylated proteins. In fact, compared to the 100-33 cells, Lck expression often led to a decrease in the

Figure 1.6 Anti-IgM-Induced Increase in Protein Tyrosine Phosphorylation in AtT20 Transfectants Expressing Wild Type but not Catalytically Inactive Syk

(A) The indicated cell lines were left unstimulated or stimulated for 5 minutes with 20 μg/ml anti-IgM. Cell lysates (10 μg protein) were separated on a 7.5% SDS-PAGE gel and immunoblotted with the anti-phosphotyrosine mAb, 4G10 (upper panel). The blot was stripped of bound antibodies and reprobed with anti-Ig-α antisera to examine loading integrity (lower panel). (B) Cell lysates (400 μg protein) used in (A) were immunoprecipitated with anti-Ig-α antisera, and the precipitates were separated on a 10% SDS-PAGE gel and immunoblotted with 4G10 (upper panel). The positions of Ig-α and Ig-β are indicated. The blot was then stripped and reprobed with anti-Ig-α as in (A) (lower panel). (C) Cell lysates were prepared and analyzed as in (A), except that 18 μg of protein were loaded in each of the first 4 lanes, and 15 μg in the last 6 lanes.


A
100-33
lck10
syk13
syk38
syk41
Anti-IgM
- + - + - + - + - +
Mr 200
(kDa)
105
69
43
28
Ig-α
B
100-33
lck10
syk13
syk38
syk41
Anti-IgM
- + - + - + - + - +
Mr (kDa)
43
28
18
Ig-β
Ig-α
Ig-α
C
100-33
syk13
kd16
kd17
kd21
Anti-IgM
- + - + - + - + - +
Mr 200
(kDa)
105
69
43
28
Ig-α

extent of tyrosine phosphorylation. Thus, Syk fulfills a unique role in BCR signalling that cannot be performed by Lck.

The increased tyrosine phosphorylation of the 30-40 kDa proteins suggested that upon anti-IgM stimulation, Ig-α and Ig-β were more highly phosphorylated in the Syk-expressing transfectants than in the parental 100-33 cells. To confirm this interpretation, the Ig-α/Ig-β heterodimer was immunoprecipitated from these cells and their level of tyrosine phosphorylation was determined by immunoblotting (Figure 1.6B). This experiment demonstrated that the tyrosine phosphorylation of Ig-α and Ig-β was increased by BCR crosslinking, and that this response was enhanced in the Syk-expressing clones.

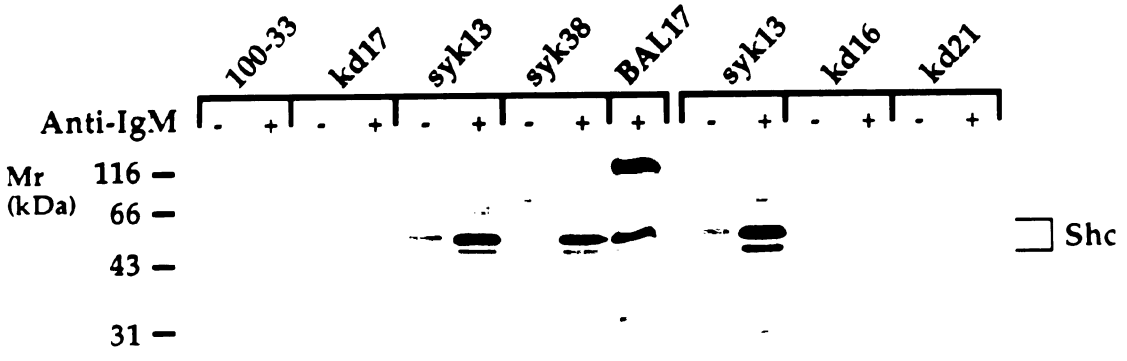
Although tyrosine kinases generally require their kinase activity to promote signalling, their ability to interact with other proteins may allow them to perform distinct signalling functions that do not require a functional kinase domain. For example, a kinase-independent activity of Lck in coreceptor-assisted T cell activation has been observed (Xu and Littman, 1993). Therefore, we tested whether Syk kinase activity was required for the tyrosine phosphorylation events seen in the AtT20 cells. AtT20 transfectants expressing the BCR and catalytically inactive Syk were stimulated with anti-IgM antibodies and examined for protein tyrosine phosphorylation (Figure 1.6C). To better visualize the anti-IgM-induced increase in tyrosine phosphorylation of the 30-40 kDa proteins seen in the 100-33 cells, more protein was loaded in each lane than in the experiment shown in Figure 1.6A. In contrast to the syk13 transfectant, the kd16, kd17, and kd21 cells did not exhibit a general anti-IgM-induced increase in protein tyrosine phosphorylation. Transfectants expressing catalytically inactive Syk did show an increase in tyrosine phosphorylation of the 30-40 kDa proteins. However,

unlike with the wild-type Syk-expressing clones, this increase was similar in magnitude to the increase seen in the 100-33 cells. BCR-stimulation of the catalytically inactive Syk-expressing transfectants also induced the tyrosine phosphorylation of another band just above the 69 kDa marker, which was not seen in the 100-33 cells. This upper band was probably the catalytically inactive Syk protein (see above). Thus, Syk expression reconstituted the tyrosine phosphorylation of a variety of cellular proteins upon BCR stimulation, and the kinase activity of Syk was required for this reconstitution.

BCR-Induced Tyrosine Phosphorylation of Shc. We next wanted to determine whether Syk expression would also reconstitute the activation of BCR-associated signalling pathways. Stimulation by a variety of receptors, including the BCR, leads to extensive tyrosine phosphorylation of the adapter protein Shc (Saxton et al., 1994; Smit et al., 1994), which allows Shc to participate in the activation of Ras (Pawson, 1995). We therefore examined the ability of BCR stimulation to induce the tyrosine phosphorylation of Shc in the AtT20 transfectants. Shc immunoprecipitates from lysates of unstimulated and anti-IgM stimulated AtT20 cells were examined by immunoblotting with anti-phosphotyrosine antibodies (Figure 1.7). As was seen in B cells, anti-IgM treatment induced Shc tyrosine phosphorylation in the transfectants expressing wild type Syk. In contrast, BCR crosslinking did not induce Shc phosphorylation in the 100-33 cells or in the transfectants expressing catalytically inactive Syk. The lck10 cells also failed to tyrosine phosphorylate Shc upon stimulation (data not shown). Thus, Syk kinase activity was required to reconstitute the BCR-induced tyrosine phosphorylation of Shc.

Figure 1.7 BCR-Induced Tyrosine Phosphorylation of Shc

The indicated cells were incubated with or without 20 μg/ml anti-IgM for 5 minutes. The Shc in cell lysates (1 mg protein) was immunoprecipitated with anti-Shc antibodies, resolved on 10.5% SDS-PAGE gels and immunoblotted with the anti-phosphotyrosine mAb, 4G10. The position of Shc is indicated.

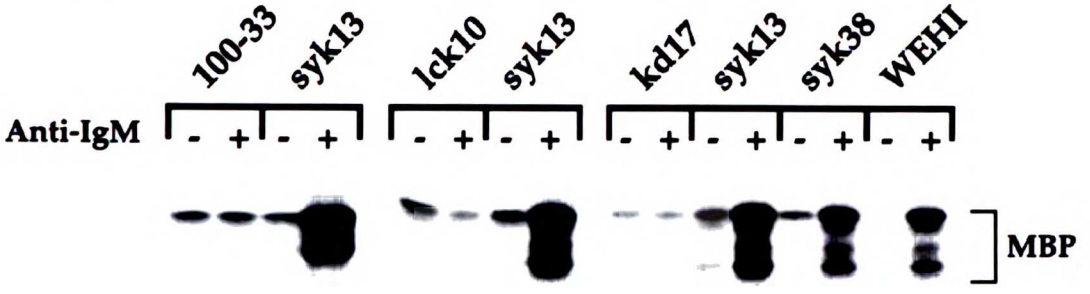

100-33
kd17
syk13
syk38
BAL17
syk13
kd16
kd21
Anti-IgM
- + - + - + - + + - + - + - +
Mr (kDa)
116
66
43
31
Shc

Reconstitution of BCR-Induced MAP kinase Activation by Wild Type Syk. The MAP kinases are a family of serine/threonine kinases that are activated through multiple signalling pathways by a variety of receptors (Marshall, 1994), including the BCR (Casillas et al., 1991; Gold et al., 1992). These kinases phosphorylate a number of transcription factors, including $p62^{TCF}$ (Gille et al., 1992) and NF-IL6 (Nakajima et al., 1993), and may therefore mediate anti-Ig induced changes in gene expression. To examine whether Syk expression would reconstitute MAP kinase activation, the p42 ERK2 form of MAP kinase was immunoprecipitated from lysates of unstimulated or anti-IgM stimulated cells, and was subjected to an *in vitro* kinase assay using myelin basic protein (MBP) as an exogenous substrate. MBP phosphorylation was visualized by SDS-PAGE followed by autoradiography (Figure 1.8A) and quantified by scintillation counting (Figure 1.8B). The 100-33 cell line did not exhibit a significant increase in p42 MAP kinase activity upon anti-IgM stimulation. In contrast, anti-IgM treatment of the syk13 and syk38 clones increased MAP kinase activity to an average of 6.9 and 3.4 times background, respectively. Higher Syk expression and/or BCR association in the syk13 cells than in the syk38 cells may explain the difference in the magnitude of MAP kinase activation between these two transfectants. The activity of p42 MAP kinase in the anti-IgM-stimulated syk13 and syk38 clones was similar in magnitude to that in anti-IgM-stimulated WEHI-231 cells. In contrast to the wild type Syk-expressing transfectants, anti-IgM caused little or no increase in p42 MAP kinase activity in the cells expressing catalytically inactive Syk. The lck10 clone also failed to activate MAPK upon stimulation. Additionally, anti-IgM stimulation of wild type Syk-expressing transfectants resulted in decreased electrophoretic mobility of both the p42

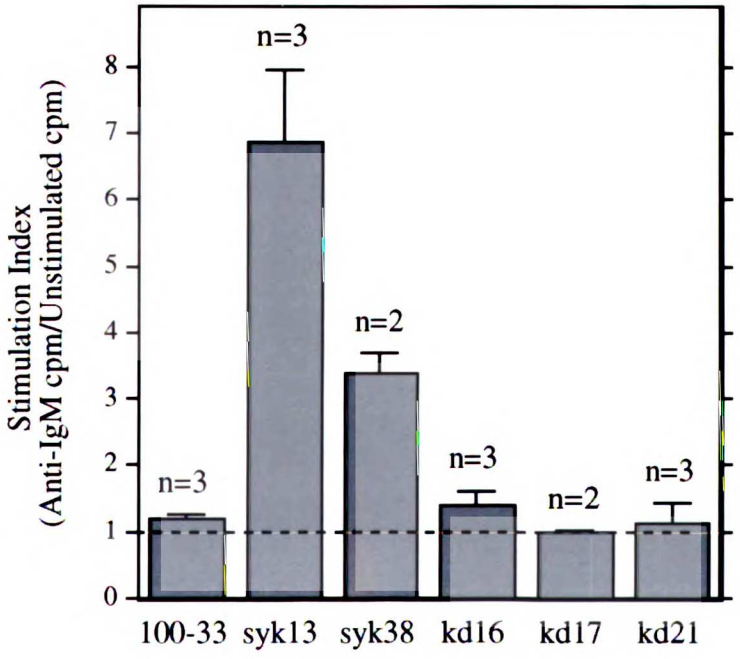
Figure 1.8 BCR-Induced Activation of p42 MAP Kinase in AtT20 Transfectants Expressing Wild Type Syk

(A) The indicated cell lines were incubated with or without 20 μg/ml anti-IgM for 5 minutes. The p42 ERK2 isoform of MAP kinase was immunoprecipitated from cell lysates (0.5 mg protein). The activity of the immunoprecipitated p42 MAP kinase was assessed by *in vitro* kinase assay using myelin basic protein (MBP) as a substrate. Reaction products were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. Autoradiograms from 3 representative experiments are shown. Immunoblotting with anti-MAP kinase antibodies indicated that similar amounts of MAP kinase were precipitated from each sample (not shown). (B) To quantify the effects of BCR crosslinking o p42 MAP kinase activity, the MBP bands were excised and counted. The anti-IgM-induced increases in MAP kinase activity for the various cell lines are shown (mean +/- S.E., n= number of independent cell stimulations assayed in separate experiments).

A


100-33
syk13
lck10
syk13
kd17
syk13
syk38
WEHI
Anti-IgM
- + - + - + - + - + - + - + - +
MBP

B


Stimulation Index
(Anti-IgM cpm/Unstimulated cpm)
0
1
2
3
4
5
6
7
8
n=3
n=3
n=2
n=3
n=2
n=3
100-33
syk13
syk38
kd16
kd17
kd21

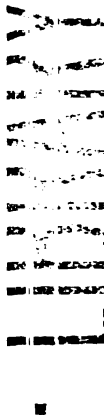
and p44 forms of MAP kinase, which is a characteristic of MAP kinase phosphorylation. This change in mobility was not observed in the 100-33 cells, the lck10 cells, or in the transfectants expressing kinase inactive Syk (data not shown).

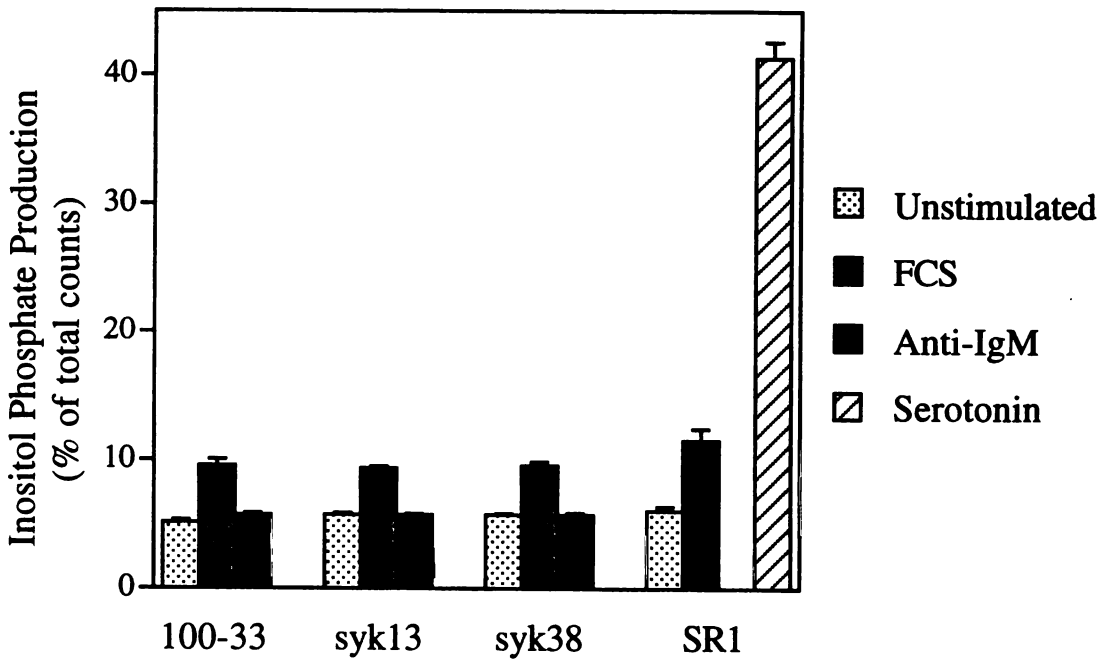
Failure of Syk to Reconstitute Inositol Phosphate Production or [Ca^{++}] Elevation in AtT20 Cells. The activation of the phosphoinositide signalling pathway is another important BCR-mediated event in B cells. Inositol-containing phospholipids are hydrolyzed by phospholipase C (PLC) to generate inositol phosphates and diacylglycerol, which lead to an elevation in intracellular free Ca^{++} and the activation of protein kinase C, respectively. These signalling pathways are important for BCR-dependent gene induction (DeFranco, 1993; Mittelstadt and DeFranco, 1993) and apoptosis in an immature B cell line (DeFranco, 1992). We previously reported that BCR^+ Syk^- AtT20 clones do not generate inositol phosphates upon anti-IgM stimulation (Matsuuchi et al., 1992). Therefore, we tested whether Syk expression would reconstitute this pathway in response to anti-IgM. As previously reported (Matsuuchi et al., 1992), FCS stimulation of all the AtT20 cell lines, as well as serotonin treatment of the serotonin receptor-expressing transfectant, SR1, induced increases in inositol phosphate production over the level observed in unstimulated cells (Figure 1.9). In contrast, neither the 100-33 cells nor the syk13 or syk38 transfectants exhibited an increase in inositol phosphate production in response to anti-IgM stimulation.

