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B cell activation: The regulation by T cells and the induction of the protein tyrosine kinase syk

A thesis presented to the faculty of the Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

Mark S. Forman

April 1, 1994
The Rockefeller University
New York, New York

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ABBREVIATIONS

allo-B blast	allo-stimulated B lymphoblast
allo-T blast	allo-stimulated T lymphoblast
AKR	AKR1.G10
AP	alkaline phosphatase
ARAM	antigen recognition activation motif
ARH-1	antigen receptor homology-1
BBS	borate buffered saline
BCR	B cell receptor
bp	nucleotide base pair
BSA	bovine serum albumin
cAMP	cyclic AMP
CD	cluster of differentiation
CD_2F_1	[BALB/c x DBA/2]F_1
CFA	complete Freund's adjuvant
complete RPMI	Hepes buffered RPMI supplemented with antibiotics and glutamine
cpm	counts per minute
CTLA	cytotoxic T lymphocyte antigen
D	Donkey
DAG	diacylglycerol
DC	dendritic cell
dd	double distilled
DNase	deoxyribonuclease
DTT	dithiothreitol

E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EL-4 sn	lymphokine-rich supernatant from phorbol-ester-induced EL-4 thymoma cell line
ELISA	enzyme-linked immunosorbent assay
FcR	Fc receptor
FDC	follicular dendritic cell
G	Goat
GM/CSF	Granulocyte/macrophage colony stimulating factor
[^{3}H]TdR	tritiated thymidine
Ham	hamster
Hepes	N-2-hydroxyethylpiperazine-N'-2'ethane sulfonic acid
HI-FCS	heat inactivated fetal calf serum
HRP	horse radish peroxidase
ICAM	intercellular adhesion molecule
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
αIg blast	anti-Ig stimulated B lymphoblast
αIg/EL-4 blast	anti-Ig and EL-4 sn stimulated B lymphoblast
IL	interleukin

IP_3	1,4,5-inositol triphosphate
kD	kilodalton
KLH	keyhole limpet hemocyanin
LFA	leukocyte function antigen
LPS	lipopolysaccharride
M	mouse
mAb	monoclonal antibody
MHC	major histocompatibility
mIg	membrane immunoglobulin
MLR	mixed leukocyte reaction
N_3	sodium azide
NP-40	nonidet P-40 detergent
nt	nucleotide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline [without Ca^{2+} and Mg^{2+}]
PCR	polymerase chain reaction
PD	Dulbecco's modified phosphate buffered saline
PdBu	phorbol dibutyrate
PI	phosphatidyl inositol
PKC	protein kinase C
PNA	peanut agglutinin
PNPP	para-nitrophenylphosphate
PTK	protein tyrosine kinase

R	rabbit
r	recombinant
RNase	ribonuclease
SDS	sodium dodecyl sulfate
SH2	*src* homology-2
SH3	*src* homology-3
TAM	tyrosine-based activation motif
TCR	T cell receptor
Tdt	terminal deoxy transferase
TE	10 mM Tris/1 mM EDTA
TGF	transforming growth factor
Th	helper T cell
TLC	thin layer chromatography
U	unit
v	volume

ABSTRACT

During the activation and differentiation of B cells, there are critical stages of regulation which determine the fate of the B cell. Resting B cells are initially stimulated by either native antigen or T cells via antigen-specific cell surface receptors. The context and timing of this signal induces either activation, tolerance or apoptosis of the B cell. If the initial signal productively activates the B cell, it acquires enhanced responsiveness to antigen independent signals, i.e. cytokines. The array of cytokines with which the B lymphoblast interacts induces proliferation and/or differentiation. We have studied several aspects of each of these stages in the B cell immune response.

We compared the requirements for membrane immunoglobulin-mediated tyrosine phosphorylation with the requirements for downstream signaling events. Our results demonstrate that receptor crosslinking is required for the accumulation of tyrosine phosphorylated proteins and for the induction of *in vitro* phosphotransferase activity of at least one kinase. The protein tyrosine kinase syk is one of the major endogenous substrates of this kinase activity. Moreover, we demonstrated that the *in vitro* phosphorylation of syk is induced synergistically by IL-4 and anti-immunoglobulin. We subsequently isolated a cDNA clone of human *syk* that, along with ZAP-70, defines a novel family of protein tyrosine

kinases. We modeled the SH2 domains of syk to design a mutational analysis of the structure and function of syk.

To facilitate the study of B cell responses to membrane immunoglobulin-independent signals, we devised a system of B cell activation dependent on a cognate interaction between B cells and allogeneic T helper cells. These allo-activated B blasts were unique in that IL-2 proved to be the predominant cytokine promoting their proliferation and Ig secretion, including high levels of Ig utilizing downstream heavy chain constant region genes. Both phenotypically and functionally, these B lymphoblasts resembled the germinal center B cell.

INTRODUCTION

A. The humoral immune response.

In 1890, von Behring and Kitasato described the production of a neutralizing agent in the blood of animals immunized with diptheria and tetanus toxin that could prevent disease (1). Serum from experimental animals was able to cure childen with diptheria or tetanus if administered early during the course of the illness. Von Behring coined the term "antibody" to describe this newfound substance (2). It was almost 80 years before the cellular basis for the antibody response was determined (3). These cells were named B lymphocytes to denote their origin in the bursa of Fabricius in birds (4). These pioneering experiments on antibody responses by von Behring and later by Ehrlich (5) paved the way for our understanding of antibody responses today.

Antibody responses are classified based on previous exposure to antigen [reviewed in (6)]. A prototypic "primary response" [eg. to a novel protein antigen] is characterized by low affinity IgM antibodies, with low levels of immunoglobulin [Ig] of other isotypes observed late [10 - 14 days] after antigen exposure. IgM titres usually peak in about 7 days. Upon re-exposure to antigen, the "secondary" response is both quantitatively and qualitatively different. Immunoglobulin secretion occurs more rapidly with peak levels of IgG [or IgA

or IgE] reached about 7 days after re-exposure to antigen. The amount of antigen-specific IgG secreted is 100-1000 times higher then during the primary response. Moreover, the Ig secreted has greater affinity for the immunizing antigen. The increase in Ig affinity as well as the secretion of IgG, IgA, and IgE are the result of two phenomenon restricted to B cells and Ig genes: somatic mutation (7,8) and isotype switching (9,10).

In vivo, specific histologic changes characterize a humoral immune response. Following exposure to antigen, germinal centers form in specific regions of peripheral lymphoid tissue as a consequence of rapid proliferation of antigen-specific B cells (11,12). These germinal centers are oligoclonal (13) and their formation is T cell-dependent (14,15). Somatic mutation and isotype switching of Ig genes probably occurs within the germinal centers (12,16,17).

Follicular dendritic cells [FDC], found predominantly within germinal centers, sequester native antigen in the form of immune complexes on their cell surface and thus, provide for long-term antigen exposure to B cells (18-20). The "clonal selection theory" (21) purports that B cells with Ig of increased affinity are selected/stimulated by antigen. Interestingly, *in vitro*, germinal center B cells will undergo apoptosis unless restimulated (22). Thus, B cells undergo both differentiation and selection within germinal centers.

B. *In vitro* models of B cell activation.

In vitro models of B cell activation have relied on both T cell-independent and T cell-dependent means of stimulation. B cell mitogens, such as LPS, activate resting B cells polyclonally (23-25). The addition of T cell- or macrophage-derived products [i.e. cytokines] promotes B cell differentiation and proliferation (24,26,27). However, the use of LPS is not physiological given the μg/ml quantities required for B cell activation (24) nor does it allow the discrete steps of B cell activation to be studied independently. Moreover, the mode of action and the putative "LPS receptor" on B cells remain unclear.

B cells can be activated independently of T cells with anti-immunoglobulin [anti-Ig] antibodies (28-30). Antibody mediated crosslinking of membrane Ig [mIg] on mature resting B cells results in their activation, as measured by their entry into the cell cycle and blast transformation (31,32). However, anti-Ig mediated activation, even when administered as a multivalent ligand, induces neither extensive B cell growth nor antibody secretion unless T cells or T cell-derived cytokines are included. Anti-Ig activated B lymphoblasts can be isolated and studied for their subsequent requirements for growth and differentiation (31,33,34). This is reminiscent of the fact that antibody responses to most protein antigens are dependent on T cell help (35,36).

B cells and T cells interact through both antigen-dependent and antigen-independent mechanisms [reviewed in (37,38)], both of which may induce signal transduction events in B cells. Several models of T cell-dependent B cell activation have been developed which exploit these B cell-T cell interactions. Membranes isolated from activated T helper [Th] cells will stimulate resting B cells to enter the cell cycle in an Ag-nonspecific, MHC class II unrestricted manner (39). The addition of lymphokines to the Th membrane-B cell cultures stimulates the B cell to proliferate and secrete Ig (39). However, the physiological relevance of this system is unclear since antibody responses to most antigens *in vivo* are dependent on MHC class II-restricted interactions between T cells and B cells (40-43).

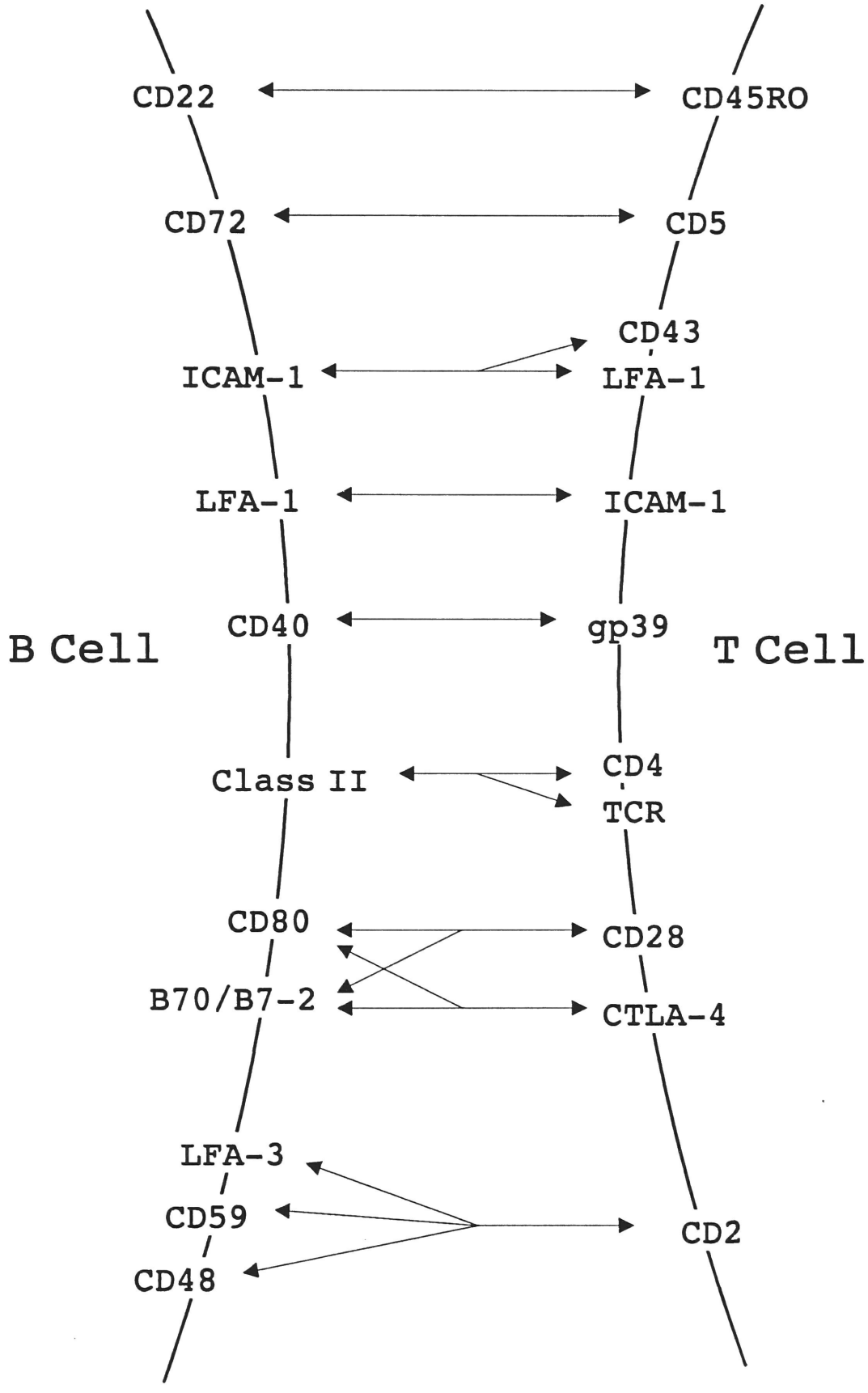
Three experimental systems for the activation of resting B cells that require a cognate interaction between T cells and B cells have been developed. In one system, antigen-specific B cells were activated by histocompatible, antigen-specific T cells or T cell clones plus antigen (44-46). However, the large numbers of antigen-specific B cells needed to study the biochemical and molecular basis of B cell activation and differentiation are not readily obtainable. Tony and Parker and colleagues developed an elegant system whereby B cells were incubated with suboptimal doses of soluble rabbit-anti-mouse Ig and provided help with MHC-restricted Th cell lines specific for rabbit Ig (47). The limitation of this system is

that it does not distinguish between the Ig and T cell-mediated signals. Alternatively, systems have been developed whereby class II-reactive T cells activate allogeneic B cells in a polyclonal fashion (48-51). Some B cell growth and Ig secretion was observed, but these systems were not characterized extensively. In Chapter I of this thesis, I describe a T cell contact-dependent system of activating murine B cells based on this latter model. We found that B cells activated by direct interaction with Th cells are remarkable in that interleukin-2 [IL-2] induces both proliferation and production of IgM, as well as high levels of IgG_1 and IgA. Moreover, in several respects these B lymphoblasts resemble germinal center B cells.

C. T cell-B cell interactions.

B cells and T cells interact in an antigen-specific manner through a trimolecular complex consisting of MHC class II on the B cell, the T cell receptor [TCR] and peptide (52-54). The MHC class II/TCR/peptide interaction is of relatively low affinity (55) and thus dependent on the contribution of proteins expressed on both the T cell and B cell [Figure 1] (37). The T cell co-receptor CD4 may contribute to the affinity of this complex by binding to the conserved regions of MHC class II (56-58). In addition, CD4 contributes to the MHC-mediated TCR signal by virtue of its

Figure 1. Molecules that mediate interactions between B cells and T cells.


CD22
CD45RO
CD72
CD5
CD43
ICAM-1
LFA-1
LFA-1
ICAM-1
B Cell
CD40
gp39
T Cell
Class II
CD4
TCR
CD80
CD28
B70/B7-2
CTLA-4
LFA-3
CD59
CD2
CD48

association with the protein tyrosine kinase [PTK], *lck* (59,60).

Cell surface molecules induced upon B cell or T cell activation mediate interactions between these cells [Figure 1]. CD40, a member of the nerve growth factor family of receptors (61), was originally described as a B cell surface molecule that upon ligation with monoclonal antibodies [mAb] induced B cell growth and Ig secretion (62,63), and prevented apoptosis of germinal center B cells (22,64). The CD40 ligand, gp39, is expressed on activated, but not resting, T cells (65-67). The interaction of gp39 with CD40 induces B cell proliferation and Ig secretion (68,69), and is required for efficient T cell-dependent B cell responses *in vivo* (70). This is dramatically demonstrated by the fact that gp39 is defective in the hyper-IgM syndrome (71-73). Individuals with hyper-IgM syndrome, manisfested by the loss of production of downstream isotypes, are characterized by their lack of T cell-dependent B cell responses (74). This provides evidence for the central role of the CD40/gp39 interaction in T cell-dependent B cell immune responses.

Co-crosslinking of CD28 and the TCR, with either antibody or ligand, activates resting T cells to proliferate and secrete IL-2 (75,76). The ligands for CD28, CD80 (77) and the related molecules B70 (78) and B7-2 (79), are expressed on B cells, DC, and activated macrophages. However, CD80 expression is enhanced upon B cell activation thereby

facilitating its interaction with CD28 (80,81). In addition, a second ligand for CD80, CTLA-4, is only expressed on activated T cells (82). Engagement of CTLA-4 is required for both efficient T cell signaling *in vitro* (82) and primary antibody responses *in vivo* (83). Thus, the activation of either the B cell or the T cell promotes the interaction between the two cell populations.

CD5 is a co-stimulatory molecule expressed on T cells and a subset of B cells (84). Crosslinking of CD5 induces an increase in intracellular calcium and, in conjunction with TCR crosslinking, stimulates T cell proliferation (85,86). The ligand for CD5 is the B cell restricted glycoprotein, CD72 (87). Anti-CD72 antibodies stimulate B cell proliferation, MHC class II expression, and increases in intracellular calcium (88,89). Furthermore, antibodies to murine CD72 block antibody responses to T cell-dependent antigens *in vivo* (90).

The B cell-specific molecule, CD22, associates with mIg (91) and is phosphorylated on tyrosine following mIg crosslinking (91-93). The ligands for CD22 are several T cell and B cell α2-6 linked sialylproteins, including CD75 and the receptor tyrosine phosphatase CD45RO (94,95). The T cell accessory molecule, CD2, facilitates B cell-T cell interaction by virtue of its ligands LFA-3, [CD58] (96), CD59 (97), and CD48 (98). Engagement of CD2 with either antibodies or ligand promotes T cell activation via a TCR-dependent mechanism (99,100).

Several other cell surface adhesion molecules/receptors, with a wider tissue distribution, also contribute to B cell-T cell interaction and activation. CD11a/CD18 [LFA-1] and its ligand, CD54 [ICAM-1] (101) are upregulated upon activation of both B cells and T cells (102,103). Antigen specific interactions between resting B cells and T cells is blocked by antibodies to LFA-1 (45), and efficient signaling in B cells requires ICAM-1/LFA-1 interaction (104). ICAM-1 also binds to sialophorin [CD43] which is normally expressed on T cells and defective in individuals with the X chromosome-linked disease, Wiskott-Aldrich syndrome (105). People with Wiskott-Aldrich syndrome have aberrant T cell function and as a consequence, impaired humoral immune responses (106), implicating the CD43/ICAM-1 interaction as the source of the immune deficiency.

D. T cell-derived cytokines and B cell growth and differentiation.

T cells transduce two types of signals upon interacting with B cells. The first category consists of the contact dependent interaction mediated by many of the molecules described above. The second is mediated by the cytokines that are produced by the activated T cells. Numerous cytokines are secreted by activated T cells including those with well-defined effects on B cells such as IL-2, IL-4, IL-5, IL-6, IL-

10, IL-13, and IFNγ (107).

Several cytokines have been touted as B cell growth factors, although no cytokines have been identified to date that promote extensive or long term B cell proliferation. IL-4 was independently described both as a B cell stimulatory factor that costimulates proliferation of anti-Ig activated B cells, and as a differentiation factor that promotes isotype switching to IgG_1 (108,109). Although by itself IL-4 does not stimulate proliferation of resting or activated B cells, it efficiently costimulates entry of B cells into the cell cycle with anti-Ig, anti-CD40, *Staphylococcus aureus Cowan* [SAC], cell membranes from activated T cells, or antigen (108,110-112). In addition, IL-4 can facilitate B cell-T cell interactions by upregulating class II MHC on B cells (113,114).

IL-5 was also originally described as a murine B cell stimulatory factor (115). IL-5 does not induce proliferation of B cells, but rather, induces murine B cells to differentiate and secrete Ig (116-118). IL-6 is a cytokine with a broad spectrum of effects on various target cells, [reviewed in (119)], but its first immunological description was as a hybridoma/plasmacytoma growth factor (120-122). IL-6 does not, however, induce proliferation of normal B cells, but promotes Ig secretion (123,124). IL-10 induces proliferation of pre-activated human B cells (125) and increases viability of murine B cells (126). The recently described IL-13 has

several functions in common with IL-4, including the stimulation of human B cell proliferation in combination with anti-Ig and anti-CD40 antibodies (127).

IL-2, originally described as a T cell growth factor (128), promotes proliferation of activated T cells *in vitro* (128,129). IL-2 was subsequently shown to induce both the proliferation of and Ig secretion by preactivated human B cells (130-132). In the murine system, B cells activated with high levels of LPS plus anti-Ig proliferated in response to IL-2 (133). However, IL-2 stimulation of murine B cells activated with anti-Ig alone is minimal and quite variable (31). Thus, when we began our studies, the ability of murine B cells to respond to IL-2 under more physiological conditions was unclear. Other T cell-derived cytokines such as IL-4, IL-5 and IL-10 may confer B cell responsiveness to IL-2 by inducing IL-2 receptor [IL-2R] expression (134,135).

T cell-derived cytokines are also instrumental in regulating the quality of the antibody response either by promoting isotype switching or by selecting for previously switched B cells. IL-4 promotes the secretion of IgG_1 (109) and IgG4 (136,137) in murine and human B cells, respectively, as well as IgE in both species (137-140). Recently, IL-13 was also demonstrated to promote IgG4 and IgE production by human B cells (141). IFNγ stimulates murine B cells to secrete both IgG_{2a} (138,142) and IgG_3 (143) while inhibiting the production of IL-4-induced isotypes (138,140). IgA production is

stimulated by TGFß in IgA^{neg} B cells (144) and by IL-5 in IgA^{pos} B cells (145,146). Finally, IgG1 and IgG3 secretion by human IgD^{pos} B cells is induced by IL-10 (147). Although the mechanism of action of cytokines remains unknown, in almost all cases, isotype switching requires an activating signal which promotes entry into the cell cycle as well as specific cytokines which promote secretion of Ig utilizing downstream heavy chain constant regions.

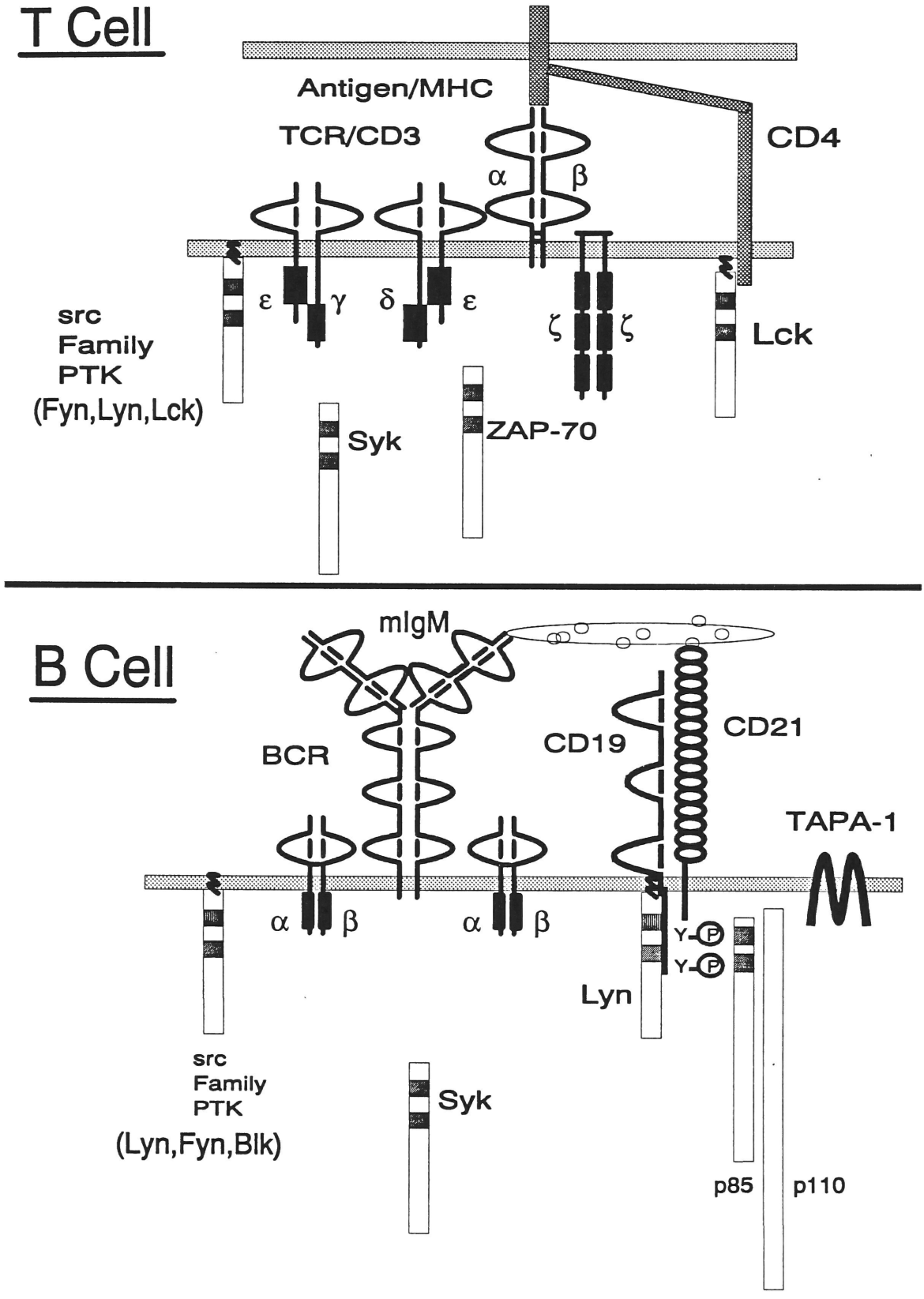
Th cells are distinguished functionally based on their pattern of cytokine production (148). The subset of Th1 cells produce primarily IL-2 and IFNγ, with low levels of IL-4 and IL-5, while the converse is true for Th2 cells (148). Subsequent analysis has revealed additional differences in cytokine production [Th1, TNFβ; and Th2, IL-6,IL-9 and IL-10] as well as a number of cytokines that are produced by both subsets [TNFα, GM-CSF, and IL-3] (149). During the immune response, the generation of predominantly Th1 or Th2 cells will effect the quality of the humoral immune response by promoting different patterns of Ig isotype secretion. For instance, viruses promote Th1 responses and IFNγ secretion which induces IgG_{2a} production (138,142). The effector functions of IgG_{2a} are mediated by its ability to fix complement (150) and bind $Fc_{\gamma}R$ (151). On the other hand, parasitic infections tend to promote Th2 responses and IL-4 secretion which induces IgG_1 and IgE secretion (109,137-140). These isotypes promote antibody-dependent cellular

cytotoxicity mediated by Fc receptors on eosinophils (152). Moreover, IL-5, [also secreted by Th2 cells] is the predominant regulator of eosinophilia (153,154). Thus, the differentiation into specific Th subsets regulates the effector mechanisms utilized by B cells.

E. The B cell receptor [BCR] complex.

Engagement of mIg leads either to B cell activation or tolerance. The internalization of mIg-bound antigen allows for subsequent processing and presentation to T cells, a process mediated by the wide array of molecules descibed above. However, mIg has only a short [3 - 28 amino acids] cytoplasmic tail which is probably not sufficient to mediate signal transduction (155). Instead, like the TCR, mIg associates with transmembrane glycoproteins, specifically Ig_α and Ig_β (156-160), the products of the B cell-specific genes, mb-1 (159,161) and B29 (161), respectively [Figure 2]. Ig_α and Ig_β have extensive cytoplasmic tails (162,163) which, when associated with mIg, form a disulfide linked heterodimer (159,160), although the precise stoichiometry remains unclear [Figure 2]. At least Ig_α is required for surface expression of mIg, but the requirement for Ig_β varies with Ig isotype (158,164). However, neither Ig_α nor Ig_β is necessary for mIg internalization (165,166). These Ig-associated molecules have no known intrinsic enzymatic activity but couple the BCR to

Figure 2. BCR and TCR with putative coreceptor complexes. mIg and CD21 are bound to antigen and the C3 fragments of complement. ARH-1 motifs are indicated by black boxes. SH2 domains are indicated as gray boxes and SH3 domains are indicated as striped boxes.


T Cell
Antigen/MHC
TCR/CD3
CD4
α
β
ε
γ
δ
ε
ζ
ζ
Lck
src
Family
PTK
(Fyn,Lyn,Lck)
Syk
ZAP-70
B Cell
mIgM
BCR
CD19
CD21
TAPA-1
α
β
α
β
Y-P
Y-P
Lyn
src
Family
PTK
(Lyn,Fyn,Blk)
Syk
p85
p110

the cytoplasmic signaling apparatus. When either Ig_α or Ig_β is expressed as a fusion protein independent of mIg, each is capable of mediating early and late signal transduction events characteristic of the intact receptor complex (167-169).

The ability of Ig_α and Ig_β to mediate signal transduction is explained, in part, by the presence of a motif that was first described by Reth (170). It consists of two repeats of the sequence YXXL separated by 6 to 8 amino acids within the minimum functional consensus sequence $[D/E]XXYXXL[X]_{6-8}YXXL$. This motif has been found in numerous molecules that are associated with various receptor complexes including the BCR, TCR, $Fc_\gamma R_{III}$, and $Fc_\epsilon R_I$ (170) as well as components of two viruses, bovine leukemia virus and Epstein-Barr virus, which are capable of transforming B cells (171). When coupled to cell surface receptor molecules, this consensus sequence is sufficient to couple extracellular events to signaling within the cell (172-174). Phosphorylation of the tyrosine residues within this motif links the receptor with downstream signaling events by association with src homology-2 [SH2] containing proteins (175,176). This motif is alternatively referred to as the antigen recognition activation motif [ARAM] (177), tyrosine-based activation motif [TAM] (178), and antigen receptor homology-1 [ARH1] motif (179).

F. BCR coreceptor function.

Coreceptor function [analogous to CD4 or CD8 in T cells] in B cells is beginning to be elucidated [Figure 2]. CD22, [described above], is one possible candidate for this function (91), although a more likely candidate is the B cell specific molecule CD19 (180,181), which associates with mIg (182). Antibody crosslinking of CD19 lowers the threshold for mIg-mediated activation (183). No ligand for CD19 has been identified, but CD19 does form a complex with several other molecules including CD21 [complement receptor 2 -CR2] (184), TAPA-1 and Leu-13 (185). CD21 binds numerous ligands including the C3 fragments of complement [iC3b and C3dg] (186,187), and CD23 (188,189). This complex co-caps with the BCR following anti-Ig crosslinking (182), while co-ligation of CD21 and mIg synergistically induces a calcium response (190,191). In addition, monoclonal anti-CD21 or recombinant soluble CD21 inhibits antibody responses *in vivo* (192,193). A potential mechanism for the function of the coreceptor might involve complement fixed-antigen signaling the B cell through mIg and the CD19/CD21 complex simultaneously [Figure 2]. Alternatively, FDC might crosslink both mIg and CD21 via immune complexes and CD23 expressed on the cell surface (194).

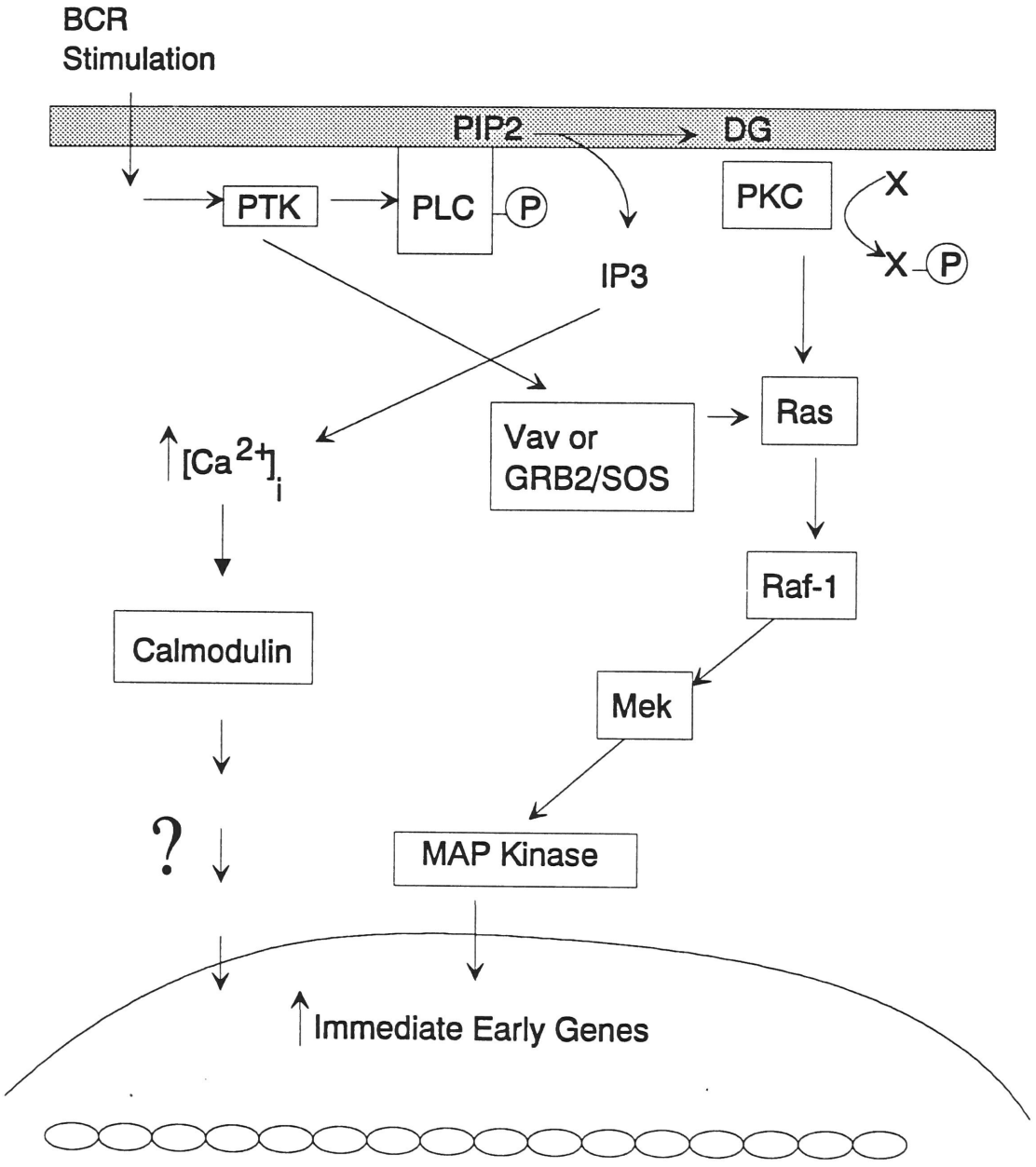
G. mIg mediated activation of phosphatidylinositol and ras second messenger pathways.

mIg must be engaged by multivalent ligand for induction of subsequent growth and Ig secretion. Receptor crosslinking by polyclonal anti-Ig initiates several well-documented immediate responses in B cells [Figure 3]. One of the first responses observed following receptor engagement is an increase in intracellular calcium (195-197). Two calcium peaks are observed. The first is mediated by release of intracellular calcium, while the latter, sustained response is mediated by extracellular calcium (197). Anti-Ig subsequently has been demonstrated to induce phosphatidyl inositol [PI] hydrolysis and the generation of diacylglycerol [DAG] and inositol 1,4,5-triphosphate [IP_3] (198,199) along with activation of protein kinase C [PKC] (200,201) and phosphorylation of MARCKS (201). IP_3 and DAG induce the increase in calcium and activation of PKC, respectively (202). The increase in IP_3 can be enhanced by the addition of a nonhydrolyzable analog of GTP thus implicating a G protein in the signaling cascade (203-205). At least one of these G-proteins is ras as anti-Ig crosslinking induces ras activity in B cells (206) and co-capping of ras with mIg (207).

H. mIg mediated tyrosine phosphorylation.

Engagement of the BCR rapidly induces [within 30 seconds] the tyrosine phosphorylation of many cytoplasmic and membrane proteins (208-210). Tyrosine phosphorylation is the first

Figure 3. Downstream signaling pathways induced following BCR stimulation. Note that the regulation of ras by GAP, as well as the phosphorylation of MARCKS is not depicted.


BCR
Stimulation
PIP2
DG
PTK
PLC
P
PKC
X
X
P
IP3
Vav or
GRB2/SOS
Ras
[Ca2+]i
Raf-1
Calmodulin
Mek
?
MAP Kinase
Immediate Early Genes

response detectable following receptor engagement and is required for the anti-Ig induced calcium response (210). Moreover, tyrosine phosphorylated proteins co-localize with mIg caps (211) and mIg internalization depends on tyrosine phosphorylation (212). Tyrosine phosphorylated proteins include the Ig-associated proteins, Ig_{α} and Ig_{β} (160,213), phospholipase $C_{\gamma 1}$ [$PLC_{\gamma 1}$] (214,215) and $PLC_{\gamma 2}$ (214,216,217), CD19 (218), CD22 (91,92), MAP-2 kinase (219), PI-3 kinase (220), vav (221), ras.GTPase activating protein [GAP] and the GAP-associated proteins p62 and p190 (222).

Anti-Ig induces the kinase activity and phosphorylation of several members of the src-related family of PTK including lyn, blk, and fyn, and under certain conditions, lck (223-225). At least two of these PTK, blk (226) and lyn (227), are preferentially expressed in B cells. Engagment of mIg induces these PTK to associate with the BCR (223-225), although it is not clear what molecules mediate these interactions. The Ig-associated molecules are likely candidates. Phosphorylation of the tyrosines within the ARH-1 motif of Ig_{α} and Ig_{β} mediates their association with several non-receptor PTK and serine/threonine kinases (179,228).

A second possibility is the BCR coreceptor complex, CD19/CD21/TAPA-1,Leu-13. Upon tyrosine phosphorylation, CD19 associates with PI-3 kinase (218). In addition, crosslinking of mIg induces PI-3 kinase to associate with the PTK, lyn (229). Thus, co-ligation of mIg with the coreceptor complex

may bring both lyn and PI-3 kinase into the BCR complex [Figure 2].