We also tested these cell lines for increases in intracellular free Ca^{++} in response to FCS, serotonin, and anti-IgM treatment. The AtT20 cells were grown in coverglass chambers, loaded with the calcium-sensitive dye indo-1, and stimulation-induced changes in intracellular free calcium were monitored at the single-cell level by image cytometry (Szöllösi et al., 1991).

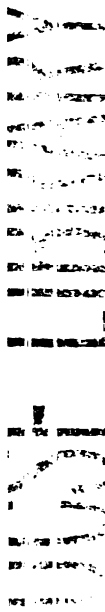
Figure 1.9 Failure of Syk to Reconstitute BCR-Induced Inositol Phosphate Production

The inositol-containing phospholipids of AtT20 transfectants were labelled overnight with ^{3}H-inositol. Cells were left unstimulated, or stimulated for 30 minutes with 10% dialyzed FCS, 20 μg/ml anti-IgM or 1 μM serotonin, as indicated. Cells were lysed and total inositol phosphate generation was measured, as described in Experimental Procedures. Inositol phosphate production is calculated as the ^{3}H present in the inositol phosphates divided by the ^{3}H present in inositol phosphates plus phosholipids, and is expressed as a percentage. Values shown are the mean and SEM of triplicate samples. The experiment shown is representative of between 3 and 6 experiments, depending on the cell line.




Inositol Phosphate Production
(% of total counts)
40
30
20
10
0
100-33
syk13
syk38
SR1
Unstimulated
FCS
Anti-IgM
Serotonin

Small Ca^{++} increases were observed after stimulation of all the AtT20 cell lines with FCS or after stimulation of the SR1 cells with serotonin. In contrast, we did not observe a Ca^{++} increase in the 100-33, syk13 or syk38 lines in response to anti-IgM antibodies (data not shown).



Discussion

As a strategy for determining the requirements for signal transduction by the BCR, we expressed this receptor in the non-lymphoid AtT20 cell line. Signalling events observed in B cells, such as rapid tyrosine phosphorylation of many cellular proteins, did not occur in the BCR^+ AtT20 transfectants. We found that AtT20 cells expressed a Src-family tyrosine kinase implicated in BCR signalling, Fyn, but did not express the Syk tyrosine kinase. Therefore, we isolated a full length murine *syk* cDNA, and used it to express Syk in the BCR^+ AtT20 cells. Syk expression was sufficient to reconstitute in a stimulation-dependent manner numerous BCR-induced signalling events, including the association of Syk with the BCR, the tyrosine phosphorylation of numerous cellular proteins including Syk and Shc, and the activation of MAP kinase. In contrast, BCR crosslinking of these cells failed to activate the phosphoinositide signalling pathway. The kinase activity of Syk was required for the reconstitution of downstream signalling events, although it was not required to recruit Syk to the BCR or for Syk tyrosine phosphorylation. Unlike Syk expression, Lck expression could not reconstitute any of these signalling events. These results demonstrate that Syk is a key component in BCR signalling, and suggest that other lymphoid-specific components are not required for many signalling events triggered by BCR ligation in B cells. In addition, these results are complementary to data from a chicken B cell line which was rendered deficient for Syk expression, in that these Syk^- B cells exhibited profound defects in BCR signalling (Takata et al., 1994).

Several features of our results are consistent with a model of how antigen receptors activate tyrosine kinases and thus initiate signalling (Law et

al., 1993; Weiss, 1993). Clustering of antigen receptors leads to phosphorylation of the cytoplasmic domains of receptor subunits on tyrosines present in a conserved sequence now called the Immunoreceptor Tyrosine-based Activation Motif (ITAM) (Cambier, 1995). According to the model, this initial phosphorylation is mediated by one or more Src-family kinases. In B cells, Syk then binds via its SH2 domains to phosphorylated Ig-α/Ig-β ITAMs, while in T cells the Syk homologue ZAP-70 binds to phosphorylated TCR subunit ITAMs. Syk or ZAP-70 becomes activated by this ITAM binding and/or by subsequent tyrosine phosphorylation (Rowley et al., 1995; Shiue et al., 1995), and phosphorylates downstream signalling targets.

Consistent with this model, we have found substantial Ig-α and Ig-β phosphorylation upon BCR crosslinking in both the Syk^- and Syk^+ AtT20 cells. The AtT20 cells were found to express Fyn, so it is possible that Fyn is responsible for this phosphorylation. This possibility is supported by observations that Fyn can associate with Ig-α and Ig-β *in vivo*, even before BCR crosslinking (Law et al., 1993), and can phosphorylate a GST-Ig-α fusion protein *in vitro* (Flaswinkel and Reth, 1994). Also in agreement with the model, whereas a small amount of Syk often could be coprecipitated with Ig-α/Ig-β prior to stimulation, the amount of Syk bound to the BCR clearly increased following anti-IgM treatment. This increase occurred with both wild type Syk and catalytically inactive Syk, and both forms of BCR-associated Syk also became tyrosine phosphorylated upon stimulation. This last observation suggests that the initial tyrosine phosphorylation of Syk was mediated by a separate kinase, possibly Fyn. This interpretation is also supported by the observation that Src-family kinases can phosphorylate Syk and ZAP-70 in COS cells (Iwashima et al., 1994; Kurosaki et al., 1994) and *in vitro* (Watts et al., 1994). In addition, Syk has been shown to interact directly

with the Src-family kinase Lyn *in vivo* (Sidorenko et al., 1995). In our experiments, BCR-associated wild type and kinase inactive Syk were tyrosine phosphorylated to a similar extent following BCR crosslinking. In contrast, when total cellular Syk was examined, it was found that wild type Syk was tyrosine phosphorylated much more extensively upon stimulation than was catalytically inactive Syk. The simplest explanation for this difference is that wild type Syk can undergo autophosphorylation either immediately before dissociating from the BCR, or after dissociation occurs. Alternatively, the increased tyrosine phosphorylation of total cellular Syk may have been an indirect result of a Syk-mediated event, such as the inhibition of a protein tyrosine phosphatase.

A key feature of the model described above is that Syk phosphorylates downstream signalling targets. Consistent with this prediction, anti-IgM stimulation led to the increased tyrosine phosphorylation of numerous cellular proteins, including Shc, in transfectants expressing wild type but not catalytically inactive Syk. These results are consistent with recently reported experiments which showed that Syk kinase activity greatly enhanced Shc phosphorylation over what was seen in Syk$^-$ chicken B cells (Nagai et al., 1995). In addition, our experiments demonstrated that no additional lymphoid-specific components were required for BCR-induced tyrosine phosphorylation of cellular proteins.

One consequence of the BCR-induced tyrosine phosphorylation of cellular target proteins is the activation of multiple signalling pathways. One important signalling pathway activated by BCR crosslinking in B cells is the MAP kinase pathway (Casillas et al., 1991; Gold et al., 1992). MAP kinase can phosphorylate a number of transcription factors, and may thus tie receptor-mediated signalling events to changes in gene expression and biological

activity. Indeed, in many cell types MAP kinase activation has been associated with the regulation of cellular proliferation, differentiation, secretion, and metabolic activity (Marshall, 1994). Expression of wild type but not catalytically inactive Syk enabled the BCR to activate p42 MAP kinase in AtT20 cells. The level of activation of MAP kinase in the AtT20 transfectants was similar to that seen in the WEHI-231 B cells. The mechanism by which the BCR activates MAP kinase in B cells is not known at this time, although there is evidence for both protein kinase C (PKC)-dependent and PKC-independent mechanisms of activation (Gold et al., 1992). BCR signalling leads to a PKC-independent increase in the active form of Ras in B cells (Harwood and Cambier, 1993; Lazarus et al., 1993), and Ras can initiate a kinase cascade that activates MAP kinase. Therefore, it seems likely that BCR-induced activation of MAP kinase in B cells occurs at least in part via Ras. Although we have not determined how BCR stimulation activates MAP kinase in AtT20 cells, we have shown that BCR stimulation of these cells leads to tyrosine phosphorylation of Shc. Shc can form a phosphorylation-dependent complex with both the adapter protein Grb2 and the Ras guanine nucleotide exchange factor mSOS, and can thereby participate in the activation of Ras (Pawson, 1995). Thus, it seems likely that MAP kinase was activated through the Ras pathway in the AtT20 cells as well. In contrast, it seems unlikely that MAP kinase was activated in the AtT20 cells in a PKC-dependent manner, since BCR crosslinking of these cells apparently did not activate phospholipase C (see below), which is upstream of PKC.

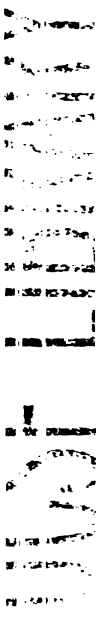
Despite its ability to lead to the activation of MAP kinase, Syk expression in AtT20 cells was not sufficient to allow BCR-induced activation of the phosphoinositide signalling pathway. In B cells, PLC-γ1 and/or PLC-γ2 is activated by tyrosine phosphorylation to hydrolyze inositol-containing

phospholipids to generate inositol phosphates and diacylglycerol (DeFranco, 1993). These two second messengers then mediate the release of intracellular calcium stores and the activation of PKC, respectively. Although this pathway was not activated in the AtT20 cells, PLC-γ1 was tyrosine phosphorylated upon anti-IgM treatment in the wild type Syk-expressing transfectants (data not shown). It is possible that the AtT20 PLC-γ1 was not phosphorylated extensively enough or on the proper tyrosine residues to become activated, or that it failed to localize to the cell membrane where its substrate resides. It has been reported previously that chicken B cells lacking Syk activity do not activate PLC-γ2 in response to BCR crosslinking, demonstrating that Syk is necessary for this activation to occur (Takata et al., 1994). Our experiments indicate that Syk activity, while perhaps necessary, is not sufficient to link the BCR to the activation of PLC. Other B cell proteins in addition to Syk may be needed to couple the BCR to PLC-γ1, perhaps by localizing PLC-γ1 to the cell membrane. We are currently investigating what these additional proteins may be.

The conclusion that Syk plays a unique role in BCR signalling events was underscored by the observation that Lck did not functionally substitute for Syk to restore BCR-induced signalling. Presumably, the inability of Lck to substitute for Syk reflects important structural and functional differences between Src-family kinases and Syk. However, since AtT20 cells do not express CD45 (data not shown), a protein tyrosine phosphatase that can dephosphorylate the negative regulatory tyrosines of Src-family kinases (Weiss and Littman, 1994), it is also possible that Lck could not be fully activated in these cells.

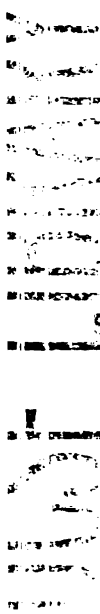
We have generated a heterologous system that allows us to identify, via a gain-of-function strategy, the lymphoid-specific proteins that are

sufficient to link the BCR to the activation of downstream signalling events. Thus far, we have focused on the Syk tyrosine kinase. This reconstitution strategy is complementary to the loss-of-function gene knockout approach, which can identify proteins that are necessary for BCR signalling, but which does not address whether these proteins are sufficient. In previous reconstitution experiments performed in COS cells (Chan et al., 1992; Couture et al., 1994; Iwashima et al., 1994; Kurosaki et al., 1994), Src-family kinases and either Syk or ZAP-70 were expressed at very high levels, and could interact and become activated even in the absence of receptor expression or stimulation. In contrast, in the system employed here, both Syk and the BCR were expressed at levels comparable to what is seen in B cells. Moreover, Syk/BCR association, Syk activation and BCR signalling all occurred in a stimulation-dependent manner, indicating that physiologically relevant interactions between Syk, Fyn, and the BCR were likely occurring as they would in B cells. Thus, the experiments described here further establish that Syk is necessary for BCR signalling. We have also demonstrated that activation of Syk is sufficient to couple the BCR to downstream signalling events such as tyrosine phosphorylation of Shc and activation of MAP kinase, but is not sufficient for activation of the phosphoinositide signalling pathway.



Acknowledgments:

I thank Andy Chan and Art Weiss for PCR primers, Vivien Chan for the affinity purified anti-Lyn and anti-Blk antibodies, Bill Hyun for assistance with the ACAS 470, and Graham Redgrave for assistance with sequence analysis. I am also grateful to Vivien Chan, Mary Crowley, Sandip Datta, Julie Hambleton, Stacey Harmer, Janice Kim, Debbie Law, Trish Roth and Steve Weinstein for their critical reading of the manuscript. This work was supported by the National Institute of Health grants R01AI20038 and T32AI07334-07, the British Columbia Health Research Foundation grant #99 (94-1), the Medical Research Council of Canada grant MT11528, the National Science and Engineering Research Council of Canada grant OGP0121457 and funds from the University of British Columbia.



Chapter 2: Further attempts to reconstitute BCR-mediated signaling in AtT20 cells by expressing a CD19 chimera and/or the Btk protein tyrosine kinase

Introduction

The data in the previous chapter demonstrated that the Syk tyrosine kinase is sufficient to functionally link the BCR to the Shc-Ras-MAP kinase pathway, but not to the phospholipase C (PLC) pathway. These results suggested that the AtT20 cells may lack additional B cell signaling machinery that links the BCR to PLC. This chapter describes experiments that attempted to further reconstitute BCR-induced signaling events in the AtT20 cell system. Specifically, reconstitution of BCR-induced activation of PI 3-kinase and PLC in the BCR- and Syk-expressing AtT20 cells was attempted by expressing a CD19 chimera with or without the Btk tyrosine kinase.