Tyrosine phosphorylation and signal transduction through both the TCR and BCR is potentially regulated by CD45, the major cell surface protein tyrosine phosphatase on lymphocytes (230,231). Although the ligand for CD45 remains elusive, several experiments suggest a role for CD45 in B cell activation. First, CD45 regulates the basal level of tyrosine phosphorylation in B cells (232). Second, CD45 co-caps with mIg (233). In addition, CD45 is required for calcium fluxes, at least in plasmacytoma cell lines (233) and its activity is regulated by changes in the intracellular concentration of calcium (234). Lastly, CD45 can dephosphorylate Ig_{α} and Ig_{β} *in vitro* (233). There is more direct evidence for the role of CD45 in T cells, where TCR mediated signaling is impaired in cells expressing enzymatically inactive CD45 (235,236).

I. PTK72/spleen tyrosine kinase [syk].

B cell activation is also accompanied by phosphorylation of a 72 kD PTK referred to as PTK72 (237). PTK72 was first purified by Gaehlen and co-workers as a 40 kD catalytically active fragment from bovine thymus (238). A polyclonal antibody specific for this fragment immunoblotted a 72 kD PTK that was preferentially expressed in lymphoid cells (239). Tyrosine phosphorylation of PTK72 is induced in B cells

activated by anti-Ig (237) and PTK72 is co-precipitated with mIg in digitonin lysates of anti-Ig stimulated B cells (240,241). PTK72 is also co-precipitated with two other receptor complexes, $Fc_{\epsilon}R_I$ (242) and $Fc_{\gamma}R$ types I and II (243,244). Taniguchi and co-workers independently cloned spleen tyrosine kinase [*syk*] from porcine spleen cells (245). Gaehlen and co-workers established the identity of PTK72 and *syk* by immunoprecipitation of PTK72 with an antibody generated against a peptide of the deduced amino acid sequence of syk [personal communication]. The predicted amino acid sequence of syk contains a C-terminal PTK domain, two tandem N-terminal SH2 domains and no N-terminal myristoylation site which distinguishes it from other families of PTK (245). Recently, a PTK referred to a ZAP-70 [zeta-associated-protein] that is induced by crosslinking the TCR was cloned and found to be closely related to *syk* (246).

The objective of this thesis.

At the time I began this work, the laboratory was interested in two specific aspects of B cell activation. The first was setting up a system of B cell activation dependent on a cognate interaction between B cells and T cells. To this end, we set up a system whereby allogeneic T lymphoblasts specifically activate resting murine B cells. These lymphoblasts are phenotypically and functionally distinct from

B lymphoblasts generated in the absence of contact dependent-T cell help.

A second interest in the lab was investigating the role of tyrosine phosphorylation in B cell signaling and receptor internalization. The activity of the PTK, syk, is one of the predominant kinases induced by mIg crosslinking of both murine B cells and human B cell lines. This activity is induced synergistically by IL-4. We isolated a full length full length clone of human *syk* and have generated polyclonal antibodies specific for syk. These reagents will facilitate further study of the role of syk in BCR-mediated signaling and BCR-specific internalization. Finally, we have generated models of the SH2 domains of syk to facilitate a mutational analysis of the structure and function of syk.

MATERIALS AND METHODS

A. Materials

1. Animals: 8-20 week old [BALB/c x DBA/2]F_1 [CD_2F_1], Balb/c, B6.$H2^k$, and C57BL/6 female mice were purchased from the Trudeau Institute [Saranac Lake, NY]. 8-12 week old New Zealand white female rabbits were purchased from Buckshire Corp. [Lansdale, PA].

2. Media: Primary cells were cultured in Hepes-buffered RPMI 1640 [Gibco Laboratories, Grand Island, NY] containing 10% heat inactivated fetal calf serum [HI-FCS] [Hazelton Research Products, Inc., Lenexa, KA or Atlanta Biologicals, Norcross, GA], 2 mM glutamine, 100 μg/ml streptomycin, 10 μg/ml gentamicin, 100 μg/ml penicillin [complete RPMI/10], and 50 μM β-mercaptoethanol [β-ME]. Hybridomas and the cell lines, AKR1.G10 [AKR], RAMOS and Daudi, were cultured in complete RPMI supplemented with 5-20% HI-FCS and L10A6.2 were cultured in complete RPMI/10 plus 50 μM β-ME and 1 mM sodium pyruvate.

3. Monoclonal antibodies [mAb]: The mAb utilized are described in Table 1. 11-4.1, M5/114.15.2, B21-2, C3PO, HO-2.2, M1/70, 3C7, 7D4, B5-5, and 33D1 were generously provided by Dr. Ralph Steinman, [Rockefeller University, New York, NY].

Table 1. Monoclonal Antibodies

Specificity	Hybridoma*	Isotype	Ref.
H-2K^{k,q,r,p}	11-4.1/TIB 95	Mouse γ_{2a}	(247)
I-A^{b,d,q}, I-E^{d,k}	M5/114.15.2/TIB 120	Rat γ_{2b}	(248)
I-A^{b,d}	B21-2/TIB 229	Rat γ_{2b}	(249)
IgM	Bet2/HB 88	Rat γ_1	(250)
IgD	JA12.5	Rat γ_{2b}	(251)
Igδ^a	Hδ^a/1	Rat γ_{2b}	(252)
$TCR_{\alpha\beta}$	H57	Hamster γ	(253)
$CD3_\epsilon$	2C11	Hamster γ	(254)
CD4/Lyt1.2	C3PO	Mouse μ	(255)
CD8/Lyt2.2	HO-2.2/TIB 150	Mouse μ	(256)
CD11a/LFA-1	FD441.8/TIB 213	Rat γ_{2b}	(257)
CD11b/Mac-1	M1/70/TIB 128	Rat γ_{2b}	(258)
CD23/$Fc_\epsilon R_{II}$	B3B4	Rat	(259)
CD25/IL-2Rp55	3C7/TIB 222	Rat γ_1	(260)
CD25/IL-2Rp55	7D4/CRL 1698	Rat μ	(260)
CD32/$Fc_\gamma R_{II}$	2.4G2/HB 197	Rat γ_{2b}	(261)
CD44/Pgp-1	18C8	Rat μ	(262)
CD45/LCA	M1/9.3.4/TIB 122	Rat γ_{2a}	(263)
$CD45R_A$/B220	RA3-3A1/6.1/TIB 146	Rat μ	(264)
$CD45R_B$	23G2/HB 220	Rat γ_{2a}	(265)
CD54/ICAM-1	YN1/1.7/CRL 1878	Rat γ_{2a}	(266)
CD62L/L-selectin	Mel-14/HB 132	Rat γ_{2a}	(267)
CDw90/Thy-1	B5-5	Rat γ_{2b}	(268)
CDw90/Thy-1.2	HO-13.4/TIB 99	Mouse μ	(269)
Heat Stable Antigen	J11d/TIB 183	Rat μ	(270)
33D1	33D1/TIB 227	Rat γ_{2b}	(271)
F4/80	F4/80/HB 198	Rat γ_{2b}	(272)
IL-2	S4B6.1/HB 8794	Rat γ	(148)
IL-4	11B11/HB 188	Rat γ_1	(273)
IL-5	TRFK-5	Rat γ	(274)
Tyr-PO_4	PY69	Mouse γ	(275)

* TIB [Tumor Immunology Bank], HB [Hybridoma Bank], and CRL [Cell Repository Line Bank].purchased from the American Type Culture Collection [Rockville, MD]. JA12.5 and HO-13.4 were

Bet2, Mel-14, RA3-3A1/6.1, M1/9.3.4, YN1/1.7 and Mel-14 were provided by Dr. E. Vitetta, [University of Texas, Southwestern Medical Center, Dallas, TX]. Hδ^a/1 was provided by Dr. F. Finkelman [Uniformed Health Services, Rockville, MD]. H57 was provided by Dr. R. Kubo [Cytel Corp., San Diego, CA]. 2C11 was provided by Dr. J. Bluestone [University of Chicago, Chicago, IL]. FD441.8 was provided by Dr. F. Fitch [University of Chicago, Chicago, IL]. B3B4 was provided by Dr. D. Conrad [Virginia Commonwealth University, Richmond, VA]. 2.4G2 was provided by Dr. J. Unkeless [Mount Sinai School of Medicine, New York, NY]. J11d was provided by Dr. J. Sprent [Scripps Research Institute, San Diego, CA]. F4/80 was provided by Dr. S. Gordon [University of Oxford, Oxford, Great Britain]. S4B6.1 and TRFK-5 were provided by Dr. T Mosmann [University of Alberta, Alberta, Edmonton, Canada]. 11B11 was provided by Dr. W. Paul [National Institute of Allergy and Infectious Disease, Bethesda, MD]. PY69 was purchased from ICN [Cleveland, OH]. mAb were used in the form of hybridoma culture supernatants or 50% saturating ammonium sulfate precipitates of serum-free hybridoma supernatants supplemented with 1% Neutridoma SP [Boehringer Mannheim Biochemicals, Indianapolis, IN].

4. Polyclonal antibodies: Isotype specific [μ, γ_1, γ_{2a}, γ_{2b}, γ_3, and α] goat-anti-mouse [GαM] antibodies [unconjugated and alkaline phosphatase [AP]-conjugated] were purchased from

Southern Biotechnology Associates [Birmingham, AL]. Fluoresceinated antibodies [MαRat Ig, GαM Ig, GαM μ, and rabbit-anti-hamster [RαHam] Ig] were purchased from Jackson Immunoresearch [West Grove, PA]. Intact and F[ab']$_2$ fragments of RαM Ig, GαM Ig, MαR Ig, MαRat Ig, and RαG Ig were also purchased from Jackson Immunoresearach. Fab' fragments of RαM Ig and intact Rα ova were generously provided by Dr. E. S. Vitetta [University of Texas, Southwestern Medical Center]. A.P. conjugated and horse radish peroxidase [HRP]-conjugated donkey [D]αR Ig were purchase from Jackson Immunoresearch. Rα Salmonella Typhimurium O antigen was purchased from Gibco Laboratories. Rα phosphotyrosine was purchased from Pharmingen, [San Diego, CA], and Upstate Biotechnology Institute [Lake Placid, NY] or provided by L. Zeltser/E. Puré.

5. Myeloma proteins: MOPC 104E [μ], MOPC 21 [γ_1], RPC-5 [γ_{2a}], MPC-141 [γ_{2b}], J606 [γ_3] and TEPC 15 [α] were obtained from Litton Bionetics, [Kensington, MD].

6. Cytokines and mitogens: Purified murine recombinant interleukin-1 [rIL-1] was provided by P. Lomedico [Hoffman-La Roche, Inc., Nutley, NJ]. Purified human rIL-2 was generously provided by Cetus Corp. [Emeryville, CA]. Purified murine rIL-4 was generously provided by either Dr. P.C. Isakson [University of Virginia, Charlottesville, VA] or Dr. E.S. Vitetta [University of Texas Southwestern Medical Center].

Murine rIL-5 was purchased from Genzyme [Boston, MA]. Purified murine rIL-6 was kindly provided by Dr. J. Van Snick [Ludwig Institute for Cancer Research, Brussels, Belgium]. Purified murine rIL-7 was purchased from Biosource International [Westlake Village, CA]. Purified human rIL-7 was generously provided by Sterling Drug, Inc. [Malvern, PA]. rIL-10 was generously provided by Dr. M. Howard [DNAX, Seattle, WA]. Murine recombinant interferon-γ [rIFNγ] was purchased from Amgen Biologicals [Thousand Oaks, CA]. Granulocyte/macrophage-colony stimulating factor [GM-CSF] was kindly provided by S. Gillis [Immunex, Seattle, WA]. Lipopolysaccharide [LPS] from Salmonella Typhimurium was purchased from Difco Laboratories [Detroit, MI]. Phorbol dibutyrate [PdBu] was purchased from Sigma Chemical Co. [St. Louis, MO].

7. Plasmid vectors and bacterial strains: pCDNAI, pCDNAII, pCRII, and pRC-CMV were purchased from Invitrogen [San Diego, CA]. The bacterial expression vector, pD10, was generously provided by Dr. F. Rauscher [Wistar Institute, Philadelphia, PA]. pCDNAII, pCRII, and pRC-CMV were amplified in the *Escherichia coli* [*E. coli*] strains NM522 or INVαF' [Invitrogen]. pCDNAI was grown in the *E. coli* strain MC1061.p3 [Invitrogen]. pD10 was grown in the *E. coli* strain S9, a generous gift from Dr. C. Buck [Wistar Institute, Philadelphia, PA].

B. Preparation of purified cell populations.

1. B cells: B cells were prepared from erythrocyte-depleted, single cell suspensions of splenic tissue by treatment with a cocktail of antibodies including HO-13,4, C3PO, and HO-2.2 [αThy-1.2, αLyt-1.2, and αLyt-2.2, respectively] followed by complement-mediated lysis using baby rabbit serum as a source of complement [Pel Freez Biologicals, Rogers, AR]. Adherent cells were removed by passage over a Sephadex G-10 column (276). High density B cells were obtained by density fractionation on a discontinuous Percoll gradient (31). High density B cells were those harvested from bands that formed above the 1.09 and 1.085 g/ml layers.

2. T cells: T cells were prepared from erythrocyte-depleted suspensions of mesenteric lymph nodes and spleen by passage through nylon wool columns. CD4-enriched T cells were prepared by treating the nylon wool nonadherent leukocytes with HO-2.2 and B21-2 [αLyt-2.2 and αI-$A^{b,d}$ respectively] followed by complement-mediated lysis.

3. Dendritic cells DC: Single cell suspensions were obtained by collagenase [Worthington Biochemical Corp., Freehold, NJ] digestion of splenic tissue . Low density splenic cells, obtained by flotation on dense bovine serum albumin [BSA] gradients [density = 1.08], were adhered to

tissue culture dishes [series 3003; Falcon Labware, Oxnard, CA]. After 1-1.5 hours, the nonadherent cells were removed by vigorous pipetting with pre-warmed complete RPMI/10. The adherent cells were cultured overnight, whereupon the DC became nonadherent. The overnight released cells were collected and readhered to tissue culture dishes for 1 hour to remove contaminating macrophages.

C. Generating lymphoblasts *in vitro*.

1. Allo-stimulated T lymphoblasts [allo-T blasts]: CD_2F_1 [H-2^d] T cells at 5 x 10^6/ml were co-cultured with DC from B6.H-2^k at a concentration of 5-10 x 10^4/ml in complete media in a humidified 7% CO_2 environment at 37°C. DC were exposed to 1,500 rad from a ^{137}Cs source before addition to the cultures. After 48 hours, DC-T cell clusters were harvested from the high density fractions of a continuous Percoll gradient. The clusters were recultured at 4-5 x 10^5/ml. After an additional 48 hours, the allo-T blasts released from the clusters were harvested from the low density [top] layer of a continuous Percoll gradient. Residual DC were removed by antibody mediated complement cytolysis [33D1 and M5/114.15.2, anti-I-E^k] (277).

2. Allo-stimulated B lymphoblasts [allo-B blasts]: B cells at 10^6/ml [B6.H-2^k unless otherwise indicated] were co-

cultured with irradiated [1500 rad], I-A^k reactive allo-T blasts at a concentration of 2 x 10^5/ml in a total volume of 10 ml. At the times indicated, the cells were harvested, the T cells depleted as described above, and the dead cells and debris were removed by flotation on a dense BSA gradient.

3. Anti-Ig B blasts [αIg blasts]: B cells at 2 x 10^6/ml were cultured with 5 μg/ml GαM Ig-coupled Sepharose [Pharmacia Fine Chemicals, Piscataway, NJ] in complete media. At the times indicated, the αIg blasts were isolated free of the Sepharose beads by centrifugation through Ficoll-Paque [Pharmacia Fine Chemicals] (31).

4. Anti-Ig/EL-4 B blasts [αIg/EL-4 blasts]: B cells at 2 x 10^6/ml were cultured with 5 μg/ml GαM Ig-coupled Sepharose in complete media. During the final 24 hours, phorbol dibutyrate [PdBu]-induced EL-4-conditioned media [partially purified, concentrated, and depleted of PdBu by sequential precipitation in 50% and then 85% saturating ammonium sulfate, followed by extensive dialysis [EL-4 sn]] was added at a 1% final concentration [v/v]. This lymphokine enriched preparation contains IL-2, IL-4 and IL-5. At the times indicated, the αIg/EL-4 blasts were isolated free of the Sepharose beads by centrifugation through Ficoll-Paque.

D. *In vitro* assays of B cell and B lymphoblast phenotype and

function.

1. Cell cycle analysis: Cells were resuspended in Dulbecco's modified phosphate buffered saline containing 1% BSA and 0.02% NaN_3 [PD/BSA/N_3] at 5 x 10^6/ml. Triton X-100 [Sigma Chemical Corp.] was added to 1% followed by the addition of propidium iodide to 0.1 mg/ml [Molecular Probes, Eugene, OR]. The cells were analyzed on a FacSCAN^R [Becton Dickinson & Co., Mountain View, CA] using the program entitled "Sum of Broadened Rectangles" (278).

2. Fluorescent flow cytometric analysis: The antibodies used for staining are described in Table 1. 2-10 x 10^5 cells/sample were incubated with either directly fluoresceinated reagent or hybridoma supernatant diluted in PD/BSA/N_3 at 4°C for 40 minutes. Samples were washed three times in PD/BSA/N_3. Samples initially incubated with hybridoma supernatant were resuspended in the appropriate fluoresceinated secondary reagent as indicated. The samples were washed again three times in PD/BSA/N_3. Cells were fixed in 3.7% formaldehyde in PD and analyzed on a FACScan^R.

3. Proliferative responses: Cells were cultured with the indicated additions in flat bottom 96-well microtiter plates for 24-48 hours in a 200 μl final volume. Cells were pulsed with [^{3}H]thymidine [^{3}H]TdR; New England Nuclear, Boston, MA]

with a specific activity of 20 Ci/mmol for 12 hours, harvested onto glass fiber filters, and counted on a scintillation counter [1205 Betaplate; Pharmacia-LKB]. Data are represented as the mean counts per minute [cpm] of triplicate cultures. Viable cell recovery was determined by counting in the presence of 0.04% Trypan blue.

4. Ig isotype specific ELISA [enzyme-linked immunosorbent assay]: Immulon 1 plates [Dynatech Laboratories, Inc., Chantilly, VA] were coated with 0.5 μg/well of isotype specific antibodies in 0.1 M borate buffered saline [BBS], pH 8.3, overnight at 4°C. The plates were washed and blocked with BBS-1% BSA [BBS/BSA]. Culture supernatant diluted in BBS/BSA was added and incubated for 4 hours at room temperature or overnight at 4°C. Plates were washed and 20 ng of the appropriate AP-conjugated isotype-specific antibody in BBS/BSA was added to each well for 4 hours at room temperature. Plates were washed and 100 μg of p-nitrophenyl phosphate [PNPP; Xymed Laboratories, San Francisco, CA] in 0.1 M 2-amino-2-methyl-1,3-propanediol, pH 10.3, was added per well. The plates were read at 410 nM on a microplate reader [MR700; Dynatech Laboratories, Inc.]. Standard curves were generated using myeloma proteins of the appropriate isotypes. Data are represented as the mean Ig concentration of quadruplicate cultures.