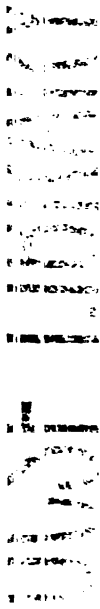
CD19 is a potent enhancer of BCR stimulation, and has been proposed by many to be a "co-receptor" for the BCR similar to CD4 and CD8 for the TCR (Carter and Fearon, 1992; Carter et al., 1991; Chalupny et al., 1993; Fearon and Carter, 1995; Tedder et al., 1994; Weiss and Littman, 1994). CD19 in mature B cells can exist in a complex with the complement receptor CR2 (CD21), and complement-bound antigens could therefore co-ligate the BCR with CD19 (Fearon and Carter, 1995; Tedder et al., 1994). Co-ligating CD19 (Carter et al., 1991) or CD21(Carter et al., 1988)with the BCR synergistically enhances activation of PLC. The CD19 transmembrane and cytoplasmic domains are sufficient for this synergy (Rigley et al., 1989). Moreover, CD19-BCR crosslinking decreases the threshold for BCR-dependent stimulation of proliferation by 100 fold (Carter and Fearon, 1992).

CD19 also participates in BCR signaling even in the absence of co-crosslinking. A fraction of CD19 associates constitutively with the BCR (Fearon and Carter, 1995), and CD19 becomes heavily tyrosine phosphorylated

upon BCR crosslinking (Chalupny et al., 1993; Uckun et al., 1993). Recent evidence demonstrates that in J558 B cells, CD19 expression is required for optimal BCR-induced activation of the PLC pathway. In these cells, CD19 participates in both the release of calcium from intracellular stores, and in calcium influx (Buhl et al., 1997). Data in this paper and elsewhere (Bolland et al., 1998) suggest that CD19 may participate in PLC activation by promoting the activation of PI 3-kinase. Indeed, BCR crosslinking induces CD19 association with p85 PI 3-kinase (Chalupny et al., 1995; Tuveson et al., 1993). Moreover, CD19 binds to Src-family kinases (Uckun et al., 1993), which are required for PLC activation (Takata et al., 1994), and may also associate with PLC itself (Chalupny et al., 1993; Zhang et al., 1998).

The Btk tyrosine kinase also participates in BCR signaling (Rawlings and Witte, 1995; Tarakhovsky, 1997) In contrast to the Src-family kinases and Syk, Btk does not appear to associate with the BCR. Rather, it appears to be activated by membrane-associated Src-family kinases (Rawlings et al., 1996) and by binding to PI 3,4,5-P_3, a lipid product of PI 3-kinase activity, through its PH domain (Bolland et al., 1998; Salim et al., 1996; Turner et al., 1997). It's role in the PLC pathway has been well demonstrated in the DT40 chicken B cell system. DT40 cells lacking Btk expression fail to activate the PLC pathway upon stimulation (Takata and Kurosaki, 1996). More recent work has also demonstrated the cooperation between PI 3-kinase and Btk in mediating PLC activation, and the role of Btk in sustained increases in intracellular Ca^{2+} following BCR crosslinking (Fluckinger et al., 1998; Scharenberg et al., 1998). In addition, a Btk mutation in mice is responsible for the X-linked immunodefiency (XID) syndrome (Khan et al., 1995). In the XID mice, B cells are produced in substantial numbers, but exhibit decreased PIP_2 hydrolysis and calcium flux in response to BCR crosslinking compared to wild type B

cells (Rigley et al., 1989). Moreover, XID B cells are unable to respond to T cell-independent type 2 (TI-2) antigens, such as many polysaccharides. Btk mutations have also been linked to the human disease X-linked agammaglobulinemia (XLA), which is characterized by greatly decreased numbers of B cells and poor antibody responses (Tsukada et al., 1994; Tsukada et al., 1993).



Materials and Methods

Cell lines, antibodies and other reagents. The AtT20 and B cell lines in this chapter were grown as described in Chapter 1, except that transfectants expressing Tac-CD19, tailless Tac, and/or Btk were also grown in puromycin (0.4-0.75 μg/ml). The 7G7 anti-Tac monoclonal antibody was provided by Dr. Patricia Roth, as was the plasmids LPCX-TacIgα, LPCX-TacT, and LPCX-Btk. LPCX-TacIgα encodes the extracellular and transmembrane domains of Tac and the cytoplasmic domain of Ig-α. LPCX-TacT encodes the extracellular and transmembrane domains of Tac. LPCX-Btk encodes the Btk tyrosine kinase. Anti-Tac-FITC (ACT-1) for FACS was purchased from DAKO. Anti-p85 polyclonal antibodies were a gift from Dr. L. Williams (UCSF). Anti-p85 N-SH2 monoclonal antibodies were purchased from Upstate Biotechnology Inc. Rabbit anti-CD19 antibodies were a gift from Drs. I. Krop and D. Fearon. Rabbit anti-Btk was generated by Drs. Janice Kim and Patricia Roth by immunizing rabbits with a GST fusion protein containing the N-terminal region of Btk. Other antibodies were described in Chapter 1.

Construction of the Tac-CD19 chimera. A cDNA clone encoding the transmembrane and cytoplasmic domain of murine CD19 was generated by PCR using the 5' primer 5'-GCTGTTGAGAGATCTTGGATGGATAGTC-3' (includes Bgl II site) and the 3' primer, 5'-CAGGCTAATCGATTGGGAGTCACGTG-3' (includes Cla I site). Separate PCR reactions were carried out using cDNA from WEHI-231 cells and 2PK/3 cells (2PK/3 cDNA and some of the WEHI-231 cDNA a gift from Dr. D. Law). Reaction products were pooled and digested with Bgl II and Cla I. LPCX-

TacIgα was also digested with these enzymes to drop out the Tac transmembrane and Igα sequences. The digested PCR products were ligated into the vector to form LPCX-TacCD19, encoding the extracellular domain of Tac and the transmembrane and cytoplasmic domains of CD19. The nucleotide sequence of the Tac-CD19 junction and the transmembrane and cytoplasmic domains of CD19 were verified by sequencing, using the Sequenase method (United States Biochemical Corp.).

Generation of AtT20 transfectants. All cells expressed the BCR. To generate the Tac-CD19-expressing cells and Tac-tailless control cells, 100-33 and syk13 cells (Chapter 1) were each transfected separately with LPCX-TacCD19 and LPCX-TacT, as described (Chapter 1). Cells were selected in puromycin (0.4 - 0.75 μg/ml). Drug resistant colonies were picked and screened for Tac expression by FACS analysis. Cells expressing both Syk and Btk were generated by Dr. P. Roth by transfection of syk13 cells with LPCX-Btk. Cells expressing Syk, Btk and the CD19 chimera were also generated in collaboration with Dr. P. Roth. Syk38 cells (Chapter 1) were co-transfected with LPCX-TacCD19 and LPCX-Btk using lipofectamine, and selected in puromycin as above. Drug-resistant colonies were screened by Dr. P. Roth for Btk expression by Western blot and/or *in vitro* kinase assay, and for Tac expression by FACS (data not shown).

Cell stimulation and preparation of lysates, immunoprecipitations, Western blotting, and phosphoinositide breakdown assays. These were performed essentially as described in Chapter 1.

PI 3-kinase activity assays. These assays were performed by A. Finn, essentially as described (Matsuuchi et al., 1992), except that PI 3-kinase was immunoprecipitated using anti-p85 antibodies (UBI).

Results and Discussion

Generation of AtT20 clones expressing a CD19 chimera, Btk, or CD19 and Btk. Syk13 and 100-33 clonal cells were transfected to express a chimeric CD19 protein containing the extracellular domain of Tac (human IL-2Rα, CD25), and the transmembrane and cytoplasmic domains of murine CD19, as described in Materials and Methods. Chimera expression was evaluated by FACS. The syk^+ clones 1-1, 2-5, and 17, and the syk^- clones 9, 12, and 19 were selected for study (Figure 2.1). Syk13 clones which were transfected to express Btk, SB26 and SB27, were provided by Dr. Patricia Roth. In addition, in collaboration with Dr. Roth, syk38 cells were transfected with constructs encoding both the CD19 chimera and Btk. The clones SBT19-68 and SBT19-78, which expressed the BCR, Syk, Btk and the CD19 chimera were selected (data not shown).

CD19 tyrosine phosphorylation requires Syk. CD19 becomes heavily tyrosine phosphorylated following BCR stimulation in B cells (Chalupny et al., 1993; Uckun et al., 1993). The CD19 phosphotyrosines provide binding sites for SH2 domain-containing signaling proteins, such as PI 3-kinase (Tuveson et al., 1993) and vav (Weng et al., 1994). We wished to determine whether the CD19 chimera would become tyrosine phosphorylated in the AtT20 cells upon BCR crosslinking. Prior to BCR crosslinking, the CD19 chimera was unphosphorylated, or only lightly phosphorylated on tyrosine residues (Figure 2.2). However, upon anti-IgM treatment, CD19 became robustly phosphorylated in the cells expressing Syk (Figure 2.2A), but not in the Syk-negative cells (Figure 2.2B). Therefore, CD19 is tyrosine

Figure 2.1. Surface expression of the Tac-CD19 chimera in AtT20 transfectants. Syk13 parental cells (A), syk13 cells transfected with the Tac-CD19 chimera (B), or syk$^-$ 100-33 cells transfected with the Tac-CD19 chimera (C) were analyzed by FACS for expression of Tac using anti-Tac antibodies.

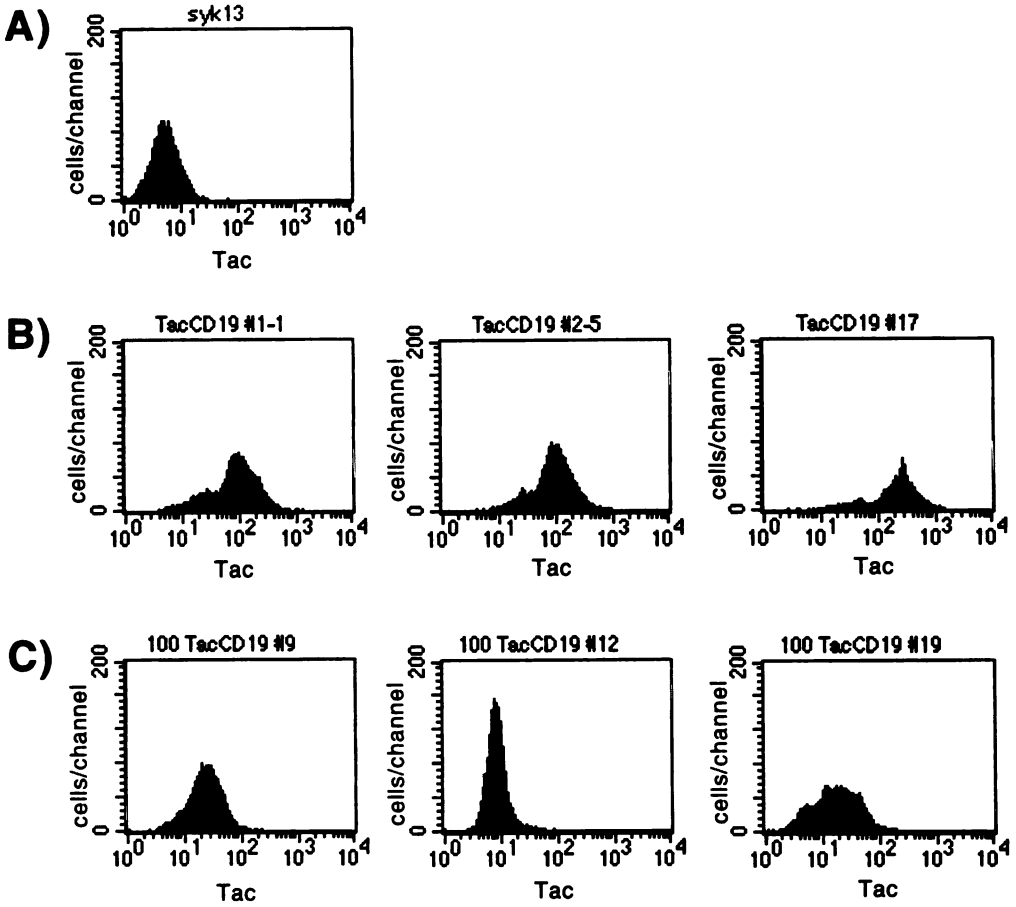
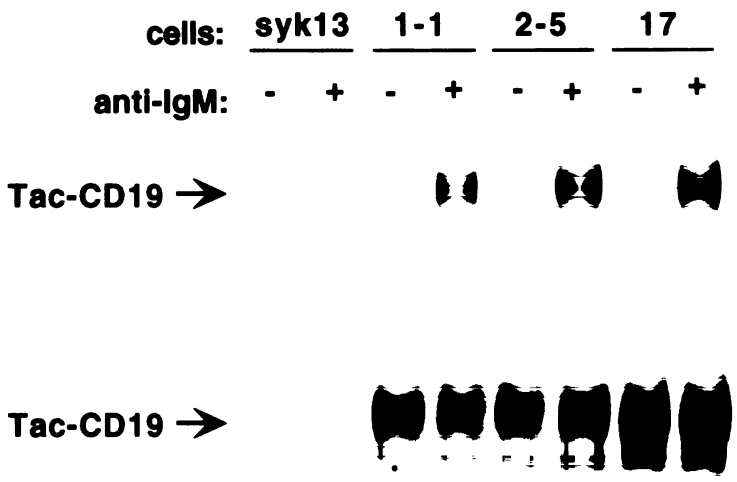
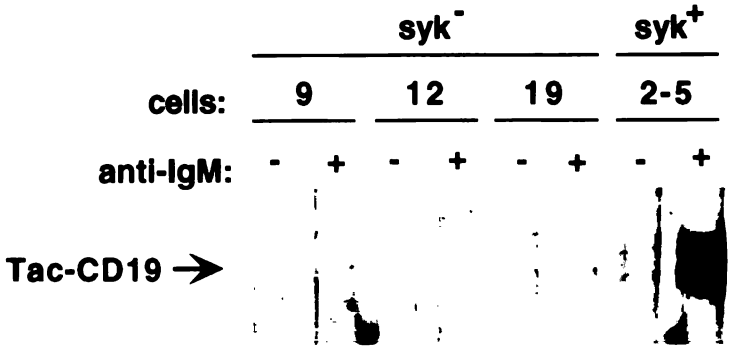

A)
syk13
cells/channel
200
0
10^0 10^1 10^2 10^3 10^4
Tac
B)
TacCD19 #1-1
TacCD19 #2-5
TacCD19 #17
C)
100 TacCD19 #9
100 TacCD19 #12
100 TacCD19 #19

Figure 2.2. Tyrosine phosphorylation of the Tac-CD19 chimera requires expression of Syk. (A) The indicated Syk^+ cell lines were left unstimulated, or stimulated with anti-IgM for 5 minutes. Cells were lysed and the Tac-CD19 chimera was immunoprecipitated. Tac-CD19 tyrosine phosphorylation was determined by Western blot analysis, using the 4G10 antiphosphotyrosine antibody (upper panel). The membrane was stripped and reprobed with anti-CD19 antibodies as a loading control (lower panel). (B) The ability of BCR crosslinking to induce Tac-CD19 tyrosine phosphorylation in AtT20 cells which were either Syk^- or Syk^+ was evaluated as in (A), (reprobe not shown).

A)


cells:
syk13
1-1
2-5
17
anti-IgM:
- + - + - + - +
Tac-CD19
Tac-CD19

B)


syk-
syk+
cells:
9
12
19
2-5
anti-IgM:
- + - + - + - +
Tac-CD19

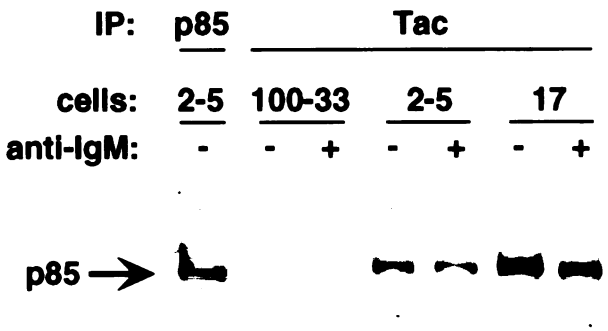
phosphorylated in the AtT20 cells in a stimulation-dependent manner, as it is in B cells, and this phosphorylation requires Syk.

CD19 association with PI 3-kinase. In B cells, tyrosine phosphorylation of CD19 leads to its association with the p85 subunit of PI 3-kinase and PI 3-kinase activity (Tuveson et al., 1993). We therefore determined whether tyrosine phosphorylated CD19 in AtT20 cells associated with the p85 subunit of PI 3-kinase. As predicted, p85 did co-immunoprecipitate with CD19 from BCR-stimulated, Syk-positive AtT20 cells (Figure 2.3A). Unexpectedly, p85 associated with the CD19 chimera from unstimulated cells as well. Moreover, there was no increase in CD19-p85 association upon stimulation, and there may have been a slight decrease. This was despite the fact that CD19 was phosphorylated much more extensively in the stimulated cells (data not shown for this particular experiment). We hypothesized that the association between CD19 and p85 in the unstimulated cells could be due to the light phosphorylation of CD19 sometimes seen prior to BCR-crosslinking in the Syk-expressing cells. We therefore tested the ability of p85 to associate with the CD19 chimera in the Syk-negative clones, where CD19 phosphorylation is undetectable. Similar to the situation in the Syk-positive cells, however, p85 associated with CD19 in both stimulated and unstimulated Syk-negative cells (Figure 2.3B). This result raises the possibility that the association between p85 and the CD19 chimera in the AtT20 cells may be indirect.

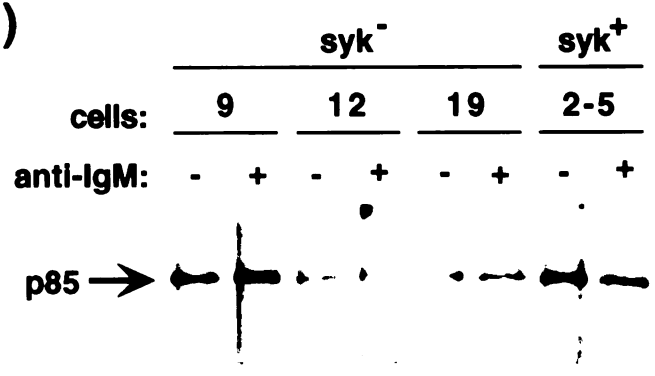
These results suggested that the p85 subunit of PI 3-kinase was associating with the CD19 chimera in a phosphotyrosine-independent fashion in the AtT20 cells. We decided to test whether this would also be the case in B cells. Bal17 B cells were transfected with the Tac-CD19 chimera. Only one Tac-positive Bal17 clone was generated. However, based on one experiment with this one transfectant, it appears that p85-CD19 association in this

Figure 2.3. p85 PI 3-kinase association with the Tac-CD19 chimera. (A) The indicated cell lines were left unstimulated or stimulated with anti-IgM antibodies and lysed. p85 or Tac-CD19 was immunoprecipitated from 0.5 or 1.5 mg of lysates, respectively, and the immunoprecipitations were separated by SDS-PAGE. p85-Tac-CD19 association was evalulated by Western blotting, using antibodies specific for p85. (B) The Syk^- $Tac\text{-}CD19^+$ cell lines 9, 12. and 19, as well as the Syk^+ $Tac\text{-}CD19^+$ cell line 2-5 were left unstimulated or stimulated with anti-IgM antibodies, as shown. Tac-CD19 was immunoprecipitated from all of the lysates, and p85 association was determined by Western blotting, as in (A).

A)


IP:
p85
Tac
cells:
2-5
100-33
2-5
17
anti-IgM:
- - + - + - +
p85

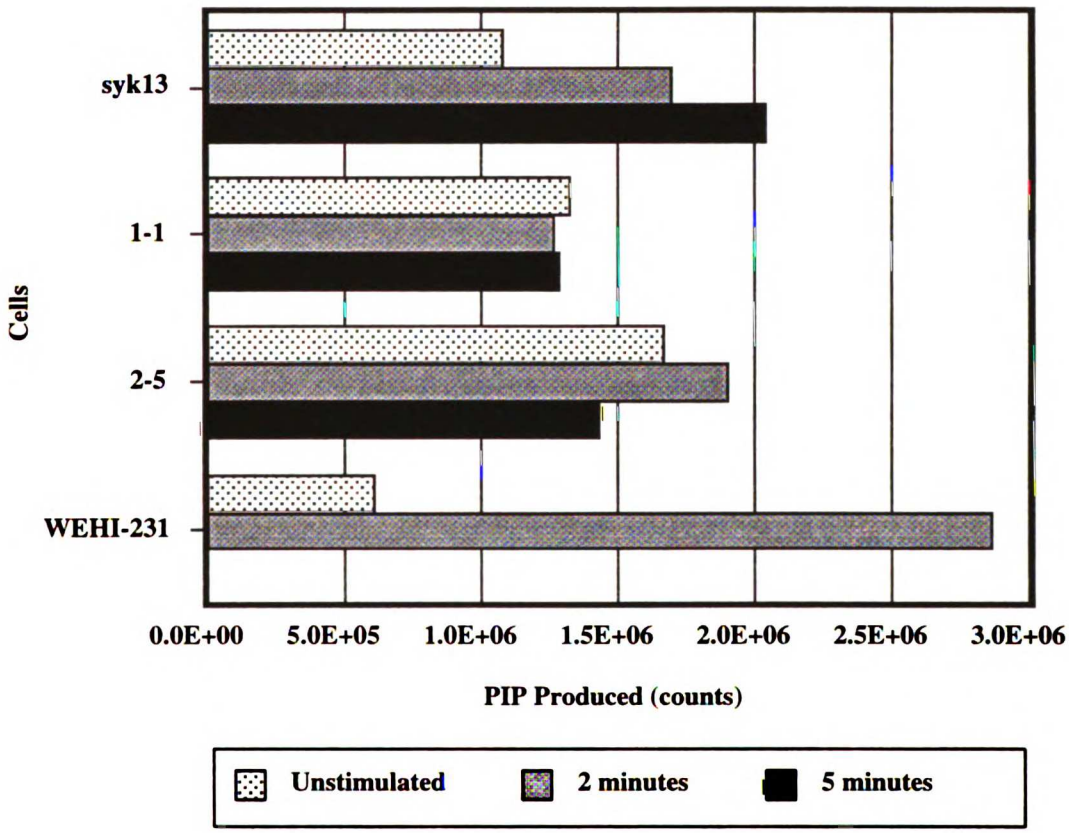
B)


syk-
syk+
cells:
9
12
19
2-5
anti-IgM:
- + - + - + - +
p85

transfectant occurred only upon stimulation through the BCR (data not shown). Although this result is based on limited data, it implies that the unexpected p85-CD19 association seen in the AtT20 cells is not due to an inherent defect in the chimera. Rather, it seems more likely that the AtT20 cells lack negative regulatory elements present in B cells which prevent PI 3-kinase from associating with CD19 prior to antigenic stimulation.

PI 3-kinase activity in the AtT20 cells. We also tested the ability of BCR crosslinking to stimulate PI 3-kinase activity in the AtT20 cells. WEHI-231 B cells, syk13 cells, and the syk-positive CD19-positive cell lines 1-1 and 2-5 were stimulated with anti-IgM antibodies and lysed. p85 was immunoprecipitated, and PI 3-kinase activity assays were performed. BCR crosslinking increased PI 3-kinase activity to almost 5 times background in WEHI-231 cells (Figure 2.4). Moreover, PI 3-kinase activity doubled in the syk13 cells, demonstrating that BCR-induced PI 3-kinase can be at least modestly activated in a CD19-independent manner. Basal PI 3-kinase activity in the 1-1 and 2-5 cells was slightly higher than in the syk13 cells, perhaps due to increased localization of PI 3-kinase to the membrane via its association with CD19. However, PI 3-kinase activity did not increase upon stimulation in these cells. In addition to data from earlier reports (Tuveson et al., 1993), recently published data from experiments using J558 B cells has demonstrated that CD19 expression is critical for optimal activation of PI 3-kinase (Buhl et al., 1997). $CD19^-$ J558 cells exhibited only a modest increase in BCR-mediated PI 3-kinase activity, similar to what was seen in the syk13 AtT20 cells, whereas the $CD19^+$ J558 cells activated PI 3-kinase to a much greater extent. However, because CD19-p85 association did not increase upon BCR crosslinking in the AtT20 cells, the lack of BCR-induced PI 3-kinase activity in these cells is not surprising.

Figure 2.4. PI 3-kinase activity in Syk-expressing AtT20 transfectants. Syk-expressing AtT20 transfectants which either did not (syk13) or did (1-1, 2-5) express Tac-CD19 were stimulated for 2 or 5 minutes with anti-IgM antibodies. WEHI-231 B cells were stimulated for 2 minutes, also with anti-IgM. p85 was immunoprecipitated, and PI 3-kinase activity assays were performed.

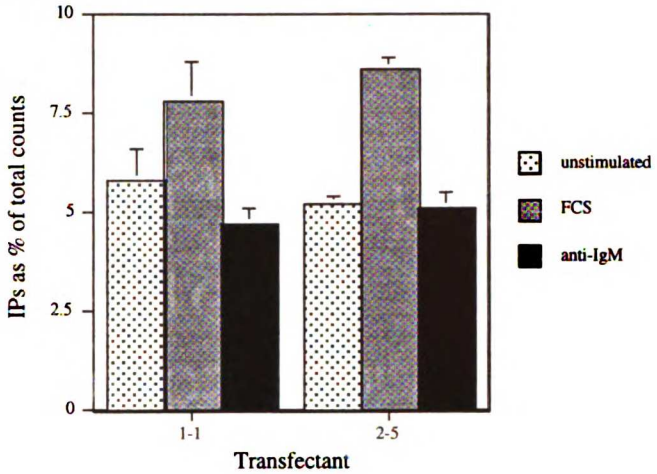


Phosphoinositide hydrolysis in the AtT20 cells. We also wished to determine whether CD19 and/or Btk expression would reconstitute BCR-induced activation of the PLC pathway. Indeed, recently-published data provide evidence that Btk, CD19 and PI 3-kinase all function to activate PLC (Bolland et al., 1998; Buhl et al., 1997; Takata and Kurosaki, 1996). Inositol phosphate production assays were performed on BCR-expressing AtT20 cells which also expressed Syk, Syk plus CD19, Syk plus Btk, and Syk plus CD19 and Btk (Figure 2.5). In all cases, anti-IgM treatment failed to activate this pathway.

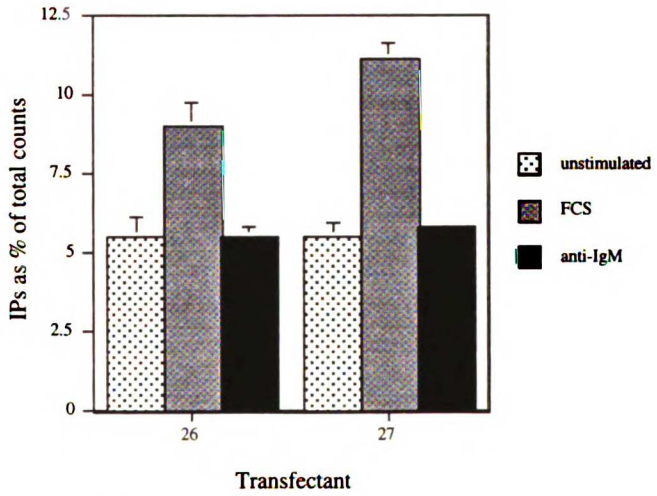
It appears from the work of other labs (Bolland et al., 1998; Buhl et al., 1997; Takata and Kurosaki, 1996) that we were on the right track. Evidence is mounting that CD19 participates in BCR-induced PI 3-kinase activation; that PIP_3, a product of PI 3-kinase activity, helps activate Btk (Bolland et al., 1998; Salim et al., 1996); and that PI 3-kinase and Btk, as well as Src-family kinases and Syk (Takata et al., 1994) are required to activate the PLC pathway in response to antigenic stimulation in B cells. In the studies detailed here, we have introduced the BCR, Syk, CD19 and Btk in a variety of combinations into AtT20 cells, which endogenously express the Src-family kinase Fyn (Chapter 1). Nevertheless, we were unable to reconstitute BCR-induced activation of the PI 3-kinase or PLC pathways in these cells (except for the modest increase in PI 3-kinase activity in the syk13 cells). This failure may be due to the lack of additional lymphoid-specific elements in the AtT20 cells which are required for the activation of these enzymes. For example, a T cell transmembrane protein, LAT, has recently been cloned which associates with PLC-γ upon T cell receptor stimulation, and may therefore act to localize PLC-γ to its substrate in the cell membrane (Zhang et al., 1998). It is possible that a B cell version of LAT may function in a similar manner upon BCR

Figure 2.5. Phosphoinositide production in the AtT20 transfectants. The inositol-containing phospholipids of AtT20 transfectants were labelled overnight with ^{3}H-inositol. Cells were left unstimulated, or stimulated for 30 minutes with 10% dialyzed FCS or 20 μg/ml anti-IgM, as indicated. Cells were lysed and total inositol phosphate generation was measured. Inositol phosphate production is calculated as the radioactivity present in the inositol phosphates divided by the radioactivity present in inositol phosphates plus phospholipids, and is expressed as a percentage. Values shown are the mean and SEM of triplicate samples. These experiments were performed on BCR^{+} Syk^{+} AtT20 transfectants which also expressed (A) Tac-CD19, (B) Btk, or (C) Tac-CD19 and Btk (except for syk13 parental cells, which do not express Tac-CD19 or Btk).