5. IL-2 binding assays: The number of binding sites and binding affinities was determined by IL-2 binding assays as described previously (279). Briefly, IL-2 was radioiodinated using lactose peroxidase/glucose oxidase [Enzymobeads; Bio-Rad Laboratories, Richmond, VA] according to the manufacturer's instructions. The radioiodinated IL-2 was mixed with 10^6 cells in 0.2 ml of complete media at IL-2 concentrations ranging from 1 pM to 100 nM. The cell suspension and IL-2 was overlayed onto a 0.2 ml mixture of 80% silicone oil [Dexter Hysol 550 fluid; Dexter Corp., Orlean, NY] and 20% paraffin oil [Fisher Scientific, Pittsburgh, PA] and incubated for 20 minutes at 37°C. Subsequently, the cells were pelleted by centrifugation at 10,000g. The tips of the tubes containing the cell pellet were cut, and the cell-bound and free radioactivity were determined in a scintillation counter. The calculated values for the number of binding sites/cell and the binding affinity were derived by Scatchard analysis of equilibrium binding data after subtraction of nonspecific binding determined in the presence of a 100 molar excess of unlabeled ligand.

6. Induction of tyrosine phosphorylation and tyrosine kinase activity: B cells were stimulated at 5 x 10^7/ml on ice for 1 hour in complete media with additions as indicated. For crosslinking of Fab' fragments of anti-Ig and mAb, B cells were incubated with a saturating concentration of primary

antibody for 30 minutes on ice, spun down to remove unbound antibody, and resuspended in 50 μg/ml secondary antibody for 30 minutes on ice.

a. Immunoblotting of tyrosine phosphorylated proteins: B cells stimulated as described above were lysed at a cell density of 1 x 10^8/ml in 150 mM NaCl, 20 mM Tris, pH 7.5, 1% NP-40, protease inhibitors [5 mM EDTA, 1 mM PMSF, 1 U/ml aprotonin, 50 μg/ml leupeptin; Sigma Chemical Corp.], and phosphatase inhibitors [200 mM sodium vanadate; Sigma Chemical Corp., 50 mM NaH_2PO_4, 50 mM KF, 10 mM sodium pyrophosphate] for 10 minutes on ice. The post nuclear extract was isolated by centrifugation and immediately diluted in an equal volume of 2x Laemmli buffer (280). Lysate representing 1 x 10^6 cell equivalents per lane were resolved by electrophoresis through 10% sodium dodecyl sulfate [SDS]-polyacrylamide gels. Proteins were transferred onto PVDF membranes [New England Nuclear] in 25 mM Tris base, pH 9, 200 mM glyine, 20% methanol [v/v] for 45 minutes at 400 mAmp. The membranes were quenched for 30 minutes in 3% BSA. Blots were incubated with 0.3 μg/ml PY69 [Mα phosphotyrosine] for one hour at room temperature in PD, 0.02% Tween, 0.1% BSA, washed and subsequently, incubated with 1 μg/ml HRP-conjugated, affinity purified, GαM IgG for 1 hour at room temperature. After washing, blots were developed by exposure to either 1 mg/ml 4-chloro-1-napthol or chemiluminescence reagent [New England Nuclear] according to the manufacturer's instructions.

b. *In vitro* assay for phosphotransferase activity of tyrosine phosphorylated proteins: Cell lysates were prepared as described above. The lysates were normalized for protein content and 200 μg of protein was used per reaction. The lysates were precleared with normal rabbit serum [50 μg/ml] followed by protein A Sepharose [10% volume] [Pharmacia Fine Chemical]. Precleared lysates were immune precipitated with an affinity purified, polyclonal Rα phosphotyrosine antibody, Rα ova or pre-immune serum preadsorbed to protein A Sepharose at an optimal dose of 100 μg/ml packed sepharose beads. Immune complexes were washed in 150 mM NaCl, 50 mM Tris, pH 6.8, 0.1% NaN_3, 0.5% NP-40, 10 mM EDTA. The kinase reaction was performed at 30°C for 10 minutes in 50 mM Hepes, pH 7.4, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 5% glycerol, 10 μCi ^{32}P-γATP [Amersham, Arlington Heights, IL]. Tyrosine phosphorylated proteins were eluted with phosphotyrosine conjugated keyhole limpet hemocyanin [KLH]. Eluates were diluted in Laemmli buffer as described above and resolved on 5-12% SDS-polyacrylamide gradient gels. Dried gels were exposed to Fuji RX film.

E. Phosphoamino acid analysis

Bands observed in the *in vitro* phosphotransferase assay were excised from dried gels. The gel slices were incubated in 30% methanol and 0.75% acetic acid for 2-3 hours to

facilitate removal of the paper. The gel slices were washed in 50% methanol for 4-6 hours to remove residual SDS and then dried. The bands were incubated in 0.1 mg/ml thermolysin [Boehringer Mannheim] in 50 mM NH_4HCO_3 at 37°C overnight. The protein was dried, resuspended in 6N HCl, and incubated at 100°C for 2 hours. The hydrolyzed phosphoamino acids were resuspended in 8% acetic acid, 2% formic acid, pH 1.9. The phosphoamino acids were resolved by electrophoresis on cellulose thin layer chromatography [TLC] plates [Eastman Kodak, Rochester, NY] at 400V in pH 1.9 for 1 hour followed by pH 3.5 for 45 minutes. 2 μg of unlabeled standards [L-O-phosphoserine, DL-O-phosphothreonine, and L-O-phosphotyrosine] were resolved on the same TLC plates and visualized by staining with 1% ninhydrin in acetone. The TLC plate was then exposed to Fuji RX film.

F. Peptide mapping of PTK72.

Peptide mapping of the 72 kD protein was performed as described by Cleveland, et al (281). Briefly, proteins were eluted from the gel as described above and dissolved in sample buffer which contained 0.125 M Tris, pH 6.8, 0.5% SDS, 10% glycerol and 0.0001% bromphenol blue. The samples were heated to 100°C for 2 minutes. Digestion was carried out at 37°C for 30 minutes using 100 μg/ml *Staphylococcal aureus* V8 protease [Sigma Chemical Corp.]. Following addition of β-ME and SDS to

a final concentration of 10% and 2% respectively, proteolysis was stopped by boiling the samples for 2 minutes. The samples were resolved on a 15% SDS/polyacrylamide gel and exposed to Fuji RX film.

G. Preparation of RNA.

1. Total RNA: Cells were washed twice in 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and subsequently lysed in 4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7 using a tissue homogenizer [Brinkman Instruments, Inc., Westbury, NY]. Cellular debris was removed by centrifugation and the supernatant was layered on a CsCl cushion [5.7 M CsCl, 25 mM sodium acetate, pH 5]. The RNA was then pelleted at 32,000 rpm for 16 hours in a SW41 rotor [Beckman Instruments, Inc., Palo Alto, CA]. The RNA was resuspended in water and evaluated for integrity on a 1% agarose gel containing 0.1 mg/ml ethidium bromide. Purity was assessed by the optical density [OD] ratio between 260 nm and 280 nm [OD_{260}/OD_{280}]. RNA from tissue and organs was prepared essentially the same way, with the exception of freezing the tissue in a dry ice/ethanol bath prior to lysis in the guanidine isothiocyanate/sodium citrate solution.

2. mRNA: Poly A+ RNA was enriched for by passage of total RNA over an oligo dT cellulose column [Boehringer

Mannheim]. Total RNA was denatured at 65°C and bound to oligo dT in 0.5 M NaCl/TE, pH 7.6, [10 mM Tris, 1 mM EDTA]. The column was washed with 0.2 M NaCl/TE and the mRNA was eluted with double distilled [dd] H_2O. The RNA was evaluated for integrity and purity as described above.

H. Preparation of cDNA libraries.

An oligo-dT primed library was constructed in the plasmid vector pCDNAII from the Epstein-Barr virus negative human B lymphoma cell line, RAMOS. Briefly, 0.5 μg oligo-$[dT]_{12-18}$ was added to 5 μg poly A+ RNA, isolated as described above, and the mixture was denatured at 70°C for 10 minutes. First strand cDNA was synthesized using 200 units SuperScript reverse transciptase [Gibco/BRL, Gaithersburg, MD] in 20 mM Tris, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml BSA, 10 mM DTT and 1 mM dNTPs at 42°C for 1 hour. The second strand cDNA was synthesized using 20 units *E. Coli* DNA Ligase, 40 units *E. Coli* DNA Polymerase I, and 2 units RNAse H [Boehringer Mannheim] in 20 mM Tris, pH 6.9, 100 mM KCl, 5 mM $MgCl_2$, 150 μM β-NAD, 10 mM $[NH_4]_2SO_4$, 400 μM dNTPs, and 5 mM DTT at 16°C for 2 hours. 50 units of T4 DNA polymerase [Boehringer Mannheim] was added and the reaction carried out for an additional 5 minutes at 16°C. The DNA was purified by phenol/chloroform extraction and ethanol precipitation and Bst XI linkers were added using T4 DNA ligase [Boehringer

Mannheim]. cDNA greater than 1 kb was size selected on a 1% agarose gel and ligated into pCDNAII at the Bst XI sites.

The λTon and the paTon were the generous gift of Dr. Lloyd Klickstein [Harvard Medical School, Boston, MA]. The λTon is an oligo-dT primed human tonsillar library in the phage vector λgt11. The λTon library was amplified in the *E. Coli* strain Y1088 [Invitrogen]. The paTon is a human tonsillar library in the eukaryotic expression vector Ap^rM8, a derivative of CDM8 (282).

I. Bacterial transfection and plasmid production.

1. Electroporation: Electrocompetent bacteria were prepared by harvesting bacteria during mid log phase growth [OD_{600} = 0.6-1.0] and resuspending gently in an equal volume of ddH_2O. The bacteria were pelleted by centrifugation and resuspended in ddH_2O in 50% of the original volume. The bacteria were pelleted and resuspended in 10% glycerol in water [v/v] two additional times in 2% and 0.2% of the original volume. The bacteria were subsequently frozen in a dry ice/ethanol bath and stored at -80°C.

Electrocompetent bacteria were transfected with plasmid DNA in a 2 mm electroporation cuvette using a Bio-Rad Gene Pulser^R apparatus [Rockville Centre, NY] set at 25 μF, 2.5 kV, and 200 ohms. 1 ml of SOC [2% bacto-tryptone, 0.5% yeast extract; Difco Laboratories, Detroit, MI, 10 mM NaCl, 2.5 mM

KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.4] was added immediately and the bacteria were incubated at 37°C for 1 hour. Subsequently, bacteria were plated on L-broth plates [1% bacto-tryptone, 0.5% yeast extract, 1.5% bacto-agar; Difco, 1% NaCl] containing the appropriate antibiotic.

2. Heat shock: Competent bacteria were the generous gift of Dr. C. Buck [Wistar Institute, Philadelphia, PA]. The competent bacteria were incubated with plasmid DNA at 4°C for 30 minutes. The bacteria were subsequently placed at 42°C for 30 seconds, and immediately transferred back to 4°C for two minutes. 1 ml of SOC was added and the bacteria were incubated and plated as described above.

3. Lipofectin. Cos-7 cells were seeded at 4 x 10^5/ml in complete Dulbecco's modified eagle media supplemented with 10% HI-FCS. After 24 hours the cells were transfected with 2 μg of plasmid DNA using lipofectin^R reagent [Gibco/BRL] according to the manufacturer's instructions.

4. Preparation of plasmid DNA: Analytical scale purifications of plasmid DNA [mini-preps] utilized alkaline lysis of the bacteria, phenol/chloroform extraction of nucleic acids, followed by RNAse A [Boehringer Mannheim] digestion as described in Maniatis et al (283). A second mini-prep method utilized digestion of the bacterial cell wall with 1 mg/ml

lysozyme [Sigma Chemical Corp.] in STET buffer [8% sucrose, 50 mM Tris, pH 8, 50 mM EDTA, 0.1% Triton X-100]. The bacteria were lysed by boiling for 45 seconds, and bacterial debris was removed by centrifugation. RNA was removed by digestion with RNAse A and plasmid DNA was precipitated with 0.5% cetyl-trimethyl ammonium bromide [Sigma Chemical Corp.]

Preparative scale purification of plasmid DNA utilized alkaline lysis and CsCl gradient centrifugation [60,000 rpm for 16 hours in a Vti 65.2 rotor; Beckman Instruments, Inc.] as described in Maniatis et al (283). A second method utilized alkaline lysis, followed by passage over Qiagen columns [Chatsworth, CA] according to the manufacturer's instructions.

J. Screening libraries.

1. Plasmid libraries: Plasmid containing bacteria were grown overnight on nitrocellulose discs [Schleicher and Schuell, Keene, NH] on LB plates supplemented with the appropriate antibiotic. Replica filters were made of the library on GeneScreen filters [New England Nuclear] and both filters were regrown on LB/antibiotic plates. The bacteria on the replica filter were lysed in 0.5 M NaOH/1.5 M NaCl and subsequently neutralized with 0.5 M Tris pH 7.5/1.5 M NaCl. The nucleic acids were UV-crosslinked to the filter [1.2 x 10^5 Joules] using a Stratalinker^R [Stratagene, La Jolla, CA] and

washed with 5 x SSC/1% SDS/1 mM EDTA at 50°C for 30 minutes to remove bacterial debris. The filters were hybridized in 6 x SSPE, 10% dextran sulphate, 1% SDS, 100 μg/ml denatured salmon sperm DNA and appropriate radiolabeled probe [see below] for 16 hours at 65°C in a Hybaid hybridization oven [National Labnet Co., Woodbridge, NJ]. The filters were subsequently washed in SSC/0.1% SDS [temperature and salt concentration were adjusted to vary the stringency] and exposed to Fuji RX film. Positive colonies were picked and rescreened until single colonies were isolated.

2. Phage libraries: The appropriate bacterial strain was grown to mid log phase growth [OD_{600} = 0.6 - 0.8]. Phage was added to 200 μl of bacteria and incubated for 20 minutes at 37°C. 4 ml of NZY top agar [0.5% NaCl, 10 mM $MgSO_4$, 0.2% maltose, 0.5% yeast extract; Difco, 1% NZ Amine; Sigma Chemical Corp., 0.8% agar] at 42°C was added and the mixture was allowed to gel on LB plates supplemented with 10 mM $MgSO_4$, 0.2% maltose. The plates were incubated at 37°C until subconfluent plaque formation. The phage were transferred to nitrocellulose filters [Schleicher and Schuell] and lysed in 0.5M NaOH, 1.5 M NaCl. The filters were UV-crosslinked and hybridized as described above. Positive plaques were cut out of the top agar and eluted into SM buffer [0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris, pH 7.5, 0.01% gelatin]. Phage were rescreened until single plaques were isolated.

a. Preparation of phage DNA: 1.5 ml of an overnight culture of bacteria were pelleted and resuspended in 50 mM Tris, pH 7.5, 10 mM $MgSO_4$. 10^5-10^8 phage [1/4 of a single plaque] was added to the bacteria and incubated at 37°C for 20 minutes. The bacteria/phage mixture was added to 40 ml of LB/maltose/Mg and grown at 37°C until the bacteria were completely lysed. Bacterial debris was removed by centrifugation and bacterial nucleic acid was digested with RNAse A [10 μg/ml] and DNAse I [5 μg/ml] [Boehringer Mannheim]. Bacteriophage were pelleted, resuspended in TE, and the DNA extracted with phenol. The cDNA insert was excised by digestion with Eco RI, purified using polyacrylamide gel electrophoresis [PAGE], and subcloned into pCDNAII, previously digested with Eco RI, dephosphorylated and gel purified using PAGE.

3. Random primer labeling of DNA: 100 ng of DNA and 75 ng of six base pair [bp] randomly generated oligomer [Boehringer Mannheim] were mixed and denatured by boiling. 5 units Klenow fragment [Boehringer Mannheim] was added in 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 200 μM dATP, dGTP, and dTTP, and 10 μCi [α-^{32}P]dCTP [specific activity 3000Ci/mmole; New England Nuclear] and incubated at 37°C for 1 hour. The reaction was terminated by the addition of EDTA to 5 mM, and the DNA was precipitated to remove free nucleotides.

K. Isolation and characterization of human *syk* cDNA.

1. Generation and cloning of a partial porcine *syk* cDNA:

First strand cDNA was generated from 1 μg mRNA from porcine spleen as described above. DNA corresponding to nucleotides 934-1884 of porcine *syk* was amplified using the polymerase chain reaction [PCR] as follows. Primers corresponding to nucleotides 934-955 of the sense strand and 1861-1884 of the antisense strand were generated in the Rockefeller University DNA synthesis facility.

porcine *syk* 934-955+: 5′-TCGTACAACCCTTACGAGTCA-3′

porcine *syk* 1864-1885-: 5′-ATTAACCACATCGTAGTAGTA-3′

Taq polymerase [Promega, Madison, WI] was used according to the manufacturers instructions under the following conditions: 50 mM KCl, 10 mM Tris, pH 9, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 100 pM primers, 5 μl of the reverse transcriptase reaction, and 2.5 units Taq polymerase. The cDNA was amplified for 30 rounds as follows: denaturation 45 seconds, 95°C; annealing 1 minute, 50°C; extension 90 seconds, 75°C. The 951 bp fragment was purified using PAGE, electroelution and ethanol precipitation, and ligated into the vector pCRII at the "TA" cloning site.

2. Isolation of partial cDNA clones of human *syk*:

Probe, generated from the 951 bp fragment of porcine *syk*, was utilized to screen the RAMOS library described above.

Numerous clones were isolated the longest corresponding to nucleotides 1188-2508. The Mbo II-Xmn I fragment [nucleotides 1213-1505] of this clone was used to screen the λTon library described above. Numerous clones were again isolated, the longest corresponding to nucleotides 634-2508 of human *syk*.