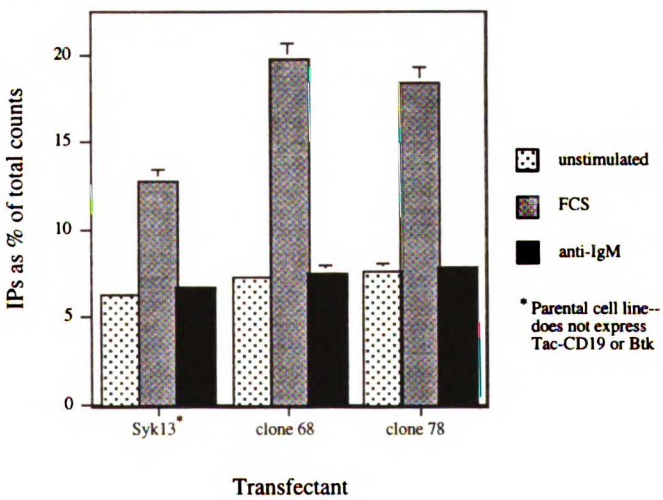
A) Tac-CD19



B) Btk



C) Tac-CD19 + Btk



crosslinking. Alternatively, it appears that the CD19-PI 3-kinase association in the AtT20 cells was dysregulated (Figure 2.3), and this dysregulation may explain the lack of PI 3-kinase activity in these cells. Because PI 3-kinase activity seems to be required for PLC activation (Bolland et al., 1998; Buhl et al., 1997; Fluckinger et al., 1998; Scharenberg et al., 1998), it is not surprising, then, that PLC was also not activated.

Chapter 3: Activation of the Ras-MAP Kinase Cascade is Required for a Subset of B Cell Responses to Antigen

Summary

Signal transduction initiated by B cell antigen receptor (BCR) crosslinking plays an important role in the development and activation of B cells. Considerable effort, therefore, has gone into determining the biochemical signalling events initiated by the BCR, and delineating which events participate in specific biological responses to antigen. We used an inhibitor of MEK 1/2, PD 98059, to assess the role of the Ras-Mitogen-activated protein (MAP) kinase pathway in several BCR-induced responses. PD 98059 treatment substantially inhibited the BCR-induced activation of the extracellular signal-regulated kinase 2 (ERK2) form of MAP kinase in both the immature B cell line WEHI-231 and in mature splenic B cells. However, PD 98059 treatment did not block BCR-induced growth arrest or apoptosis of WEHI-231 cells, indicating that the Ras-MAP kinase pathway is not required for these events. In contrast, PD 98059 treatment did inhibit the upregulation of specific BCR-induced proteins in splenic B cells, including the transcription factor Egr-1. Moreover, this inhibitor also suppressed BCR-induced proliferation of splenic B cells, both in the absence and presence of Interleukin 4 (IL-4). Therefore, activation of the Ras-MAP kinase pathway is necessary for a specific subset of B cell responses to antigen.

Introduction

The B cell antigen receptor (BCR) is composed of membrane immunoglobulin (Ig), which recognizes specific antigen, complexed to the Ig-α/Ig-β heterodimer, which mediates transmembrane signalling (DeFranco, 1998). The biochemical events induced when the BCR is crosslinked by antigen begin with the activation of Src-family and Syk protein tyrosine kinases. These kinases trigger a complex network of signalling pathways downstream of the receptor, including the activation of phospholipase C(PLC)-γ1 and -γ2, phosphatidylinositol 3-kinase (PI 3-kinase), the Ras-Raf-MEK-MAP kinase pathway, and phosphorylation of the Vav protooncogene product (DeFranco, 1998; DeFranco, 1997; Richards et al., 1996). The resulting signals quickly reach the nucleus and alter gene expression. The ultimate effects on the B cell are profound and vary depending on the maturation state of the cell and on the additional signals received. Antigen contact with immature B cells causes them to enter into an anergic state or undergo apoptosis, responses which promote immunological tolerance to self antigens. In contrast, mature B cells contacting antigen enter G1 phase of the cell cycle and upregulate many proteins involved in adhesion and antigen presentation to helper T cells. Strong BCR stimulation can also induce B cell proliferation (DeFranco, 1998). Helper T cells enhance this proliferation, and induce B cell differentiation into the antibody-secreting plasma cell state by providing cell-cell contact signals via CD40 and by releasing cytokines such as IL-4 and IL-5 (DeFranco, 1998).

Both genetic and pharmacologic approaches have been used to determine how individual signalling pathways participate in biological responses to antigen. For example, wortmannin, which inhibits PI 3-kinase,

has been shown to block BCR-induced apoptosis in a human immature B cell line (Beckwith et al., 1996). Experiments with pharmacologic agents that mimic the second messengers resulting from PLC-γ activation, and with mutant cell lines defective in this pathway, have demonstrated its importance for BCR-induced growth arrest and apoptosis of B cell lines (Page and DeFranco, 1990; Page and DeFranco, 1988; Page et al., 1991; Takata et al., 1995) and proliferation of mature splenic B cells (Monroe and Kass, 1985; Rothstein et al., 1986). Moreover, mice and cell lines with targeted mutations in the signalling proteins Vav (Tarakhovsky et al., 1995; Zhang et al., 1995) and HS1 (Fukuda et al., 1995; Taniuchi et al., 1995) both exhibit decreased B cell responses to antigen.

By comparison, relatively little is known about the role(s) of the Ras-Raf-MEK-MAP kinase pathway in B cell responses to antigen. This pathway has been found to be important in many cell types for receptor-induced biological responses such as proliferation, growth arrest and differentiation (Kim et al., 1991; Marshall, 1994; Marshall, 1995; Pritchard et al., 1995; Sewing et al., 1997; Woods et al., 1997). When in its active GTP-bound state, Ras activates the serine/threonine protein kinase Raf, which phosphorylates and activates the protein kinases MEK 1 and 2. The MEK 1/2 kinases in turn phosphorylate and activate the classical p44 and p42 MAP kinases, also called ERK 1 and 2, respectively (Marshall, 1995). We employed the MEK 1/2 inhibitor PD 98059 to investigate the role this pathway plays in response to BCR crosslinking. PD 98059 suppressed BCR-induced activation of ERK2, as well as upregulation of the early response gene *egr-1* and the adhesion protein CD44. This inhibitor also blocked BCR-induced proliferation of mature splenic B cells. In contrast, PD 98059 did not inhibit BCR-induced growth arrest or apoptosis of the immature B cell line WEHI-231. Thus, the

Materials and Methods

Antibodies and Other Reagents. Affinity-purified goat-anti-mouse IgM was obtained from Jackson Immunological Research (West Grove, PA). Recombinant mouse IL-4 was from Genzyme (Cambridge, MA). Hybridomas HO13.4 (anti-Thy1), 53.6.172 (anti-CD8) and GK1.5 (anti-CD4) were purchased from ATCC. Anti-ERK2 (C-14), anti-JNK1 (C-17) and anti-Egr-1 (C-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated RA3-6B2 (anti-B220), FITC-conjugated GL-1 (anti-B7-2), and FITC-conjugated 39-10-8 (anti-MHC II, I-A^d) were purchased from PharMingen (San Diego, CA). 5-Bromo-2'-deoxyuridine (BrdUrd) was purchased from Sigma (St. Louis, MO). Anti-BrdUrd-FITC was purchased from Becton-Dickinson (San Jose, CA). Avidin-TriColor was obtained from CalTag (South San Francisco, CA). PD 98059 and the GST-Elk-1 fusion protein were purchased from New England Biolabs (Beverly, MA). The GST-Jun(1-79) fusion protein was prepared as described (Hambleton et al., 1996). Splenic B cells were cultured in RPMI 1640 medium supplemented with 10% FCS (Gibco-BRL), 2 mM pyruvate, 20 mM glutamine, 50 μM 2-mercaptoethanol, and penicillin/streptomycin. WEHI-231 cells were cultured as described (Richards et al., 1996).

Isolation of Splenic B Lymphocytes and Treatment with PD 98059. Female C57Bl/6 x DBA/2 F_1 (BDF1) mice from Simonson (Gilroy, CA) or Charles River (Hollister, CA) were sacrificed at 2 to 4 months of age. Small resting B cells were isolated from the spleen as described (Chan et al., 1997). Cells were collected from the 60%/70% Percoll interphase, washed, and rested in normal medium at 37°C for 30 minutes to two hours prior to treatment

with PD 98059. In all experiments, PD 98059 or DMSO carrier was added to the splenic B cells or WEHI-231 cells one hour prior to stimulation. Cells were stimulated and cultured in media which always contained either PD 98059 or DMSO. The purity of the splenic B cell preparations was typically at least 96% $B220^+$, as verified by flow cytometry.

Cell Stimulation, Preparation of Cell Lysates and Immunoblotting. Cells (1×10^7 cells/ml) were stimulated with goat anti-mouse IgM and/or IL-4 (50 U/ml) for the indicated times and washed twice with ice cold phosphate buffered saline (PBS) containing 1 mM Na_3VO_4 before lysis. For the ERK2 and JNK kinase assays (below), cell lysis conditions were as described (Hambleton et al., 1995). For examining Egr-1 upregulation, cells (5×10^6 cells/lane) were stimulated for one hour and lysed in SDS polyacrylamide gel elecrophoresis (PAGE) sample buffer, resolved by SDS-PAGE and transferred to nitrocellulose. The anti-Egr-1 antibody was used at 1:500 for detection. General SDS-PAGE and immunoblotting procedures were as described (Richards et al., 1996).

ERK2 and JNK Immunocomplex Protein Kinase Assays. Cells were stimulated for 4 minutes (ERK2 assays) or for 15 minutes (JNK assays). Anti-JNK and anti-ERK2 immunoprecipitations were performed as described (Hambleton et al., 1996). Immunocomplex protein kinase assays were performed on the immunoprecipitates as described (Chan et al., 1997), with GST-Elk-1 as the substrate for ERK2 and GST-Jun(1-79) as the substrate for JNK. Reaction products were resolved by SDS-PAGE, transferred to nitrocellulose and detected by autoradiography. Quantitation of kinase activity was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To calculate inhibition of kinase activity by PD 98059, we

used the formula: Percent inhibition = {1 - [($stim_{pd}$ - $unstim_{pd}$) / (stim - unstim)] } x 100.

When appropriate, blots were reprobed with anti-ERK2 antibodies (1:1000) to ensure all reactions contained equal amounts of ERK2 (data not shown).

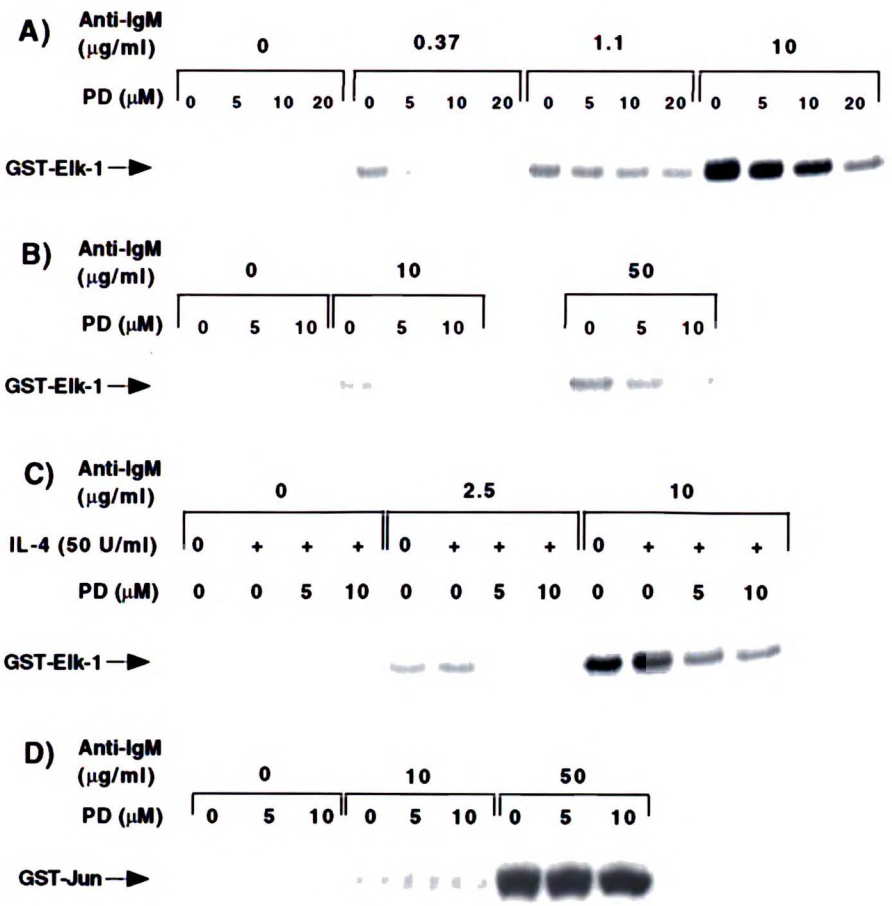
Growth Arrest, Apoptosis and Proliferation. For growth arrest, WEHI-231 cells were plated in triplicate at 2 x 10^4 cells/well in 96 well plates and stimulated with goat-anti-mouse IgM for 28 hours. Cells were pulsed with [^{3}H]dT (1μCi/well) for the last four hours, harvested onto glass fiber filters (Wallac, Gaithersburg, MD), and the amount of incorporated [^{3}H]dT was determined using a Betaplate reader (Wallac). For apoptosis, WEHI-231 cells were plated at 1.5 x 10^5 cells/ml in 12 well plates, stimulated and cultured for 48 hours. Cell survival was measured by ability to exclude 1 μg/ml propidium iodide (PI) as determined by flow cytometry. Proliferation of murine splenic B cells was measured either by incorporation of ^{3}H-thymidine or incorporation of 5-Bromo-2'-deoxyuridine (BrdUrd). For [^{3}H]dT incorporation assays, splenic B cells were plated in triplicate in 96 well plates (5 x 10^5 cells/well), in the presence of anti-IgM +/- 50 U/ml IL-4, and cultured for 45 hours. Cells were pulsed with [^{3}H]dT for the last five hours of culture, and incorporation was determined as above. Inhibition of proliferation was calculated for each concentration of PD 98059 at each concentration of anti-IgM stimulation, using the same formula as above. *In vitro* BrdUrd labeling experiments were performed as described (Chan et al., 1997). Briefly, RBC-depleted splenocytes were plated at 0.5 x 10^6 cells/ml, stimulated and cultured for 48 hours. BrdUrd (10 μM) was added during the final 24 hours. Cells were fixed, and costained with anti-B220 and anti-BrdUrd and analyzed by flow cytometry. The data are presented as the percentage of $B220^+$ cells that are $BrdUrd^+$.