3. Isolation of the 5′ end of human *syk*: Utilizing 5′ RACE [rapid amplification of cDNA ends; Gibco/BRL], a PCR product corresponding to nucleotides -99-915 was isolated as described by the manufacturer's instructions. Primers corresponding to nucleotides 864-884, 895-915, and 1132-1152 of the anti-sense strand of human syk were generated in the DNA synthesis facility at the Wistar Institute [Philadelphia, PA].

human *syk* 864-884-: 5′-TTTGGGAAGGAGTATGATTTG-3′

human *syk* 895-915-: 5′-TTGGGCAGGGGAGGACTTTCT-3′

human *syk* 1132-1152-: 5′-GTAGCCCTTTTTCACAGTTCC-3′

First strand cDNA was generated using 1 μg of mRNA from RAMOS cells as described above, except that primer 1132-1152- was used to prime the reaction. A poly dC tail was added to the first strand cDNA using terminal deoxy transferase [Tdt] under the following conditions: 10 mM Tris, pH 8.4, 25 mM KCl, 1.25 mM $MgCl_2$, 10 μg/ml BSA, 400 μM dCTP, and 0.5 units/μl Tdt. DNA corresponding to the 5′ end of human *syk* was amplified by 2 successive rounds of PCR using Taq polymerase as described above. The first round of PCR utilized the nested *syk*

specific primer 895-915- and the anchor primer [Gibco/BRL]:

5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC GGGIIGGGIIGGGIIG-3′
UDG cloning site Sal I anchor region
Mlu I Spe I

The second round of PCR utilized the further nested primer 864-884- and the anchor primer. The second round of PCR yielded a single band of DNA which was purified via PAGE, electroelution, and ethanol precipitation and was subcloned into pCRII at the TA cloning site. The Eco RI-Apa I fragment [nucleotides -99-341] generated from the RACE product was used to screen the paTon library described above. A cDNA clone corresponding to nucleotides -99-1828 was isolated.

4. Generation of a full length cDNA clone of human *syk*: The fragment of the 5′ cDNA clone corresponding to nucleotides -99-1464 of *syk* was isolated by digestion with Xho I and Bgl II, followed by PAGE, electroelution and ethanol precipitation. This fragment was ligated into the clone corresponding to the 3′ end of *syk* [nucleotides 1464-2508] in the vector pCDNAII, previously cut with the same enzymes, dephosphorylated and purified using PAGE.

5. Sequencing of human *syk*: The cDNA insert encoding human *syk* was sequenced using Sequenase™ [United States Biochemical, Cleveland, OH] as follows. One - 3 μg of mini-prep or plasmid prep DNA was added to 10 ng of sequencing

primer in 0.1 M NaOH at 68°C for 10-20 minutes to denature the DNA. Sequencing primers were either pCDNAII specific [forward and reverse primers were designed to anneal just outside the multiple cloning site of the vector on the sense [+] and anti-sense [-] strand, respectively] or human *syk* specific and were generated in either the Rockefeller Protein Sequencing Facility or the Wistar Institute DNA Synthesis Facility.

pCDNAII+:	5′-GGCCAGTGAATTGTAAT-3′
pCDNAII-:	5′-TAGAATACTCAAGCTAT-3′
15-32+:	5′-CAACCACCTGCCCTTCTT-3′
240-257+:	5′-CGCCGACCTCTGCCACTA-3′
280-297-:	5′-CTTGAGGAGGCAGACCAG-3′
895-915-:	5′-TTGGGCAGGGGAGGACTTTCT-3′
1043-1062+:	5′-CGGACCCCGAGGAGATCAGG-3′
1192-1211-:	5′-TTGGCCTCGTTTTTCAGTAT-3′
1344-1361+:	5′-TGGTCCCCTCAATAAGTA-3′
1697-1624-:	5′-AGTAGTTGATGCATTCCG-3′
2254-2271-:	5′-GTGCTCTAGGCCACTCTG-3′

In addition, Apa I, Pst I, and Nsi I fragments of the *syk* clone were isolated and subcloned into pCDNAII, previously cut with the same enzyme, dephosphorylated and purified using PAGE. The subclones were sequenced with the pCDNAII-specific primers.

The sequencing reaction was carried out according to the manufacturers's instructions [United States Biochemical] with the exception of the reaction buffer which was 0.28 M TES [Sigma Chemical Corp.], 0.12 M HCl, 0.05 M DTT, 0.08 M $MgCl_2$ and 0.2 M NaCl. The reactions were denatured by boiling and

analyzed using 6% urea/PAGE. The gels were exposed to Fuji RX film. Nucleotide and amino acid sequences were analyzed using the computer program, PC/Gene^{R} [IntelliGenitics Inc., Mountain View, CA] and GCG Sequence Analysis Software Package, [Genetics Computer Group, Inc., Madison, WI].

L. Generation and purification of histidine-syk fusion proteins:

A Histidine-syk fusion protein were generated through the use of *syk* specific primers and PCR. The primers were engineered with restriction enzyme sites 5′ of the *syk* specific sequence to facilitate subcloning into the bacterial expression vector, pD10. In addition, a termination codon was placed 3′ of the *syk*-specific sequence on the antisense primer.

sense: 5′-CTAGA GGATCC CATGGAAAAATCTCT-3′
Bam HI nt 493 - 762

antisense: 5′-CTAGA AAGCTT TTA ACATGGGACAGTAAG-3′
Hind III nt 748 - 762
stop

The thermophilic Pfu polymerase^{R} [Stratagene, La Jolla, CA] was used under the following conditions: 20 mM Tris, pH 8.8, 10 mM KCl, 6 mM $[NH_4]_2SO_4$, 2 mM $MgCl_2$, 1% Triton X-100, 1 mg/ml BSA, 0.2 mM dNTPs, 100 pM primers, 10 ng template [human *syk* in the vector pCDNAII] and 2.5 units Pfu polymerase. The reaction was amplified for 25 cycles as follows: denaturation

45 seconds, 95°C; annealing 1 minute, 50°C; extension 2 minutes, 74°C. The amplification resulted in a 295 bp fragment corresponding to nucleotides 493-762 of *syk*. The fragment was purified by phenol/chloroform extraction and ethanol precipitation and subjected to cleavage with Bam HI and Hind III. The resulting fragment was purified using PAGE, electroelution, and ethanol precipitation and ligated into pD10, previously cut with the Bam HI and Hind III, dephosphorylated and purified using PAGE. The ligation mix was transfected into S9 bacteria, and selected on kanamycin and ampicillin LB plates. The entire length of the Bam HI/Hind III fragment was sequenced to check the fidelity of the PCR reaction.

Expression of the fusion protein in the S9 bacteria was induced for 3 hours with 1 mM IPTG [Sigma Chemical Corp.] during mid log phase growth. The bacteria were pelleted and lysed in 6 M guanidine-HCl, 50 mM sodium phosphate, pH 8 for 2 hours and the insoluble bacterial debris was removed by centrifugation.

Purification of the fusion protein was facilitated by the affinity of the histidine tail for Ni. The lysate was adsorbed to a Ni-NTA-agarose column [Qiagen], washed into 8 M urea, 100 mM sodium phosphate, and 10 mM Tris, pH 8 buffer, and eluted at pH 5.9-4.5. The fusion protein was dialyzed into phosphate buffered saline [PBS; 150 mM NaCl, 40 mM sodium phosphate, pH 7.3] and purity was assessed using SDS/PAGE.

M. Generation of polyclonal anti-syk antibodies.

New Zealand white rabbits were immunized subcutaneously with 200 μg of the his-syk fusion protein emulsified in complete Freund's adjuvant [CFA; Difco]. The rabbits were boosted at four week intervals with test bleeds obtained one week after each boost. Antibody titers were evaluated by immunoblotting against purified fusion protein using HRP-conjugated DαR Ig as secondary. After four months, the rabbits were exsanguinated and the blood clotted at 37°C for 1 hour. Cellular components were removed by centrigation and the serum was concentrated by precipitation with 50% saturating ammonium sulfate. Serum IgG was affinity purified by absorption to protein G Sepharose [Pharmacia Fine Chemicals]. The IgG fraction was eluted with 0.1 M glycine, pH 2.8, neutralized with 1/10 volume 1 M Tris, pH 9, and dialyzed into PD/N_3.

The his-syk fusion protein was coupled to AminoLink™ agarose [Pierce, Rockford, IL] according to the manufacturer's instructions. Briefly, sodium cyanoborohydride catalyzes Schiff base formation between free amine groups of protein and aldehyde groups on the agarose. Protein G purified rabbit IgG antibody was absorbed to the column in PBS and anti-syk antibody was specifically eluted with 3.5 M $MgCl_2$, 100 mM Tris, pH 8. Reactivity was checked in an ELISA as described above, with the following exceptions: purifed fusion protein

was bound to the Immulon plates [Dynatech] at 5 μg/ml and AP-conjugated DαR IgG was used as secondary.

N. Modeling of syk SH2 domains.

The structure of the N-terminal and C-terminal SH2 domains of human syk were modeled using the structure of the SH2 domain of src (284) as a template. The amino acid sequence of the syk SH2 domains were aligned with the src SH2 domain using the program Pile-Up in the GCG Sequence Analysis Software Package [Genetics Computer Group, Inc.]. Using this alignment, "dummy" coordinates were entered for each amino acid of the syk SH2 domains. These coorinates were subjected to energy minimization and molecular dynamics using XPLOR, (285). Subsequently, the coordinates were tested for compatibility with existing structures using the program Profile (286,287). Finally the modeled structures were analyzed using the software package Grasp (288).

O. Generation of human *syk* mutants.

1. Truncation mutant Δ623: The full length clone of human *syk* was subcloned into the eukaryotic expression vector pRC-CMV at the Bst XI sites. The insert was recovered from pCDNAII using Xho I and Eco RI [Boehringer Mannheim]. The resulting fragment was blunt ended by digestion with S1

nuclease [Boehringer Mannheim]. Subsequently, Bst XI linkers [Invitrogen] were attached using T4 DNA Ligase [Boehringer Mannheim] and the resulting fragment was ligated into Bst XI digested pRC-CMV which was dephosphorlated and purified using PAGE.

The truncation mutant was generated with PCR through the use of an antisense primer which contained a termination codon following the nucleotides coding for N^{623} and a sense primer specific for a region of the vector pRC-CMV 5′ of the Hind III site in the poly-linker.

Δ625 primer: 5′-TACGT TCTAGA TTA ATTGCGCAGCCGCAG-3′
Xba I stop 1855 - 1869

pRC-CMV+: 5′-TAGGAAGGCAACAGACAG-3′

The primers were used to amplify a fragment of DNA corresponding to nucleotides -99-1869 of *syk* using Vent^R polymerase under the following condition: 10 mM KCl, 20 mM Tris, pH8.8, 10 mM $[NH_4]_2SO_4$, 4 mM $MgSO_4$, 0.1% Triton X-100, 0.2 mM dNTPs, 100 pM primers, 10 ng syk/pRC-CMV and 2.5 units Vent. The reaction was amplified for 25 cycles as follows: denaturation 45 seconds, 95°C; annealing 50°C, one minute; extension 2 minutes, 74°C. The 2065 bp fragment was cut with Hind III and Xba I, purified on an 0.8% agarose gel using GeneClean [Bio 101, La Jolla, CA] according to the manufacturer's instructions and ligated into Hind III and Xba I digested pRC-CMV. The entire fragment was sequenced to check the fidelity of the PCR reaction.

2. N-myristoylated syk: An N-Myristoylation consensus sequence [G-small hydrophobic-X-X-S-not P] (289) was engineered onto the 5′ end of the full length *syk* clone as described for making the truncation mutant. The sense primer was designed with nucleotides coding for a N-myristoylation consensus sequence preceded by nucleotides coding for a Hind III site, and followed by nucleotides corresponding to the *syk* clone. The antisense primer was specific for a region of the vector pRC-CMV flanking the 3′ end of the multiple cloning site.

N-Myr: 5′-ACGTA AAGCTT ATGGGTAGT GCTGACAGCGCC-3′
Hind III M G S A D S A
nt 4 - 15

pRC-CMV-: 5′-AGGGGCAAACAACAGATG-3′

The 2588 bp fragment generated was digested with Hind III, purified on an 0.8% agarose gel, and ligated into Hind III digested *syk*/pRC-CMV. The fragment was sequenced to check the fidelity of the PCR reaction.

3. Point mutants, K^{397}->R and Y^{520}/Y^{521}->FF: The DNA fragment from the Hind III site in the poly-linker of pRC-CMV to the Hind III site [nucleotide 2396] in *syk* was isolated and religated into pCDNAI linearized with Hind-III and dephosphorylated with alkaline phosphatase [Boehringer Mannheim]. This partial fragment of *syk* cDNA was used for generating mutations to minimize the length of cDNA replicated in the PCR reactions. Two primers were generated for each

mutation, one homologous to the sense and one homologous to the antisense strand, with the exception of the nucleotides required to generate the amino acid[s] substitution indicated. The primers overlapped each other by >15 bp, and extended 3′ past the mutation by >15 bp. In addition, for each mutation, two "external" primers were designed to match the sequence of *syk* approximately 200-350 bp 5′ [sense] and 3′ [antisense] of the site of the point mutation, respectively.

K^{397}->R

syk 1176-1206: 5′-AAAACCGTGGCTGTGA**A**AATACTGAAAAACGAG-3′
3′-TTTTGGCACCGACACT**T**TTATGACTTTTTGCTC-5′

K^{397}/R+: 5′-GCTGTGA**G**AATACTGAAAAACGAG-3′
K^{397}/R-: 3′-TTTTGGCACCGACACT**C**TTATGAC-5′

external primers

819-836+: 5′-TTCCCATCCTGCGACTTG-3′
1470-1487-: 5′-AACACATTTCTTGCAGCC-3′

Y^{520}/Y^{521}->F/F

syk 1543-1578: 5′-CGTGCTGATGAAAACT**A**CT**A**CAAGGCCCAGACCCAT-3′
3′-GCACGACTACTTTTGA**T**GA**T**GTTCCGGGTCTGGGTA-5′

Y^{520}-Y^{521}/FF+: 5′-GAAAACT**T**CT**T**CAAGGCCCAGACCCAT-3′
Y^{520}-Y^{521}/FF-: 3′-GCACGACTACTTTTGA**A**GA**A**GTTCCGG-5′

external primers

1344-1361+: 5′-TGGTCCCCTCAATAAGTA-3′
1816-1833-: 5′-CCTGTTTTCCACATCGTA-3′

For each mutation, the 5′ external primer was used with the antisense mutation primer, and the 3′ external primer was used with the sense mutation primer, to amplify fragments corresponding to nucleotides 819-1206 and 1185-1487 for K^{397},

and 1344-1578 and 1552-1833 for Y^{520}/Y^{521}. Pfu polymerase was used as described above. This first amplification generated double-stranded DNA fragments extending 5′ and 3′ from the desired site of mutation. These fragments were purified by PAGE, followed by electroelution and ethanol precipitation. 50 ng of each of the fragments was used in the presence of the external primers and amplified using Taq polymerase as described above except with 5 rounds of PCR with an annealing temperature of 20°C, followed by 30 rounds of PCR using an annealing temperataure of 35°C. The resulting fragments [nucleotides 819-1487 for K^{397}, and 1344- 1833 for Y^{520}/Y^{521}] was purifed, and cut with either Bgl II and Tth 111I [K^{397}] or Bgl II and Bsp HI [Y^{520}/Y^{521}]. The resulting fragment was purified using PAGE, electroelution and ethanol precipitation and ligated into *syk*/pCDNAI with the corresponding fragment deleted by digestion with the same enzymes. The entire length of the amplified fragments were sequenced to verify the point mutation[s] as well as the fidelity of the PCR reaction.

CHAPTER I

A T-DEPENDENT MODEL OF B CELL ACTIVATION

The study of antigen induced activation and differentiation of resting B lymphocytes is facilitated by *in vitro* models which enable the process to be separated into discrete steps. In the past, models of B cell activation relied mainly on T cell-independent stimulation by anti-Ig (28-30) or LPS (23,25). Anti-Ig triggers the B cell via its antigen receptor to undergo blast transformation, but, even when administered as a multivalent ligand, it does not induce either extensive B cell growth or antibody secretion. However, relative to resting B cells, anti-Ig activated B lymphoblasts are phenotypically and functionally distinct. The B lymphoblasts express increased levels of cell surface receptors for T cell-derived cytokines that support their growth and differentiation, as well as cell surface molecules that mediate B cell interactions required for their antigen presenting cell function (31,290-292). Moreover, relative to resting B cells, the B lymphoblasts have an altered migratory pattern due to the expression of distinct adhesion molecules (102,103,293-295).

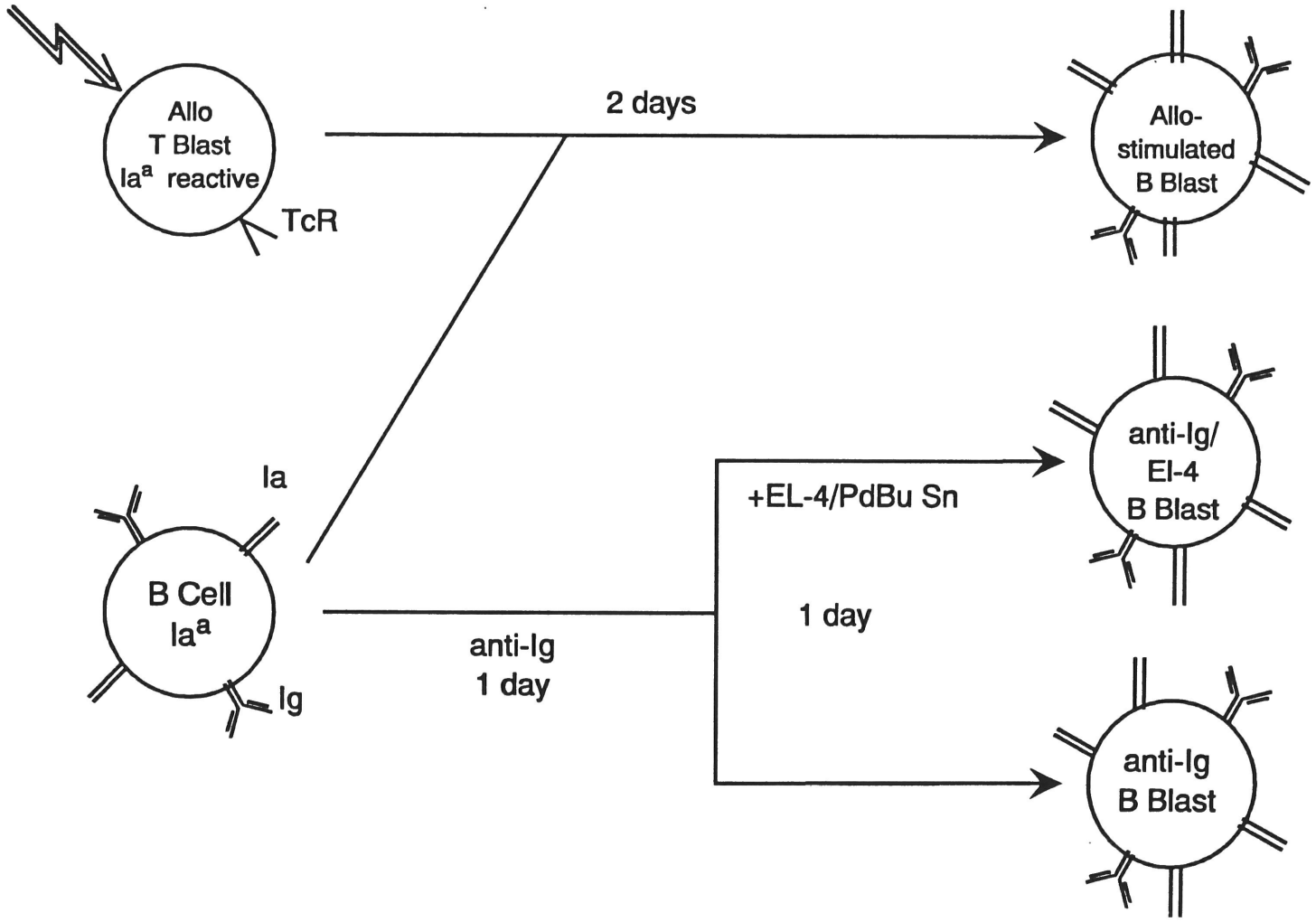
Antibody responses to most protein antigens are dependent on an MHC class II-restricted interaction between T cells and B cells (40-42). Several experimental systems for activating

resting B cells that require a cognate interaction between T cells and B cells have been developed. In one system, antigen specific B cells were activated by histocompatible T cells or T cell clones plus antigen (44-47). However, in these models, it is difficult to generate large numbers of antigen-specific B cells or to discern the Ig- and T cell-mediated signals. Alternatively, systems have been developed whereby class II-reactive T cells activate allogeneic B cells in a polyclonal fashion (48-51). Some B cell growth and Ig secretion was observed in these systems but they were not characterized extensively. We exploited this latter system to try to generate B lymphoblasts that resemble a more differentiated stage of B cell development, such as germinal center or memory B cells. The generation of a population of B cells *in vitro* that resemble these stages of differentiation might allow for the study of processes such as isotype switching and somatic mutation that are specific to this stage of development.

To this end, we set up a system in which CD4+ T cells were activated in the mixed leukocyte reaction [MLR] (277) and used to activate allogeneic B cells. These allo-B blasts were compared throughout to B cells activated with insoluble anti-Ig +/- T cell-derived cytokines [Figure 4].

A. MHC class II-restricted activation of B cells by allo-T blasts.

Figure 4. ***In vitro*** **models of polyclonal B cell activation.** Allo-T blasts were generated by co-culturing irradiated B6.H-2^k [H-2^k] dendritic cells with CD_2F_1 [H-2^d] CD4+ T cells in bulk culture. After 48 hours, the cell clusters were harvested and re-cultured for an additional 48 hours. Allo-T blasts were isolated and co-cultured with B6.H-2^k, [H-2^k], CD_2F_1 [H-2^d] or Balb/c [H-2^b] high density B cells for 2 days at a ratio of T cells:B cells of 1:5. αIg blasts and αIg/EL-4 blasts were generated by culturing high density B cells with 5 μg/ml GαM Ig-coupled Sepharose for 2 days. EL-4 sn was added during the last 24 hours at 1% v/v.


Allo
T Blast
Ia^a reactive
TcR
2 days
Allo-
stimulated
B Blast
Ia
B Cell
Ia^a
Ig
anti-Ig
1 day
+EL-4/PdBu Sn
1 day
anti-Ig/
El-4
B Blast
anti-Ig
B Blast

Allo-B blasts were generated as depicted schematically in Figure 4. Typically, T cells from H-2^d mice were stimulated with H-2^k dendritic cells in the primary culture, and then the H-2^d-reactive T blasts were cultured with allogeneic Ia^k, syngeneic Ia^d, or third party, Ia^b B cells. The allo-T blasts formed large, stable clusters with the allogeneic B cells, but not with the syngeneic or third party B cells and within 1 day of co-culture, the T blasts induced DNA synthesis in the allogeneic B cells [Figure 5]. Less than 10% of this response was seen in B cells from third party mice while no DNA synthesis was induced in B cells from syngeneic mice [Figure 5]. The function of the alloreactive T blasts was Ia^k restricted since the stimulation was blocked by anti-Ia^k antibodies [Figure 5].

By forward light scatter analysis, virtually all the B cells began to enlarge on the first day, and most were large blasts by the second day of T cell-B cell co-culture [Figure 6]. The recovery of viable B lymphoblasts was approximately 80% of the number of input cells after 48 hours. Based on flow cytometric cell cycle analysis, approximately 25% of the B cells were in the S, G_2, and M phases of the cell cycle by 24 hours and this increased to approximately 44% by 48 hours [Table 2]. The activation of B cells as measured by forward light scatter, entry into cell cycle, and DNA synthesis was similar to that of B cells stimulated by anti-Ig (31,32) and anti-Ig plus EL-4 sn [data not shown].

Figure 5. Activation of B lymphocytes by alloreactive-T blasts is MHC restricted. Freshly isolated high density splenic B cells of the indicated MHC haplotypes were cultured at 10^6/ml with irradiated allo-T blasts at 2 x 10^5/ml in a total volume of 10 ml. At the times indicated, a 200 μl aliquot of the co-culture was pulsed for 6 hours with 1 μCi [^{3}H]-TdR, harvested and counted. Data represents the average of quadruplicate cultures.

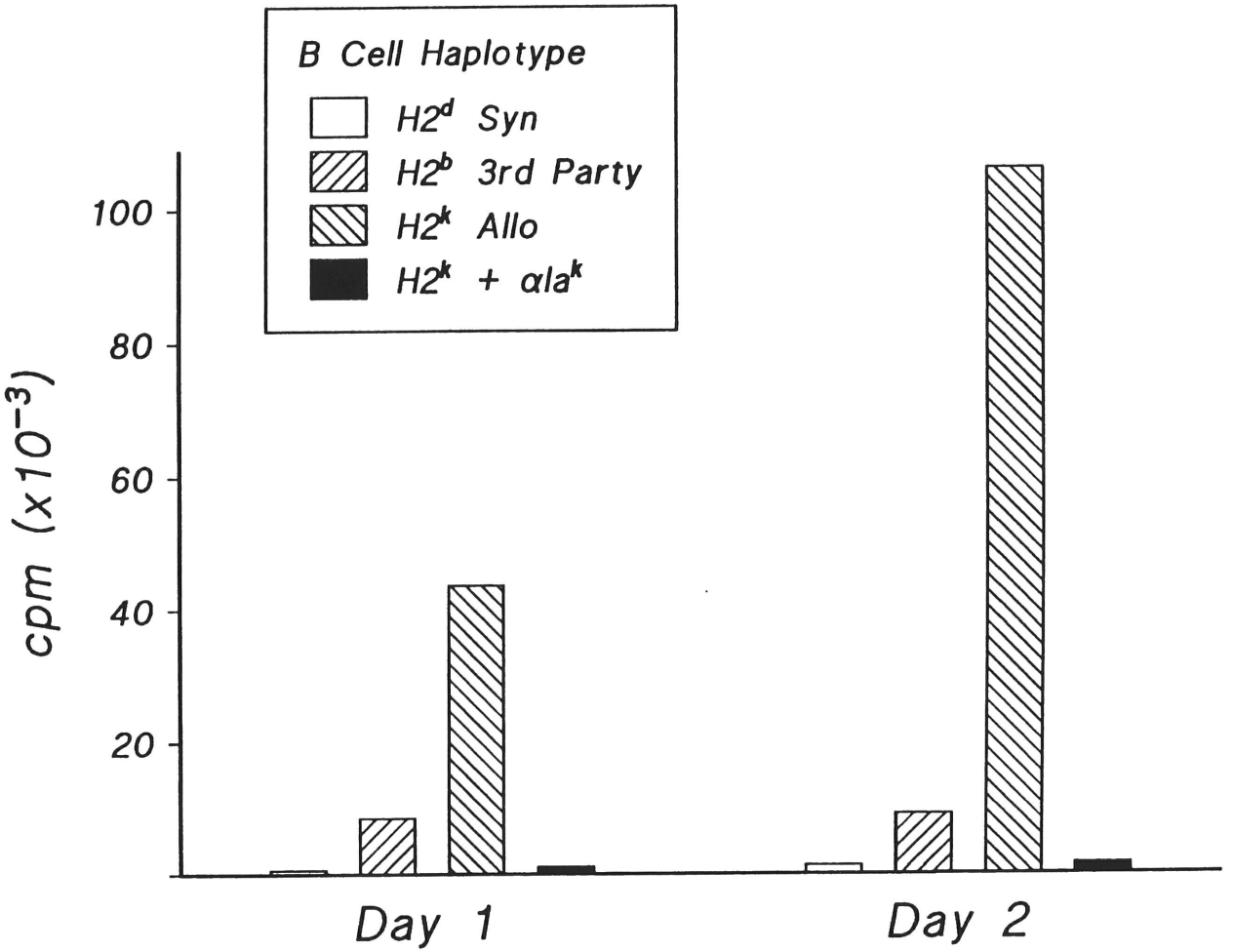

B Cell Haplotype
H2d Syn
H2b 3rd Party
H2k Allo
H2k + αIak
cpm (x10−3)
100
80
60
40
20
Day 1
Day 2

Figure 6. Kinetics of B lymphocyte blast transformation induced by allo-T blasts. Freshly isolated high density splenic B cells [B6.H2^k] at 10^6/ml were cultured with irradiated allo-T blasts [CD_2F_1] at 2 x 10^5/ml. At the times indicated, blast transformation of B cells was assessed on a FACScan^R by measuring forward light scatter. Day 0 [——]; day 1 [. . .]; day 2 [.....]; day 3 [----].

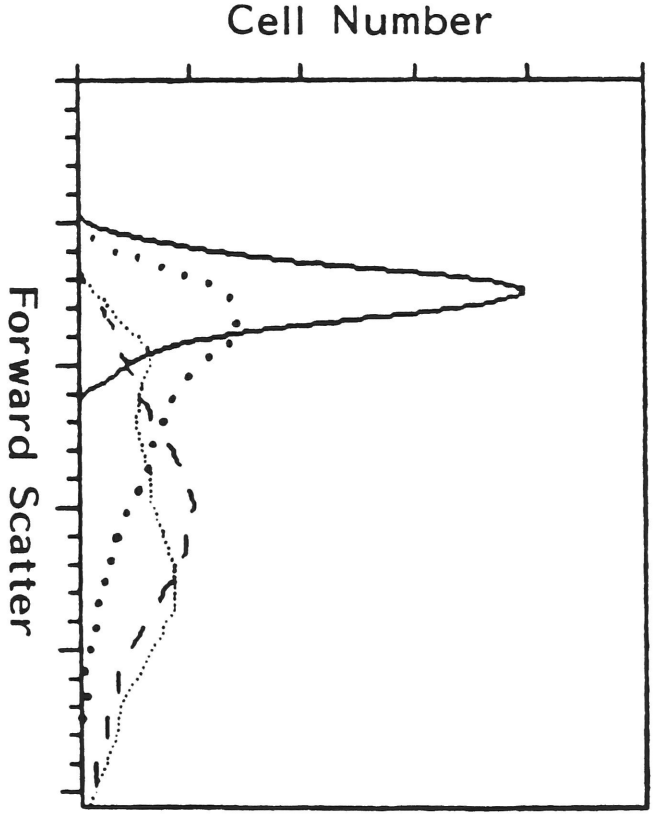

Cell Number
Forward Scatter

Table 2. Cell Cycle Analysis of Allo-B Blasts

	Day 0	Day 1	Day 2	Day 3
		%		
G_0/G_1	96	75	53	64
S	4	24	33	31
G_2 + M	0	1	14	5

Allo-B blasts were havested after 24, 48, and 72 h of T cell-B cell coculture. The numbers represent the percent of cells in G_0/G_1, S, and G_2/M at the indicated times post-stimulation.

B. Phenotype of B lymphoblasts.

Fluorescence-activated flow cytometry was used to determine the cell surface phenotype of the three types of B lymphoblasts, i.e., αIg, αIg/EL-4, or allo-B blasts [Figure 7 and Table 3]. Several cell surface molecules were upregulated to varying degrees on B lymphoblasts, relative to resting B cells, regardless of the means of activation. These included adhesion molecules [ICAM-1, Pgp-1, and LFA-1], MHC class I and II products, as well the IL-2Rα chain. These markers of cell activation were expressed on the vast majority of each of the B lymphoblast populations demonstrating the phenotypic homogeneity of the cells recovered. The amount of Ia and IL-2Rα, expressed on the lymphoblasts was consistently greater in B lymphoblasts that received T cell help. The adhesion molecules, MHC molecules and IL-2Rα were all upregulated within 24 hours after activation [Figure 8].

A decrease in surface IgD was observed on all B lymphoblasts, but was greater in those lymphoblasts generated in the presence of T cell-derived lymphokines or T cells. In contrast, surface IgM did not change significantly in any of the blast populations. The decrease in IgD was detectable on a subpopulation of blasts by day 2 but was dramatically reduced on almost all of the allo-B blasts by day 3 [Figure 8].

Interestingly, there were additional phenotypic changes

Figure 7. Phenotype of B lymphoblasts and B cells. B cells or 3 day B lymphoblasts were analyzed on a FACScan^R with antibodies to cell surface antigens or lectins as indicated. B cells [____]; αIg blasts [. . .]; αIg/EL-4 blasts [.....]; allo-B blasts [----]. The mAb used include: H57 [$TCR_{\alpha\beta}$], B5-5 [Thy-1], 11-4.1 [H-$2K^k$], M5/114.15.2 [Ia^k], 3C7 [IL-2Rα/IL-2Rp55], JA12.5 [IgD], YN1 [ICAM-1] and 18C8 [Pgp-1/CD44]. mIg was detected with FITC-GαM Ig.

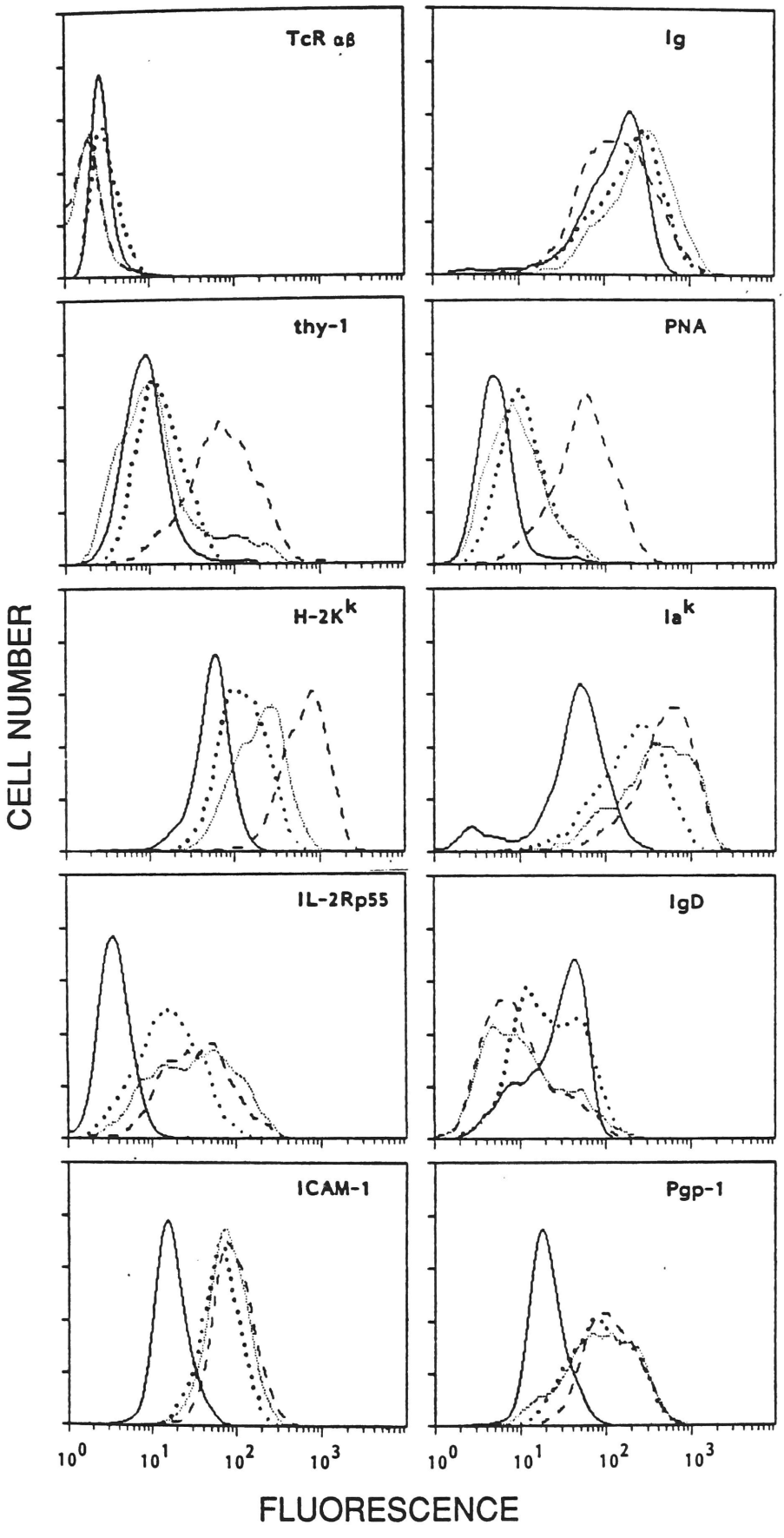

TcR αβ
Ig
thy-1
PNA
H-2K^k
Ia^k
IL-2Rp55
IgD
ICAM-1
Pgp-1
CELL NUMBER
10^0
10^1
10^2
10^3
FLUORESCENCE

Table 3. Changes in Surface Phenotype after *In Vitro* Activation of B Cells

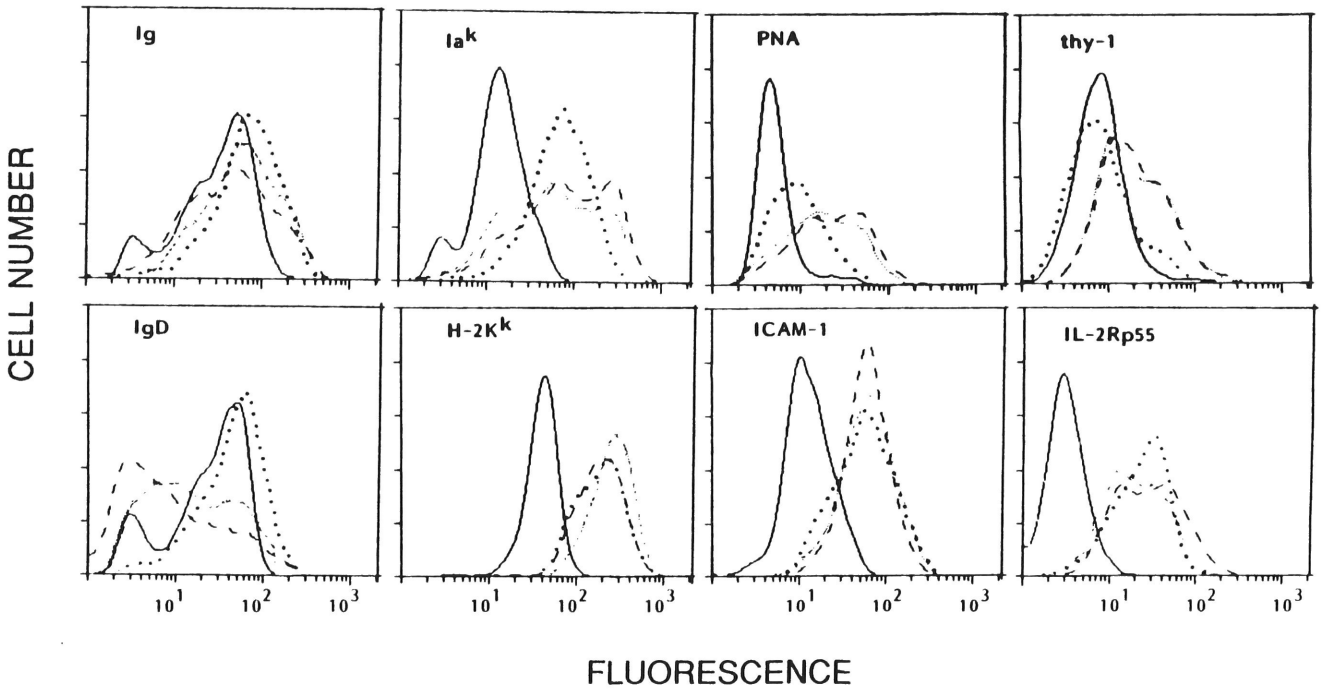
Antigen	Allo-B	αIg/EL-4	αIg
Class I MHC	3+	2+/3+	2+
Ia	2+/3+	2+	1+/2+
CD25/IL-2Rp55	2+	2+	1+
CD54/ICAM-1	2+	2+	2+
CD11a/LFA-1	2+	2+	2+
CD44/Pgp-1	2+	2+	2+
CDw90/Thy-1.2	1+/2+	NC	NC
PNA	1+/2+	NC/1+	NC/1+
Heat Stable Ag	NC	1+	1+
CD62L/L-Selectin	NC	NC	NC
CD45/LCA	NC	NC	NC
$CD45R_A$/B220	NC	NC	NC
$CD45R_B$	NC	NC	NC
$CD32/Fc_\gamma R_{II}$	NC	NC	NC
$CD23/Fc_\epsilon R_{II}$	NC*	NC*	NC
Ig	NC	NC	NC
IgM	NC	NC	NC
IgD	1-	1-	1-/NC

B cells or 3-d lymphoblasts were analyzed with antibodies to cell surface antigens or lectins. The change in surface phenotype refers to the change in mean fluorescent intensity relative to high density B cells: 1+ = 0-0.5 log increase; 2+ = 0.5-1 log increase; 3+ = >1 log increase; 1- = decrease; NC = no change.