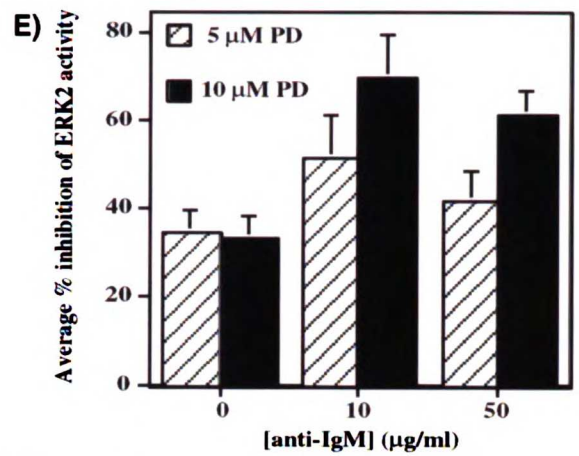
Upregulation of Cell Surface Molecules. RBC-depleted splenocytes were cultured at 5 x 10^5 cells/ml. Cells were stimulated with goat anti-mouse IgM whole antibodies or F(ab)'$_2$ fragments (for CD44 upregulation) for 18 hours. Cells were costained with anti-B220 and either anti-B7-2, anti-I-A^d, or anti-CD44 and analyzed by flow cytometry. Only live (PI-negative) B220$^+$ cells were analyzed.

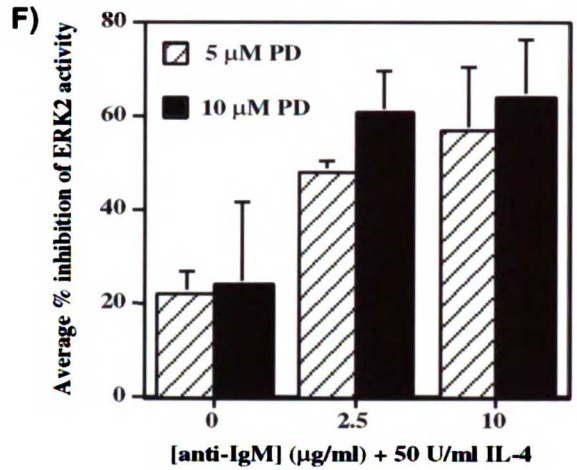
Results

PD 98059 Inhibits Activation of ERK2 but not JNK in WEHI-231 and Purified Splenic B Cells. *In vitro* protein kinase assays were performed to determine whether PD 98059 inhibited the BCR-induced activation of the ERK2 form of MAP kinase, as expected. Stimulation of the WEHI-231 immature B cell line with anti-IgM antibodies substantially increased the activity of ERK2 (Figure 3.1A). This activation was inhibited in a dose-dependent manner by PD 98059. At low concentrations of stimulating antibody, ERK2 activation was always greatly inhibited by 5-20 μM PD 98059, and in some experiments completely blocked. Inhibition by 10-20 μM PD 98059 was substantial but not absolute at higher doses of stimulating antibody, consistent with previous results in other systems (Alessi et al., 1995). Likewise, 5-10 μM PD 98059 suppressed ERK2 activation in purified murine splenic B cells stimulated with anti-IgM (Figure 3.1B) and anti-IgM plus IL-4 (Figure 3.1C). IL-4 did not reproducibly increase or decrease BCR-induced ERK2 activation at a 4 minute timepoint (Figure 3.1C), nor did it alter the magnitude and kinetics of BCR-induced ERK2 activation over a 2 hour time course (data not shown). Moreover, PD 98059 was equally as effective at blocking BCR-induced ERK2 activity in the presence or absence of IL-4 (compare Figs. 3.1E and 3.1F). In contrast, PD 98059 in this dose range did not decrease the BCR-induced activation of the c-Jun N-terminal kinase (JNK), another MAP kinase protein (Figure 3.1D), which is downstream of the MEK family member MKK4 (Treisman, 1996). PD 98059 also did not block the BCR-induced activation of the tyrosine kinase Syk, or the appearance of tyrosine phosphoproteins in the lysates of BCR-stimulated B cells (data not shown).

Figure 3.1 PD 98059 inhibits BCR-induced activation of ERK2. (A-C) WEHI-231 B cells (A) or murine splenic B cells (B and C) were treated with PD 98059 or DMSO carrier for one hour, stimulated for 4 minutes with anti-IgM antibodies +/- 50 U/ml IL-4, and lysed in a 1% Triton X-100-containing lysis buffer. ERK2 was immunoprecipitated, and its activity was assessed by *in vitro* kinase assay using GST-Elk-1 as the substrate. Reaction products were separated by SDS-PAGE and visualized by autoradiography. The position of GST-Elk-1 is indicated. (D) Murine splenic B cells were stimulated for 15 minutes and lysed, as above. JNK1 was immunoprecipitated and its activity was measured by *in vitro* kinase assay, using GST-Jun(1-79) as the substrate. (E and F) In three separate experiments, the percent inhibition of BCR-induced ERK2 activity in splenic B cells was calculated for 5 μM and 10 μM PD 98059 at the indicated concentrations of anti-IgM, for cells stimulated with anti-IgM (E), or anti-IgM plus 50 U/ml IL-4 (F). These individual values were averaged to generate the average % inhibition of ERK2 activity, which is shown. Error bars indicate standard error of the mean.


A)
Anti-IgM (μg/ml)
0
0.37
1.1
10
PD (μM)
0 5 10 20 0 5 10 20 0 5 10 20 0 5 10 20
GST-Elk-1
B)
Anti-IgM (μg/ml)
0
10
50
PD (μM)
0 5 10 0 5 10 0 5 10
GST-Elk-1
C)
Anti-IgM (μg/ml)
0
2.5
10
IL-4 (50 U/ml)
0 + + + 0 + + + 0 + + +
PD (μM)
0 0 5 10 0 0 5 10 0 0 5 10
GST-Elk-1
D)
Anti-IgM (μg/ml)
0
10
50
PD (μM)
0 5 10 0 5 10 0 5 10
GST-Jun


E)
Average % inhibition of ERK2 activity
5 μM PD
10 μM PD
0
20
40
60
80
0
10
50
[anti-IgM] (μg/ml)

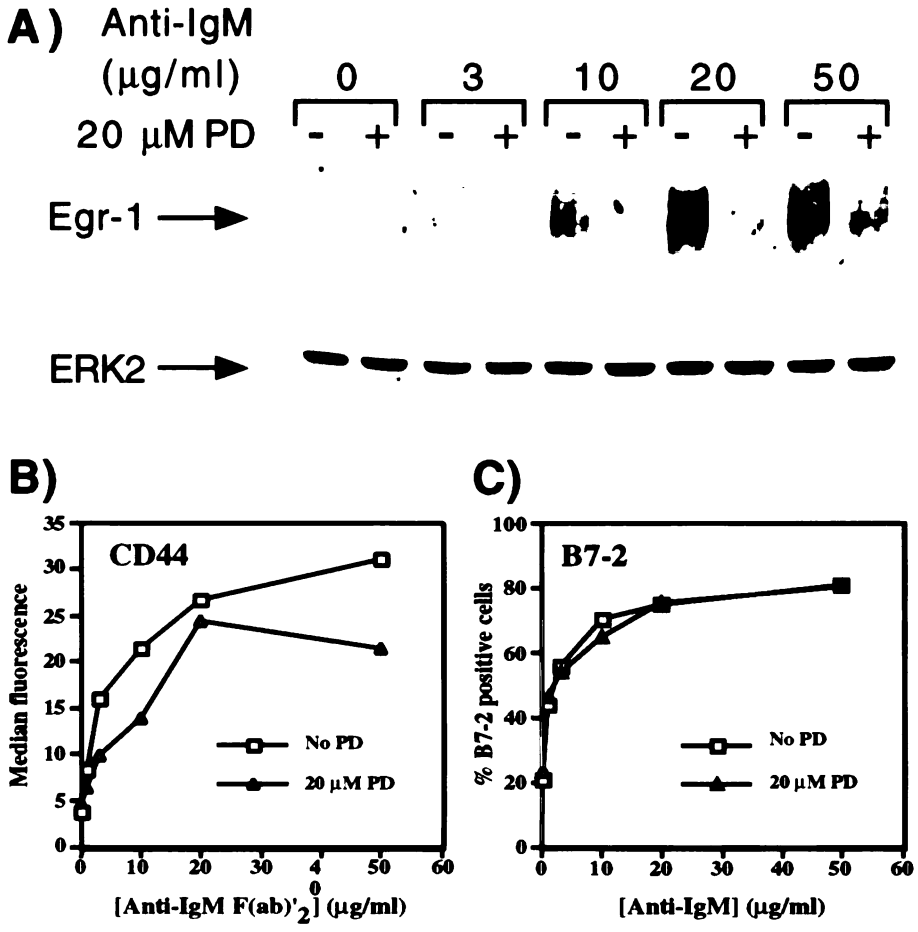

F)
Average % inhibition of ERK2 activity
5 μM PD
10 μM PD
0
20
40
60
80
0
2.5
10
[anti-IgM] (μg/ml) + 50 U/ml IL-4

Therefore, PD 98059 seemed to inhibit MEK 1/2, and thus ERK specifically in B cells, as it does in other cell types up to concentrations of at least 50 μM (Alessi et al., 1995; Dudley et al., 1995; Lazar et al., 1995; Pang et al., 1995).

PD 98059 Inhibits the BCR-induced Upregulation of Egr-1 and CD44. BCR-stimulated B cells upregulate the expression of many transcription factors (Treisman, 1996). This upregulation leads to changes in the expression of proteins important for cell cycle regulation, cell-cell adhesion, and activation of T cells. One BCR-induced transcription factor is Egr-1, which is important for the BCR-induced expression of the adhesion proteins CD44 and ICAM-1 (Maltzman and Monroe, 1996; Maltzman and Monroe, 1996). BCR-induced transcription of a reporter gene linked to the *egr-1* promoter can be blocked by dominant negative forms of Ras and Raf (McMahon and Monroe, 1995). We therefore wished to determine whether BCR-induced Egr-1 and CD44 protein expression required MEK activity. 20 μM PD 98059 treatment greatly suppressed anti-IgM-induced upregulation of Egr-1 (Figure 3.2A), and significantly decreased the upregulation of CD44 (Figure 3.2B) in mature splenic B cells. In contrast, this dose of PD 98059 did not inhibit BCR-induced expression of B7-2 (Figure 3.2C), a protein that provides an important co-stimulatory signal for helper T cells. Likewise, MHC class II expression, which also increases upon BCR crosslinking, was only minimally inhibited by high concentrations of PD 98059 (data not shown).

PD 98059 Does Not Inhibit BCR-Induced Growth Arrest or Apoptosis in WEHI-231 B Cells. BCR stimulation induces WEHI-231 B cells to arrest in the G1 phase of the cell cycle, and then undergo apoptosis (Benhamou et al., 1990; Hasbold and Klaus, 1990; Page and DeFranco, 1990; Page and DeFranco, 1988; Page et al., 1991). To determine whether the Ras-MAP kinase pathway is important for these events, we pretreated WEHI-231 cells with varying

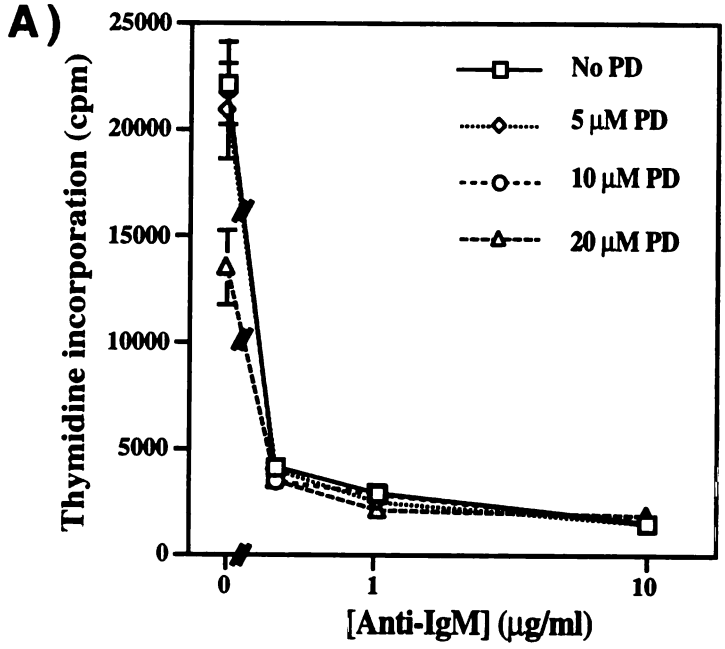
Figure 3.2 PD 98059 selectively inhibits BCR-induced upregulation of Egr-1 and CD44. (A) Murine splenic B cells (5×10^6 cells/lane) were treated with PD 98059 or DMSO carrier for one hour, stimulated with anti-IgM antibodies for one hour, and lysed in SDS-PAGE sample buffer. Lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-Egr-1 antibodies (upper panel). The membrane was also probed with anti-ERK2 antibodies to verify equal loading (lower panel). (B and C) Murine splenic B cells were incubated with PD 98059 or DMSO for one hour, then stimulated with anti-IgM antibodies for 18 hours. Expression of CD44 (B) and B7-2 (C) on $B220^+$ cells was analyzed by flow cytometry.


A)
Anti-IgM
(µg/ml)
0
3
10
20
50
20 µM PD
- + - + - + - + - +
Egr-1
ERK2
B)
CD44
Median fluorescence
No PD
20 µM PD
[Anti-IgM F(ab)'2] (µg/ml)
C)
B7-2
% B7-2 positive cells
No PD
20 µM PD
[Anti-IgM] (µg/ml)

amounts of PD 98059 before stimulation with anti-IgM antibodies. No inhibition of BCR-induced growth arrest was seen, as assessed by incorporation of ^{3}H-thymidine (Figure 3.3A). However, 20 μM PD 98059 did reproducibly decrease basal proliferation of WEHI-231 cells, suggesting that the Ras-MAP kinase pathway may be required for the proliferation of this cell line. PD 98059 also did not inhibit the BCR-induced apoptosis of these cells, as determined by their ability to exclude propidium iodide (Figure 3.3B). Because PD 98059 did not completely inhibit BCR-induced ERK activation, it is possible that only a small increase in ERK activity is sufficient to induce maximal growth arrest and apoptosis in these cells. However, because we did not detect a decrease in growth arrest or apoptosis even at low concentrations of anti-IgM when ERK activity is inhibited close to basal levels (Figure 3.1A), these data suggest that the Ras-MAP kinase pathway is not required for these events in WEHI-231 cells.

PD 98059 Inhibits Proliferation of BCR-stimulated Splenic B Cells. In contrast to its effect on immature B cells, BCR engagement causes mature B cells to enter the G1 phase of the cell cycle and to proliferate. Addition of T cell-derived cytokines such as IL-4 enhances this proliferative response (Hodgkin et al., 1991; Oliver et al., 1985; Rabin et al., 1985). We wished to determine whether the Ras-MAP kinase pathway is important for BCR-induced proliferation of murine splenic B cells. Anti-IgM treatment induced B cell proliferation, and this proliferation was blocked by PD 98059 in a dose-dependent manner (Figure 3.4A). To determine the extent to which PD 98059 blocked proliferation, the percent inhibition of proliferation was calculated for each concentration of PD 98059 and at each concentration of stimulating antibody over four separate experiments. These values were averaged together to generate Figure 3.4B. Higher concentrations of PD 98059 were

Figure 3.3 PD 98059 does not inhibit BCR-induced growth arrest or apoptosis of WEHI-231 immature B cells. WEHI-231 cells were incubated with PD 98059 or DMSO carrier for one hour. Cells were left unstimulated, or stimulated with 0.37, 1.1 or 10 μg/ml anti-IgM antibodies. (A) Cells were stimulated for 28 hours, pulsed with ^{3}H-thymidine for the last 4 hours, and ^{3}H-thymidine incorporation was measured. (B) Cells were stimulated for 48 hours. Apoptosis was measured by the inability to exclude 1 μg/ml propidium iodide, as determined by flow cytometry.