B cells and lymphoblasts were T cell marker negative [$CD3_\epsilon$, TCR, and CD4]; macrophage marker negative [CD11b and F4/80]; and DC marker negative [33D1].

* Although the mean fluorescent intensity exhibited little or no consistent change, the staining became markedly heterogeneous.

Figure 8. Time course of phenotypic changes of allo-B blasts. Allo-B blasts were harvested after 24, 48, and 72 hours of T cell-B cell co-culture. Allo-B blasts or high density, resting B cells [day 0] were stained with antibodies to cell surface antigens or lectins as indicated. Resting B cell [day 0] [———]; day 1 [. . . .]; day 2 [.....]; day 3 [----]. The same antibodies were used as in Figure 7.


Ig
Ia^k
PNA
thy-1
IgD
H-2K^k
ICAM-1
IL-2Rp55
CELL NUMBER
FLUORESCENCE
10^1
10^2
10^3

that were restricted to blasts generated by direct T cell-B cell interaction [Figure 7]. Allo-B blasts had a greater increase in MHC class I expression than αIg or αIg/EL-4 blasts. The majority of allo-B blasts also expressed low levels of Thy-1. αIg blasts were Thy-1^{neg} and only 10% of 2-3 day αIg/EL-4 blasts expressed Thy-1 [data not shown]. This phenotypic change is likely mediated in part by IL-4, presumably released by T cells during the co-culture, which has been shown to induce the expression of Thy-1 on some murine B cells (296). However, IL-4 must act in concert with either LPS (296) or T cells [as shown here] to induce Thy-1 on B cells since EL-4 sn and anti-Ig alone did not induce its expression. However, this may simply reflect a difference in the concentration of IL-4 as discussed below.

Finally, stimulation by allo-T blasts enhanced reactivity with the lectin, peanut agglutinin [PNA] [Figure 7]. High reactivity with PNA implies that T cell-B cell interaction may result in the loss of terminal sialic acid residues from some surface glycoproteins resulting in the exposure of the penultimate N-acetyl glucosamine to which PNA is specific (297). One of the major cell surface glycoproteins, $CD45R_B$ is desialylated in some activated B cells *in vivo* as determined by reactivity with the mAb 23G2 which is specific for sialylated $CD45R_B$ (298). However, the mean fluorescence intensity of the allo-B blasts stained with 23G2 did not change [Table 3] indicating that this is not the cause of the

enhanced reactivity with PNA. Increased reactivity with PNA and expression of Thy-1, was detectable by day 1 and was maximal by day 2 [Figure 8]. In summary, this analysis of phenotype indicated that the B cells were uniformly activated, and that when B cells were activated directly by Th cells, a distinct phenotype was induced, i.e. IgD^{low} (299) and PNA^{high} (297), similar to germinal center B cells *in situ*.

C. Proliferative responses of B cells and isolated B lymphoblasts.

Each population of B lymphoblasts was isolated from the primary culture and then recultured. None of the purified blast populations continued to incorporate [^{3}H]TdR nor did they divide if re-cultured in the absence of additional stimuli. In the case of B cells and B lymphoblasts activated by anti-Ig, viability dropped to 30-50% by 24 hours (31), whereas virtually all the αIg/EL-4 blasts and allo-B blasts remained viable. This enabled us to study the requirements for maintaining cell viability and growth using anti-Ig, LPS, and lymphokines as stimuli.

B cell activation, regardless of the method used, primed the blasts for subsequent restimulation with anti-Ig-Sepharose or anti-IgD-Sepharose. Priming was consistently greater in those B blasts that received T cell help [Table 4, Exp. A, and data not shown].

Table 4. Effect of Cytokines on B Lymphoblast Proliferation

Exp.	Additions	B cell	αIg	αIg/EL-4	Allo-B
			cpm x 10^{-3}		
A	None	0.2	1.6	2.4	1.8
	αIg	0.9	18.8	45.0	32.2
	IL-2	0.2	6.4	134.8	125.0
	EL-4 Sn	1.0	16.0	228.1	152.1
	αIg/EL-4 Sn	12.9	353.2	698.0	293.1
	LPS	2.5	322.9	309.6	7.5
B	None	0.2	2.0	3.2	2.0
	αIg	0.4	17.2	17.4	103.1
	IL-2	0.1	11.4	71.1	70.7
	αIg/IL-2	0.4	123.5	253.5	233.8
	IL-4	0.1	2.4	3.6	2.7
	αIg/IL-4	0.3	24.1	27.8	83.4
	IL-5	0.1	7.1	12.1	8.4
	IL-6	0.1	2.1	2.9	2.2
	IL-7	0.1	1.1	3.0	1.8

B cells or 3-d [Exp. A] or 2-d [Exp. B] B lymphoblasts were recultured at 2.5 x 10^5 in 200 μl final volume with additions as indicated. After 24 h, the cultures were pulsed with 0.5 μCi [^{3}H]TdR for 12 h and harvested. Additions were: IL-2, 10 U/ml; IL-4, 100 U/ml; IL-5, 100 U/ml; IL-6, 4 ng/ml; IL-7, 100 U/ml; αIg-Sepharose, 5 μg/ml; EL-4 Sn, 0.5% [v/v]; and LPS, 20 μg/ml.

All three types of B blasts examined were primed to respond to the lymphokine-rich supernatant from PdBu-stimulated EL-4 thymoma cells which contained at least IL-2, IL-4 and IL-5 [Table 4, Exp. A]. Responsiveness to EL-4 sn was greatest in those B lymphoblasts primed in the presence of T cell-derived lymphokines, or alloreactive T cells. Individual lymphokines were also tested for their capacity to induce B lymphoblast proliferation, including rIL-1, rIL-2, rIL-4, rIL-5, rIL-6, rIL-7, rIL-10, IFNγ, and GM-CSF. Consistent with previously published reports (31), αIg blasts were relatively insensitive to each of these lymphokines [Table 4, Exp. B]. In contrast, B lymphoblasts generated in the presence of T cell help <u>were</u> primed for responsiveness to IL-2 but remained unresponsive to the other lymphokines tested [Table 4, Exp. B]. The response to IL-2 was not augmented by addition of other lymphokines, notably rIL-4, rIL-5, rIL-6 [data not shown]. Neutralizing anti-IL-2 antibody plus anti-IL-2R antibody inhibited the response to rIL-2 and, more importantly, the antibodies inhibited the response of allo-B and αIg/EL-4 blasts to EL-4 Sn by >90% [data not shown]. This result suggested that IL-2 was the predominant growth factor in EL-4 sn responsible for the IL-2 induced proliferative response of B blasts that had been generated in the presence of T cell help. In contrast, IL-4, which was originally described as a growth factor for B cells (108), did not promote proliferation of any of the B lymphoblasts or resting

B cells. IL-4 and IL-5, even in combination with anti-Ig, had little effect on B lymphoblast proliferation. However, B lymphoblasts exhibited a synergistic response to a combination of anti-Ig or anti-IgD plus EL-4 sn or IL-2, regardless of the means of activation [Table 4 and data not shown].

When viable cell recoveries were determined, the pattern of B lymphoblast responses was similar. In the case of B lymphoblasts generated in the presence of T cell help [soluble or direct cell contact], the recovery at 24 hours were approximately 200-250% with anti-Ig or anti-IgD, approximately 200-300% with rIL-2 or EL-4 sn, and 300-350% with anti-Ig plus rIL-2 or EL-4 Sn. The recovery of αIg blasts stimulated with anti-Ig or anti-IgD [45-130%], rIL-2 [75-100%], or EL-4 sn [150%] alone suggested that either these lymphoblasts did not proliferate to the same extent as the T-dependent B blasts or that fewer cells were rescued. However, the potential of αIg blasts to divide was indicated by their response to anti-Ig plus lymphokines [200-280%]. There was no evidence of cell division by the resting B cells with any stimuli since cell recovery was always < 60%.

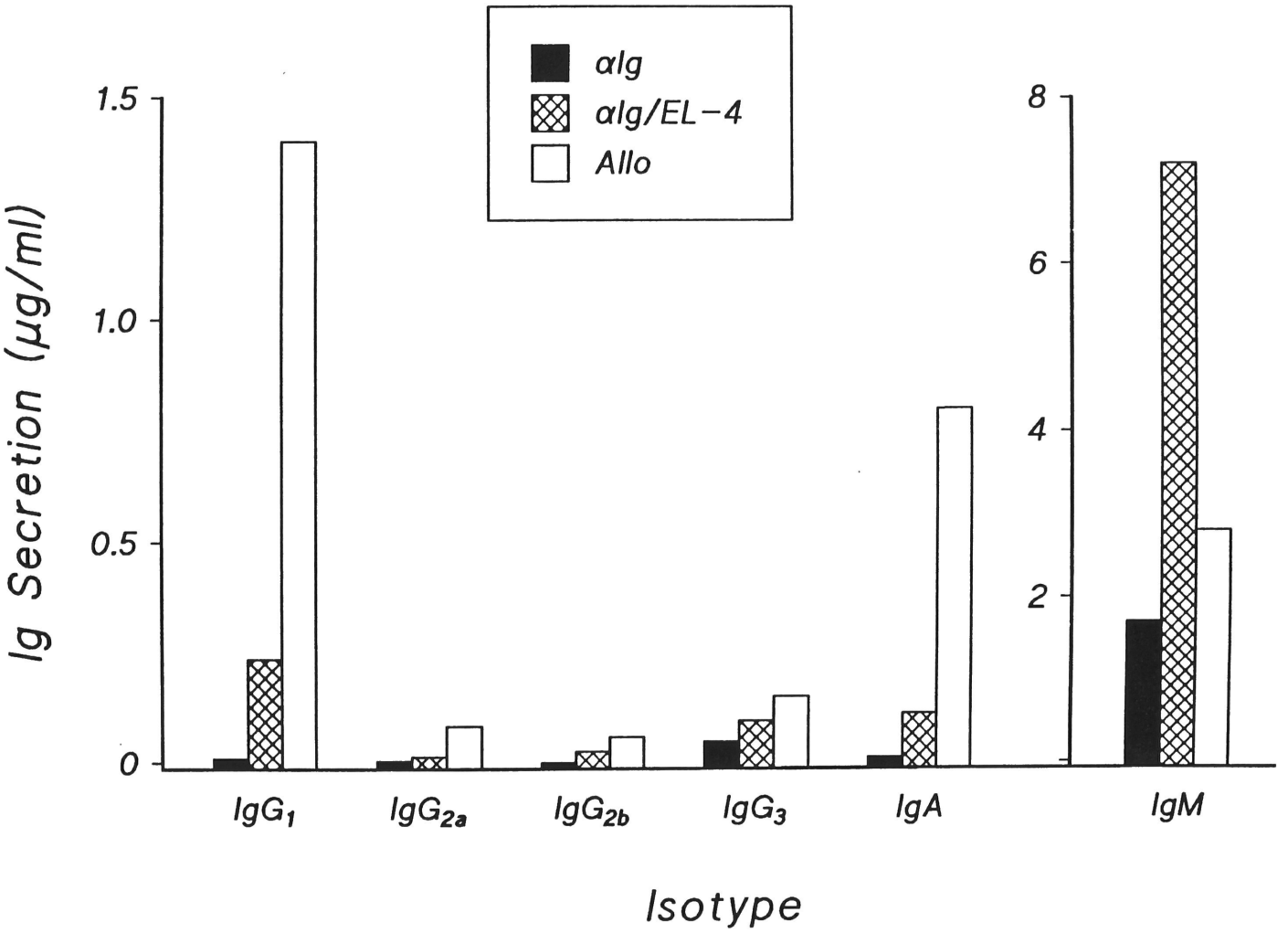
Although IL-2 stimulated T cell-dependent B lymphoblasts to proliferate, it was not sufficient to maintain B cell viability in long term cultures, [i.e. greater then two weeks] even if co-cultured with anti-Ig. This is in contrast to T cell clones which can be maintained in culture indefinitely with appropriate stimulation. To date, the only means of

maintaining long term cultures of B cells is by transformation with viruses (300). Thus, either the "B cell growth factor[s]" have not been identified to date, or long term viability of B cells requires a more complex microenvironment.

D. Ig secretion by B lymphoblasts.

An analysis of the quantity and isotype of the Ig secreted provides an additional means of assessing B cell activation and differentiation. T cell help, whether in the form of T blasts or activated T cell-conditioned medium, primed B cells for enhanced secretion of total immunoglobulin [Figure 9]. However, the activation of B cells by allo-T blasts resulted in preferential production of immunoglobulins utilizing downstream heavy chain constant regions, most notably IgG_1 and IgA, while the amount of IgM was reduced relative to αIg/EL-4 blasts. The pattern of Ig isotype secretion was the same whether the cells were restimulated with rIL-2 or with EL-4 sn +/- anti-IgD [Figure 9 and data not shown]. The priming for utilization of heavy chain constant regions γ_1 and α was not dependent on direct cell contact in the primary culture [data not shown]. If the allo-T/B culture supernatant was substituted for the EL-4 sn in the primary culture, a similar pattern of isotype secretion was observed to that of the allo-B blasts [data not shown]. This suggests that isotype switching requires two signals. These include an

Figure 9. Allo-B blasts are uniquely primed for production of Ig utilizing downstream heavy chain constant region genes. Three day B lymphoblasts, stimulated as indicated in the primary cultures, were cultured at 2.5 x 10^5/ml in 200 μl final volume with 0.5% v/v EL-4 sn. After 60 hours, the supernatants were collected and tested in an isotype-specific ELISA. IgE was tested and uniformly below the limit of detection [4 ng/ml] of the ELISA [data not shown].


αIg
αIg/EL-4
Allo
1.5
1.0
0.5
0
8
6
4
2
Ig Secretion (μg/ml)
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
Isotype

activating signal which is provided by either anti-Ig or T cell contact as well as the specific lymphokines added to or produced in the culture which promote production of the specific Ig isotypes.

E. Time dependence of B cell priming by allo-T blasts.

We investigated the kinetics of functional priming of allo-B blasts during the primary co-culture of T blasts with B cells. Allo-B blasts were primed for enhanced responsiveness to anti-Ig-Sepharose within 24 hours [Figure 10]. In contrast, allo-B blasts required 2 days of co-culture with allo-T blasts both for enhanced production of Ig as well as for maximal priming of the proliferative responses to IL-2 and EL-4 sn [Figures 10 and 11]. The 2 day requirement for priming of allo-B blasts to respond to IL-2 was unexpected because the increase in IL-2Rα subunit peaked by day 1 [Figure 8]. One possibility is that the expression of the β or γ subunits of the IL-2R that are also required for IL-2-mediated responses may be upregulated with different kinetics. However, as discussed below, there was no correlation between the number of high affinity IL-2 binding sites and IL-2 induced proliferation of the various populations of B lymphoblasts.

F. IL-5 is sufficient for acquiring IL-2 responsiveness.

Figure 10. Time course of priming of allo-B blasts for proliferation. Allo-B blasts were harvested at 24, 48 and 72 hours after initiating the primary culture with alloreactive T blasts. Isolated allo-B blasts or high density B cells were cultured at 2.5 x 10^5/ml in 200 μl final volume with the indicated stimuli. After 24 hours, the B cells were pulsed with [^{3}H]-TdR for 12 hours, harvested and counted. Additions were: αIg-Sepharose, 5 μg/ml; EL-4 sn, 0.5% v/v; IL-2, 10 U/ml.