A)
25000
20000
15000
10000
5000
0
Thymidine incorporation (cpm)
No PD
5 µM PD
10 µM PD
20 µM PD
0
1
10
[Anti-IgM] (µg/ml)

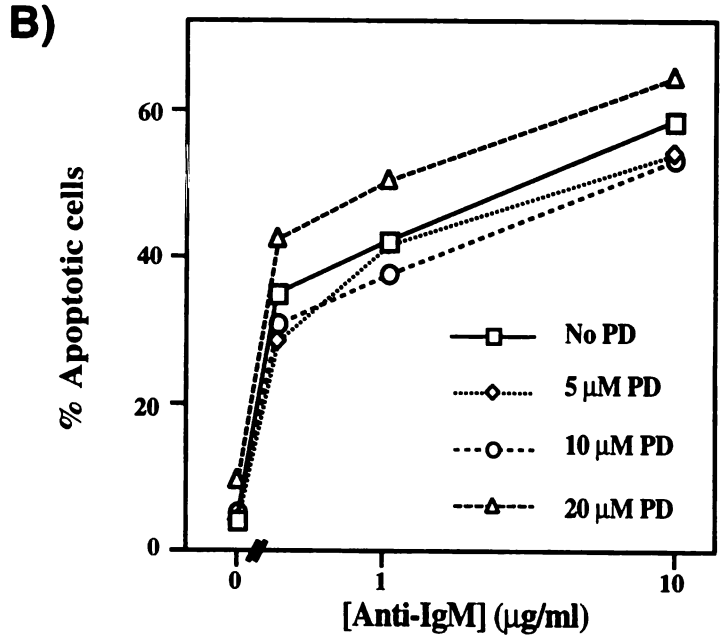
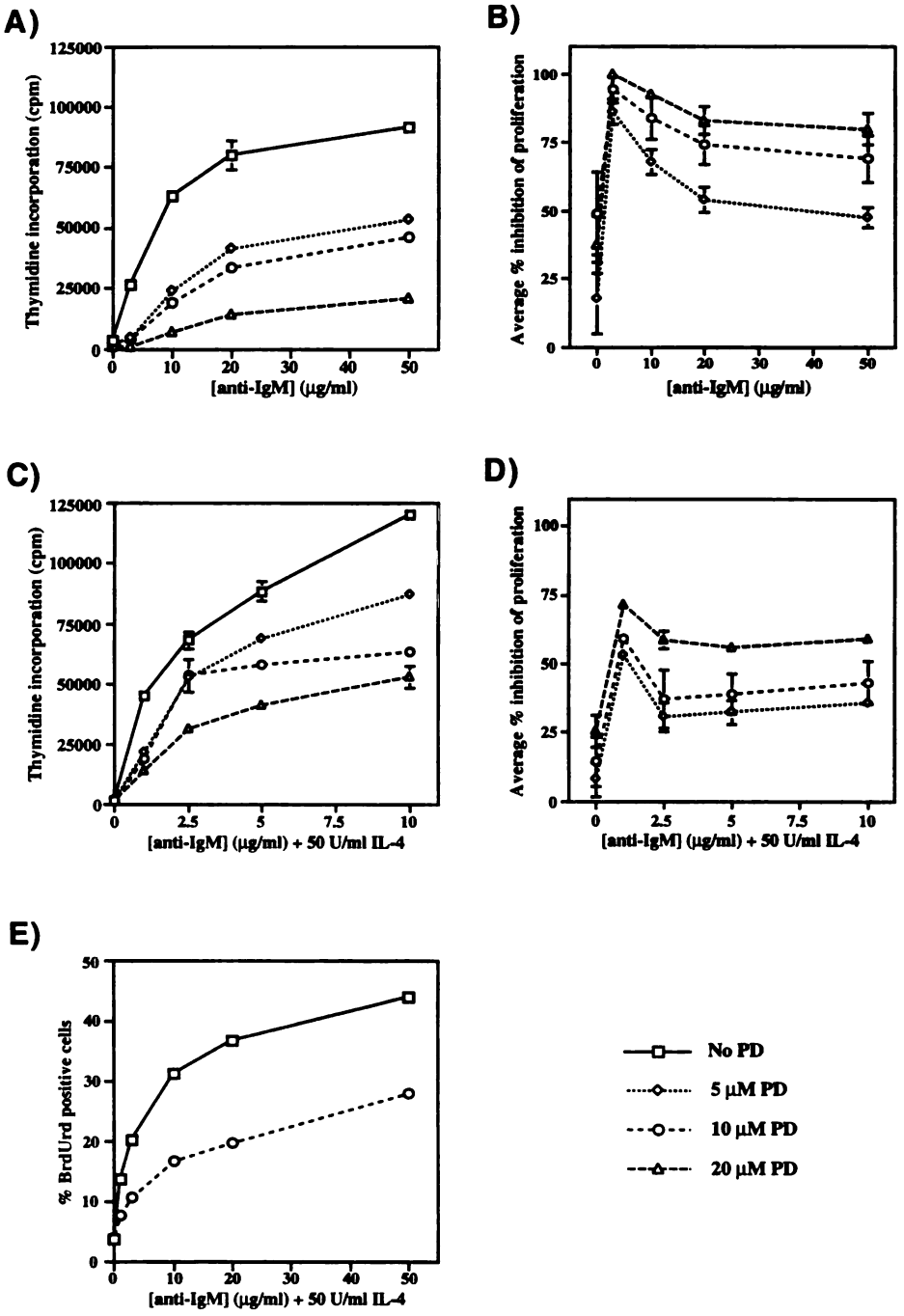

B)
60
40
20
0
% Apoptotic cells
No PD
5 µM PD
10 µM PD
20 µM PD
0
1
10
[Anti-IgM] (µg/ml)

Figure 3.4 PD 98059 inhibits BCR-induced proliferation in the absence and presence of IL-4. (A-D) Murine splenic B cells were treated with PD 98059 or DMSO and stimulated with anti-IgM (A and B) or anti-IgM plus 50 U/ml IL-4 (C and D) for 45 hours. Cells were pulsed with ^{3}H-thymidine for the last 5 hours. ^{3}H-thymidine incorporation from a representative experiment, performed on the same preparation of B cells, is shown in (A) and (C) (note different scales on x-axis). The percent inhibition of proliferation was calculated for each concentration of PD 98059 and at each concentration of stimulating antibody over 4 separate experiments for anti-IgM, and 3-5 experiments for anti-IgM plus IL-4, depending on the concentration of PD 98059. These values were averaged together to determine the average inhibition of proliferation for each concentration of PD 98059, as shown in panels (B) and (D). Error bars indicate the standard error of the mean. (E) RBC-depleted splenocytes (0.5×10^{6}/ml) were treated with PD 98059 or DMSO and stimulated with anti-IgM plus 50 U/ml IL-4 for 48 hours. BrdUrd (10 μM) was added for the final 24 hours. Shown is the percentage of B220^{+} B cells that incorporated BrdUrd, indicative of entry into S phase of the cell cycle.


A)
Thymidine incorporation (cpm)
125000
100000
75000
50000
25000
0
0
10
20
30
40
50
[anti-IgM] (µg/ml)
B)
Average % inhibition of proliferation
100
75
50
25
0
0
10
20
30
40
50
[anti-IgM] (µg/ml)
C)
Thymidine incorporation (cpm)
125000
100000
75000
50000
25000
0
0
2.5
5
7.5
10
[anti-IgM] (µg/ml) + 50 U/ml IL-4
D)
Average % inhibition of proliferation
100
75
50
25
0
0
2.5
5
7.5
10
[anti-IgM] (µg/ml) + 50 U/ml IL-4
E)
% BrdUrd positive cells
50
40
30
20
10
0
0
10
20
30
40
50
[anti-IgM] (µg/ml) + 50 U/ml IL-4
No PD
5 µM PD
10 µM PD
20 µM PD

more effective at suppressing proliferation than were lower concentrations, reflecting the observation that ERK2 activity is suppressed more completely by higher doses of PD 98059 than lower doses. In addition, PD 98059 blocked proliferation more effectively at lower doses of anti-IgM than at higher doses, presumably because increasing doses of anti-IgM induced increasing ERK2 activity, which was incompletely blocked by the inhibitor.

In agreement with earlier reports (Hodgkin et al., 1991; Oliver et al., 1985; Rabin et al., 1985), the addition of IL-4 greatly enhanced the proliferation of the anti-IgM-stimulated B cells (Figure 3.4C). PD 98059 was also able to suppress ^{3}H-thymidine incorporation in these cells, but to a lesser degree than in cells stimulated with anti-IgM alone (Figures 3.4C and 3.4D). Thus, IL-4 had two effects: it increased ^{3}H-thymidine-incorporation, and rendered the cells less susceptible to inhibition by PD 98059. IL-4 increases ^{3}H-thymidine incorporation in this assay both by acting as a B cell survival factor and by increasing the percent of B cells that enter the cell cycle (Hodgkin et al., 1991; Oliver et al., 1985; Rabin et al., 1985). PD 98059 did not substantially inhibit the ability of IL-4 to enhance cell survival (data not shown). In contrast, the percentage of B cells that entered S phase in response to anti-IgM plus IL-4 was significantly diminished by treatment with PD 98059 (Figure 3.4E).

Discussion

We wished to study the role(s) the Ras-MAP kinase pathway plays in B cell responses to antigen. To do this, we employed a pharmacologic inhibitor of MEK 1/2, PD 98059, to inhibit activation of this pathway in both WEHI-231 immature B cells and in mature splenic B cells. PD 98059 inhibits MEK 1/2 specifically, and does not block activation of the closely related MEK family members MKK3 or SEK(MKK4), nor does it inhibit numerous other serine/threonine, tyrosine, or lipid kinases (Figure 3.1D) (Alessi et al., 1995; Dudley et al., 1995; Lazar et al., 1995; Pang et al., 1995). *In vitro* immunocomplex kinase assays demonstrated that PD 98059 was able to substantially inhibit BCR-induced ERK2 activity in a dose-dependent manner. Treatment with PD 98059 also inhibited the BCR-induced expression of the early response gene *egr-1*, demonstrating the importance of the Ras-MAP kinase pathway for this gene induction. This result is consistent with previous studies demonstrating the requirement of promoter serum response elements (SREs) for *egr-1* expression (McMahon and Monroe, 1995), and the ability of dominant negative mutants of Ras and Raf to block *egr-1* promoter-driven reporter gene expression in response to BCR crosslinking (McMahon and Monroe, 1995). Anti-IgM-induced proliferation of splenic B cells was also substantially inhibited by PD 98059, both in the absence and presence of IL-4. Thus, MEK1/2 activity and presumably ERK activity play important roles in BCR-induced proliferation. In contrast, this pathway does not appear to participate in the BCR-induced growth arrest and apoptosis of the immature WEHI-231 B cell line.

Our results indicating that activation of the Ras-MAP kinase pathway is important for proliferation of splenic B cells are consistent with results obtained with splenic B cells from mice lacking the Src-family tyrosine kinase Lyn (Chan et al., 1997). Most signalling events were delayed and/or diminished in these cells, but BCR-induced activation of the Ras-MAP kinase pathway and proliferation were both increased. These results are also consistent with observations in other cell types, such as fibroblasts and PC12 cells, where this pathway also contributes to cellular proliferation (Kim et al., 1991; Marshall, 1994; Marshall, 1995; Pritchard et al., 1995; Sewing et al., 1997; Woods et al., 1997). The mechanism by which ERK promotes BCR-mediated proliferation is not yet defined, although it is likely to involve changes in gene expression; BCR stimulation rapidly induces changes in expression of several important cell cycle regulators, such as cyclins, cyclin-dependent kinases, and cell cycle inhibitors (Reid and Snow, 1996; Solvason et al., 1996; Tanguay and Chiles, 1996). The ERK kinases phosphorylate and activate several transcription factors, including Elk-1 and SAP-1a, which help enhance transcription of genes whose promoters contain SREs (Treisman, 1996). Indeed, the *egr-1* early response gene contains several SREs, is rapidly induced upon BCR crosslinking, and required ERK activity for its expression (Figure 3.2A).

In addition to the Ras-MAP kinase pathway, HS1, Vav and the PLC-γ pathway are also important for BCR-mediated proliferation (Monroe and Kass, 1985; Rothstein et al., 1986; Taniuchi et al., 1995; Tarakhovsky et al., 1995; Zhang et al., 1995). Just as ERK kinases activate Elk-1 and Sap-1a, each of the above signalling pathways likely activates other pre-existing transcription factors. For example, the PLC-γ pathway activates the serine/threonine phosphatase calcineurin, which dephosphorylates and activates the

transcription factor NF-AT (Rao et al., 1997). Together, these pre-existing but newly-activated transcription factors induce expression of early response genes, many of which, like Egr-1, are transcription factors themselves. These "secondary" transcription factors likely control the expression of many other genes important for events such as proliferation and interaction with helper T cells. Indeed, Egr-1 is required for expression of CD44 and ICAM-1 (see below). Different signalling pathways, then, could regulate the expression of different cell cycle genes by virtue of the transcription factors they activate. Some cell cycle genes may require the activation of transcription factors controlled by one signalling pathway, and others by multiple pathways.

As expected, IL-4 increased BCR-induced ^{3}H-thymidine incorporation dramatically. This proliferation was also inhibitable by PD 98059, but to a lesser degree than with BCR stimulation alone (compare Figs. 4B and 4D). IL-4 increases ^{3}H-thymidine uptake in these assays both by increasing B cell survival and by enabling lower doses of anti-IgM to induce a greater percentage of cells to divide for several cell divisions (Hodgkin et al., 1991; Oliver et al., 1985; Rabin et al., 1985). PD 98059 did not markedly affect the ability of IL-4 to enhance B cell survival (data not shown). This result is not surprising, since IL-4 enhances survival even in the absence of ERK activation, such as in B cells treated with IL-4 alone (Hodgkin et al., 1991) (data not shown). On the other hand, PD 98059 did dramatically reduce the percentage of cells that underwent DNA synthesis in response to anti-IgM plus IL-4 (Figure 3.4E), indicating that BCR plus IL-4-mediated proliferation is largely ERK-dependent.

Anti-IgM plus IL-4-induced proliferation was more resistant to inhibition by PD 98059 than proliferation induced by anti-IgM alone. One possibility to explain this result would be that IL-4 increased the activity of

ERK in BCR-stimulated cells, but this did not appear to be the case (Figure 3.1C and data not shown). Moreover, ERK2 activity was suppressed by PD 98059 to a similar extent in cells stimulated with or without IL-4 (Figures 3.1E and F). Thus, it appears that IL-4 stimulates other distinct signalling events that diminish, but do not totally relieve, the requirement for ERK activation in BCR-induced proliferation. These may include the activation of IRS-1, IRS-2 and Stat6, all of which can participate in IL-4 induced proliferation of hematopoietic cell types (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996; Wang et al., 1993; Wang et al., 1993).

In addition to proliferating, BCR-stimulated mature B cells upregulate adhesion molecules and a variety of cell surface proteins important for promoting interaction with helper T cells. PD 98059 only marginally inhibited upregulation of MHC II (data not shown), and did not at all block upregulation of B7-2 (Figure 3.2C), both of which participate in T cell activation. In contrast, BCR-induced upregulation of the transcription factor Egr-1 was suppressed by PD 98059, as mentioned above. Egr-1 expression is important for the upregulation of the adhesion proteins CD44 and ICAM-1 (Maltzman and Monroe, 1996; Maltzman and Monroe, 1996). CD44 has been proposed to facilitate B cell migration to and retention in secondary lymphoid follicles, where B cells interact with T cells (Maltzman and Monroe, 1996), and ICAM-1 expression helps mediate adhesion between antigen-presenting B cells and helper T cells during T cell activation, and subsequent stimulation of the B cells (DeFranco, 1998; Dustin and Springer, 1991). Upregulation of CD44 expression was partially blocked by PD 98059 (Figure 3.2B), consistent with its dependence on Egr-1 expression. These data provide evidence that in addition to its role in BCR-induced proliferation, the Ras-MAP kinase

pathway is important for the upregulation of specific proteins involved in cell-cell interactions and B cell trafficking.

The Ras-MAP kinase pathway has been implicated in growth arrest in murine fibroblasts (Pritchard et al., 1995; Sewing et al., 1997; Woods et al., 1997), and ERK activity also has been demonstrated to be important for apoptosis in some instances (Goillot et al., 1997; Watabe et al., 1996). However, we observed no inhibition in BCR-induced growth arrest or apoptosis in WEHI-231 B cells treated with PD 98059 (Figure 3.3). Because 20 μM PD 98059 did not completely block BCR-induced ERK activity in these cells, it is possible that only a modest increase in ERK activity is required to induce growth arrest and apoptosis. If this were true, however, we would have expected to see inhibition of BCR-induced growth arrest at low anti-IgM concentrations where ERK activity was blocked completely or nearly so, but this was not the case. It seems more likely, therefore, that other BCR-induced signalling pathways are mediators of apoptosis in immature B cells. Candidates include the JNK and p38 MAP kinases (Graves et al., 1996), as has been seen in some other cell types (Xia et al., 1995). This is an interesting point, because while BCR crosslinking in WEHI-231 cells strongly activates ERK2, it only weakly activates the JNK and p38 MAP kinases (Sutherland et al., 1996). In contrast, CD40 stimulation, which protects these cells from apoptosis, preferentially activates JNK and p38 over the ERK kinases (Berberich et al., 1996; Sutherland et al., 1996). Thus, the context in which these MAP kinase family members are activated may be important for biological outcome.

The data presented here demonstrate that the Ras-MAP kinase pathway plays an important role in a subset of B cell responses to antigen. BCR-induced proliferation, and upregulation of specific proteins important

for B cell function are dependent on activation of this pathway. In contrast, anti-IgM-induced growth arrest and apoptosis do not appear to require Ras-MAP kinase activity. BCR crosslinking activates many signalling pathways in both immature and mature B cells; however, it appears that specific biological responses to antigen require only some of these events.

Acknowledgments: We thank Vivien Chan for protocols and advice. We also thank V. Chan, A. Finn, C. Fitzer-Attas, M. L. MacKichan and T. Tian for critical review of the manuscript.

CONCLUDING REMARKS

Chapters 1 and 2 of this thesis describe experiments which sought to determine the minimal requirements for BCR-mediated signaling events. Specifically, they attempted to define the lymphoid-specific proteins which are sufficient to functionally link the BCR to common downstream signaling pathways. To this end, various B cell proteins were expressed in the AtT20 pituitary cell line, and the signaling properties of the resulting transfectants were determined. When successful, the reconstitution approach employed here can demonstrate both which proteins participate in BCR signaling, and in what capacity. This gain-of-function strategy is complementary to a loss-of-function gene knockout approach, which can identify proteins which are necessary for BCR signaling, but which does not address which proteins are sufficient. However, negative results with this approach, such as those described in Chapter 2 are less useful, as several alternative reasons for failure to reconstitute signaling may be difficult to distinguish.

The data in Chapter 1 demonstrate that the Syk protein tyrosine kinase is sufficient to reconstitute some downstream BCR signaling reactions in AtT20 cells expressing the BCR. Upon BCR crosslinking, Syk associated with the BCR, became tyrosine phosphorylated (and catalytically more activated; (Harmer and DeFranco, 1997)), directly and/or indirectly promoted the phosphorylation of many cellular proteins including Shc, and led to the activation of the ERK-MAP kinase pathway. The catalytic activity of Syk was not required for it to associate with the BCR or for it to become phosphorylated on tyrosine residues, but was required to reconstitute these downstream signaling events. These data support a now widely-accepted,

multistep model of BCR signaling (DeFranco, 1998; Law et al., 1993; Weiss, 1993; Weiss and Littman, 1994). According to this model, tyrosines in conserved cytoplasmic domain sequences of the BCR, referred to as ITAMs, (Cambier, 1995; Reth, 1989) are initially phosphorylated by Src-family kinases, in all likelihood by Fyn in the AtT20 cells. Subsequently, Syk binds to the phosphorylated ITAMs via it's two SH2 domains and becomes activated. Both ITAM binding and phosphorylation can contribute to Syk enzymatic activation (Rowley et al., 1995; Shiue et al., 1995). Syk then phosphorylates a variety of cellular proteins, including Shc (Harmer and DeFranco, 1997), promoting the activation of numerous signaling pathways. Indeed, Syk has been demonstrated to participate in activating the Ras-MAP kinase pathway (Chapter 1); in activating the phosphoinositide pathway (Takata et al., 1994); and in the phosphorylation of CD19 (chapter 2), which is instrumental in activating PI 3-kinase (Buhl et al., 1997; Tuveson et al., 1993). Syk is likely involved in activating other signaling pathways in response to BCR engagement as well. It is interesting to note that Syk also participates in ITAM-mediated signaling in a variety of other cell types, including FcεRI signaling in mast cells (Fischer et al., 1998; Oliver et al., 1994) and basophils (Holsinger et al., 1998), T cell antigen receptor (TCR) signaling in T cells (Chan et al., 1994; Couture et al., 1994), and FcγR signaling in monocytes and macrophages (Agarwal et al., 1993; Crowley et al., 1997).

Although introduction of Syk into AtT20 cells reconstituted some BCR-induced signaling pathways, it did not activate others, including the phosphoinositide pathway (chapter 1), and the PI 3-kinase pathway (chapter 2). These results suggested that additional, lymphoid-specific proteins critical for the activation of these pathways were not expressed in the AtT20 cells, and that their introduction into this system might further enhance signaling. To

this end, we introduced CD19, Btk, or both into the AtT20 cells, as described in Chapter 2.

CD19 was an attractive candidate to reconstitute BCR-mediated activation of PI 3-kinase and PLC. The CD19 cytoplasmic domain contains two YxxM motifs, which are bound by the p85 SH2 domains (Songyang et al., 1993), and previous work had demonstrated that PI 3-kinase protein and activity associates with CD19 subsequent to BCR crosslinking (Tuveson et al., 1993). Moreover, a product of PI 3-kinase activity, PIP_3, promotes Btk activation (Bolland et al., 1998; Salim et al., 1996), which in turn is required to activate PLC-γ2 (Takata and Kurosaki, 1996). Finally, recent evidence confirms CD19's role in activating PI 3-kinase and the phosphoinositide hydrolysis pathways (Buhl et al., 1997). In the CD19-expressing AtT20 cells, BCR-mediated CD19 tyrosine phosphorylation required Syk. However, BCR crosslinking in the CD19-expressing cells failed to reconstitute activation of PI 3-kinase. In these cells, the CD19-PI 3-kinase association occurred in a phosphotyrosine-independent, perhaps indirect manner even in unstimulated cells, and there was no increase in association subsequent to BCR crosslinking. The pre-association of these proteins probably explains the small increase in basal PI 3-kinase activity in these cells, compared to the syk13 cells. Also, the fact that additional PI 3-kinase was not recruited to CD19 upon stimulation likely explains the lack of further PI 3-kinase activation upon BCR crosslinking. PI 3-kinase activity seems to be required for PLC activation (Bolland et al., 1998; Buhl et al., 1997). It is possible, then, that in the CD19-expressing AtT20 cells, the small increase in basal PI 3-kinase activity was not enough to activate PLC, even in the presence of Btk, whereas a more robust activation of PI 3-kinase might have been sufficient.

It is possible that the AtT20 cells lack key regulatory elements present in B cells which prevents the phosphotyrosine-independent association of CD19 and PI 3-kinase, or which allows phosphotyrosine-dependent association between these two proteins. Moreover, it is conceivable that these cells express proteins that actively prevent the stimulation-dependent CD19-PI 3-kinase association. Perhaps one way to circumvent these potential problems would be to express Btk and a constitutively activated mutant of p110 PI 3-kinase, which has been generated (Klippel et al., 1996), rather than Btk and CD19. Targeting activated p110 to the cell membrane might provide sufficient PI 3-kinase activity in these cells to activate Btk, and then PLC. However, it is also possible that the AtT20 cells would not tolerate the activated p110. Indeed, a much higher percentage of the drug-resistant AtT20 colonies were positive for TacTailless (TacT) than for Tac-CD19, which suggested that the AtT20 cells did not tolerate the CD19 cytoplasmic domain very well. Perhaps this is because in most CD19-expressing cells, PI 3-kinase was constitutively activated, and this inhibited growth of the cells or induced their apoptosis.

It is also possible that the AtT20 expressing the BCR, Syk, Btk and the CD19 chimera still lacked additional lymphoid-specific machinery required to activate the phosphoinositide signaling pathway. For example, the T cell transmembrane protein, LAT, may localize PLC-γ to its substrate in the cell membrane upon TCR stimulation (Zhang et al., 1998). It is possible that a B cell version of LAT may function in a similar manner upon BCR crosslinking. In addition, Vav, which is expressed only in hematopoietic lineage cells, participates in TCR-mediated activation of the phosphoinositide signaling pathway, both as measured by calcium flux (Fischer et al., 1998; Holsinger et al., 1998; Turner et al., 1997), and by NF-AT activation (Wu et al.,

1995; Wu et al., 1996). How it participates in these events is currently unknown. One idea is that Vav is part of a multiprotein signaling complex that includes PLC-γ and therefore Vav helps stabilize the active state/location of PLC-γ. Also, the hematopoietic-specific protein SLP-76 appears to synergize with Vav in this regard (Wu et al., 1996), suggesting that perhaps its expression in AtT20 cells would also help reconstitute activation of PLC-γ.

It may be useful to switch to a transient expression approach to test all these possibilities. Cloning the genes encoding these proteins into vaccinia or adenovirus and infecting the AtT20 cells with these viruses may facilitate a more rapid analysis. Alternatively, it is possible that the using the AtT20 system will no longer be useful. Currently we are expressing up to seven B cell proteins in the AtT20 cells, and this might already be more than the cells can appropriately regulate. Indeed, the CD19-PI 3-kinase association was dysregulated in these cells. It is also possible that AtT20 cells might express signaling proteins that dominantly inhibit the BCR-induced events that we attempted to reconstitute. If so, the use of a reconstitution system more closely related to B cells, such as the J558 plasma cell system employed by Cambier and colleagues (Buhl et al., 1997), may be more fruitful.

Chapter 3 details experiments aimed at another goal, to define the role of the ERK MAP kinases in B cell responses to antigen. It has been known for several years that BCR crosslinking activates the ERK family of MAP kinases in both immature and mature B cells (Casillas et al., 1991; Gold et al., 1992; Tordai et al., 1994). However, the exact role(s) these kinases play in the biological responses to antigenic stimulation has not been determined. To answer this question, I employed a pharmacologic inhibitor of MEK1/2, PD 98059. MEK1 and 2 phosphorylate and activate ERK1 and 2. The use of pharmacologic inhibitors and mimics has been very beneficial in the past for

elucidating the roles of other BCR-activated signaling enzymes, including PI 3-kinase (Beckwith et al., 1996) and PLC-γ (Monroe and Kass, 1985; Page and DeFranco, 1990; Page and DeFranco, 1988; Page et al., 1991; Rothstein et al., 1986).

The role of BCR-induced MEK-ERK activity was examined in WEHI-231 immature B cells, as well as in resting primary mature splenic B cells from mice. Anti-IgM stimulation of WEHI-231 cells leads to growth arrest and apoptosis (Benhamou et al., 1990; Hasbold and Klaus, 1990; Page and DeFranco, 1990; Page and DeFranco, 1988; Page et al., 1991), neither of which were inhibited by PD 98059. Experiments are underway to determine whether the BCR-induced apoptosis of primary immature B cells is also resistant to inhibition by PD 098059. If so, it is possible that these tolerogenic responses are mediated by the JNK and p38 MAP kinases (Graves et al., 1996; Xia et al., 1995) or by other signaling reactions. For example, the phosphoinositide signaling pathway (Page and DeFranco, 1990; Page and DeFranco, 1988; Page et al., 1991; Takata et al., 1995) and the protein HS-1 (Fukuda et al., 1995) participate in BCR-mediated growth arrest and apoptosis in WEHI-231 cells.

BCR crosslinking in mature B cells induces the upregulation of a variety of cell surface proteins, entry into the cell cycle and proliferation (DeFranco, 1998). Helper T cells enhance proliferation, and induce B cell differentiation into antibody-secreting plasma cells, by providing cell-cell contact signals via CD40, and by releasing cytokines such as IL-4 and IL-5 (DeFranco, 1998). PD 98059 selectively inhibited the upregulation of the transcription factor egr-1 and the cell surface protein CD44. PD 98059 also inhibited BCR-induced proliferation, both in the presence and absence of IL-4, although the inhibition was lessened in the presence of IL-4. The latter effect could have been due to a reduction in cell survival, a decrease in the

percentage of cells that entered the cell cycle, or both. PD 98059 reduced the percentage of cells that entered the S phase in cells treated with anti-IgM + IL-4 (chapter 3) and anti-IgM alone (data not shown), whereas it appeared to have little if any effect on survival of cells treated with anti-IgM + IL-4 (data not shown). In contrast, preliminary experiments indicate that PD 98059 substantially inhibited the survival of cells treated only with anti-IgM (data not shown). This difference in survival may explain the differential inhibitory effects on proliferation of cells treated with anti-IgM versus anti-IgM + IL-4.

The experiments described in this work have attempted to determine the roles of Syk and of the MEK-ERK pathway in BCR-induced signaling events and in B cell biological responses to antigen. Clearly, there is much work left to be done, both to define specific signal transduction pathways in B cells, as well as to determine how a B cell interprets the many cues it receives from its complex environment.

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