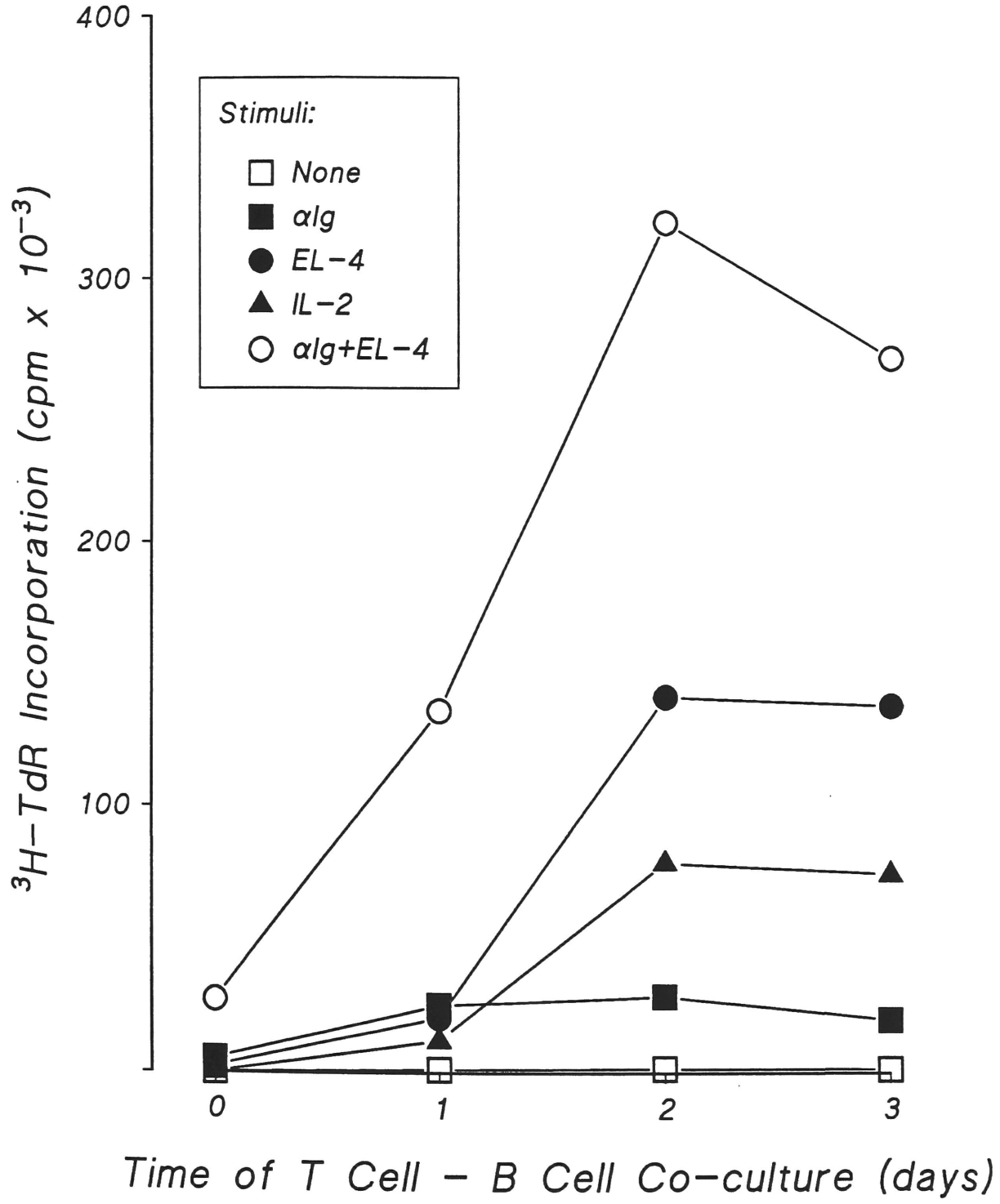
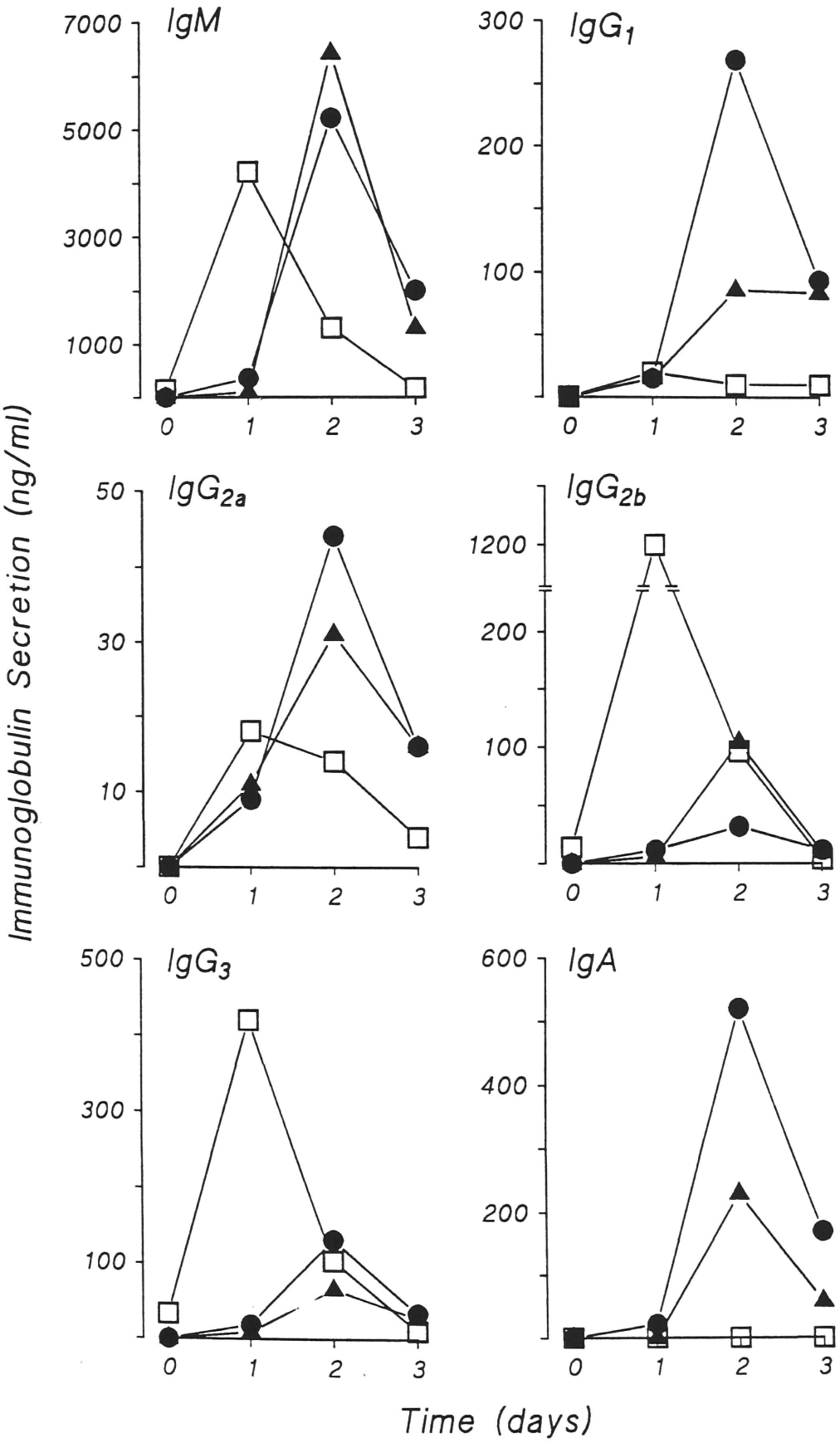

400
300
200
100
^3H–TdR Incorporation (cpm x 10^{-3})
Stimuli:
None
αIg
EL–4
IL–2
αIg+EL–4
0
1
2
3
Time of T Cell – B Cell Co–culture (days)

Figure 11. Time course of priming of allo-B blasts for Ig secretion. Allo-B blasts were harvested at 24, 48, and 72 hours after initiating the primary culture with alloreactive T blasts. Isolated allo-B blasts or high density, resting B cells [days = 0] were cultured at 2.5 x 10^5/ml in 200 μl final volume with additions as indicated. After 60 hours, the supernatants were removed and tested in an isotype-specific Ig ELISA. Additions were: EL-4 sn, 0.5% v/v [•]; IL-2, 10 U/ml [▲]; LPS, 20 μg/ml [■].


IgM
7000
5000
3000
1000
IgG1
300
200
100
IgG2a
50
30
10
IgG2b
1200
200
100
IgG3
500
300
100
IgA
600
400
200
0
1
2
3
Immunoglobulin Secretion (ng/ml)
Time (days)

The priming of B cells for responsiveness to IL-2 required T cell help either by direct contact or in the form of T cell-derived cytokines [Table 4]. Loughnan and Nossal demonstrated that IL-4 and IL-5 induced the expression of IL-2Rβ and IL-2Rα, respectively, on B cells (134). To explore the role of cytokines in priming for IL-2 responsiveness, we generated B lymphoblasts with anti-Ig-coupled Sepharose plus individual lymphokines [IL-2, IL-4 or IL-5]. The lymphoblasts were isolated and restimulated with either IL-2 or EL-4 sn [Figure 12]. The presence of IL-5 in the primary culture primed the B cells for subsequent responses to IL-2, albeit at reduced levels when compared to EL-4 Sn [data not shown]. The IL-2-induced proliferation was completely blocked by anti-IL-2 and anti-IL-2R antibodies.

To determine if IL-5 was inducing the expression of IL-2Rβ, we performed IL-2 binding assays on the various B cell populations and the data were subjected to Scatchard analysis [Table 5]. Interestingly, all of the blast populations [except for those generated in the presence of LPS], expressed both high and low affinity IL-2 receptors at 48 hours [Table 5]. Thus, expression of the high affinity IL-2 receptor was not sufficient to confer B cell responsiveness to IL-2. The difference in responses may be due to the differential expression of the IL-2Rγ or to an uncoupling of the receptor from the signaling pathway. An analysis of the allo-T/B supernatant showed high levels of IL-2, IL-4, IL-5, and IL-10

Figure 12. IL-5 primes anti-Ig activated B cells for IL-2 induced proliferation. αIg blasts generated in the presence of the indicated cytokines were cultured at 2.5 x 10^5/ml with the indicated additions [IL-2, 10 U/ml; EL-4 sn, 0.5% v/v; anti-IL-2 and anti-IL-2Rα, cocktail of 3C7, 7D4 and S4B6.1 hybridoma supernatants, 10% v/v]. After 24 hours, the cells were pulsed with 1 μCi [^{3}H]-TdR for 12 hours, harvested and counted. Data represents the average of triplicate wells.

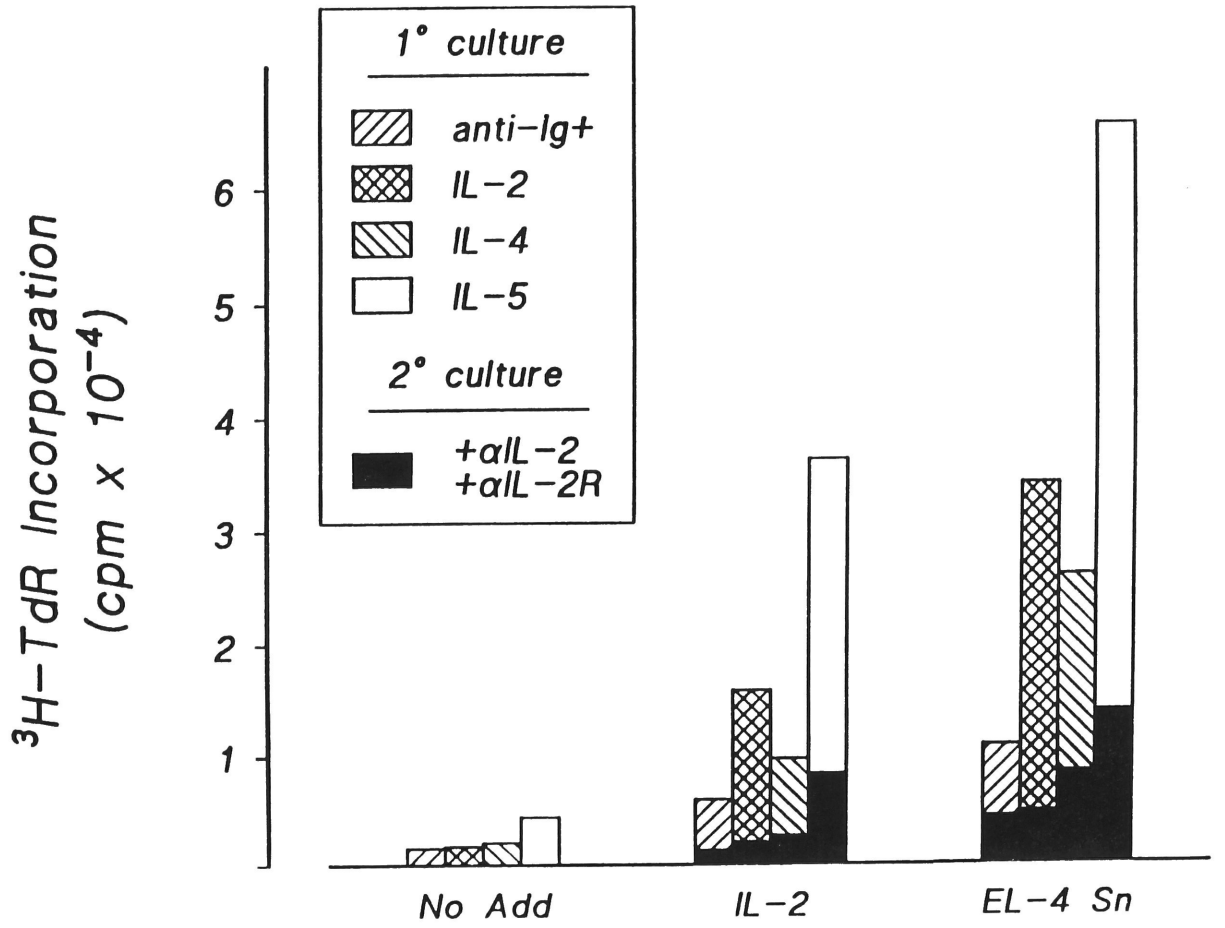

1° culture
anti-Ig+
IL-2
IL-4
IL-5
2° culture
+αIL-2
+αIL-2R
^3H-TdR Incorporation
(cpm x 10^{-4})
6
5
4
3
2
1
No Add
IL-2
EL-4 Sn

Table 5. IL-2 Receptor Expression on B Cells and B Lymphoblasts

	low affinity		high affinity	
	sites/cell	K_D [$x10^{-12}$]	sites/cell	K_D [$x10^{-12}$]
B cells	0	-	0	-
αIg Blasts	11,000	6200	200	20
αIg/IL-2 Blasts	26,000	4700	480	33
αIg/IL-4 Blasts	4,500	2900	185	60
αIg/IL-5 Blasts	33,500	5600	525	29
αIg/El-4 Blasts	24,000	7800	375	37
LPS Blasts	0	-	0	-
CTLL	240,000	5100	5500	37

10^6 B cells, B lymphoblasts or CTLL were incubated with 1 pM to 100 nM [^{125}I]IL-2 for 20 minutes. The cells were pelleted and the free and cell-bound IL-2 was determined. The number of sites per cell and K_D were determined by Scatchard analysis.

[Table 6]. Recently, IL-10 was also demonstrated to prime B cells for restimulation with IL-2 (135). In addition, IL-4 and IL-5 induce both the IL-2Rα and IL-2Rß (134). Thus, a combination of these cytokines may be required to reconstitute the IL-2 receptor on B cells.

G. Response of B lymphoblasts to LPS.

Bacterial LPS is a mitogen for murine B cells (23-25). B cell activation through the BCR by anti-Ig-Sepharose primed the B cells for enhanced responsiveness to LPS [Table 4] (31). In contrast, allo-B blasts were remarkably insensitive to LPS as measured by proliferation [Table 4], cell viability [data not shown], or Ig production [Figure 11]. This lack of response to LPS by allo-B blasts was dependent on the time B cells were co-cultured with allo-T blasts [Figure 11 and data not shown] and was inversely related to the kinetics of priming for responses to anti-Ig-Sepharose and/or lymphokines [Figures 10 and 11]. The LPS response of allo-B blasts generated by 1 day of T cell-B cell co-culture was notable for high levels of IgG_{2b} and IgG_3. These findings suggest that co-culture of B cells with allo-T blasts either preferentially expands a unique population of LPS-resistant B cells or induces a specific unresponsiveness to LPS through an unidentified pathway. One specific possibility is that allo-stimulation of B cells renders them lymphokine dependent. If

Table 6. Lymphokine Production in Allogeneic Cultures

	Lymphokines [U/ml]			
Conditioned Medium	IL-2	IL-4	IL-5	IL-10
T/allo-DC	7	0.1	100	8
T blast/allo-B	15	30	650	55

Cells from the T cell/DC or T blast/B cell mixed lymphocyte cultures were harvested and the culture supernatant was tested for the lymphokines IL-2, IL-4, IL-5, and IL-10. Data represents the average of triplicate cultures.

this were the case, it would be predicted that when stimulated with LPS alone they would not respond, but LPS plus lymphokines should have a synergistic effect. However, the restimulation of allo-B blasts with a combination of lymphokines plus LPS resulted in a response that was only additive [data not shown].

CHAPTER 2

INDUCTION OF THE PHOSPHORYLATION AND KINASE ACTIVITY OF PTK72 IN B CELLS

In the immune response, B cells play a role in both the efferent limb, by secreting immunoglobulin, as well as the afferent limb by virtue of their ability to present antigen to T cells. The requirements for B cell antigen presentation are distinct from professional antigen presenting cells [i.e. DC] (290). B cells must be activated to present antigen to resting T cells (291,292). Another difference is the high efficiency of presentation of antigen specifically bound to mIg on B cells which is dependent on receptor crosslinking (301). In addition, recent work from Dr. Puré's laboratory demonstrated that tyrosine phosphorylation is required for mIg internalization (212). To investigate the molecular basis for tyrosine phosphorylation dependent BCR internalization, we examined the conditions necessary for the induction of tyrosine phosphorylation in B cells.

A. Receptor crosslinking is required for optimal anti-Ig induced accumulation of tyrosine phosphorylated proteins and kinase activity.

We began by determining the conditions necessary for

induction of tyrosine phosphorylation and compared these requirements with those of downstream signaling events such as proliferation. To determine the requirement for crosslinking, we compared stimulation of B cells using monovalent and divalent fragments, [Fab' and $F[ab']_2$, respectively], of anti-Ig. These fragments of anti-Ig were characterized for purity based on silver staining of SDS-polyacrylamide gels and their ability to induce DNA synthesis in resting B cells. Consistent with previous reports (302-304), divalent $F[ab']_2$, but not monovalent Fab' fragments of anti-mouse Ig, induced DNA synthesis [data not shown]. $F[ab']_2$ fragments of anti-Ig induced a dose dependent accumulation of tyrosine phosphorylated proteins as detected by immunoblotting with a monoclonal anti-phosphotyrosine antibody [Figure 13A]. Equimolar or greater doses of monovalent Fab' fragments of anti-Ig were markedly less effective at inducing tyrosine phosphorylation of most of the proteins [Figure 13A]. Stimulation by Fab' fragments could be enhanced by crosslinking with a secondary $F[ab']_2$ MαR Ig [Figure 13B]. Tyrosine phosphorylation of proteins of 35, 40, 42, 52-56, 62-66, 72-80, 92, 100, 120, and 130 kD was consistently upregulated [Figure 13]. Occasionally, species of 150 and 200 kD were also induced.

The molecular weight distribution of induced tyrosine phosphorylated proteins detected here was similar to that reported by Gold et al. (209), and included all of the species

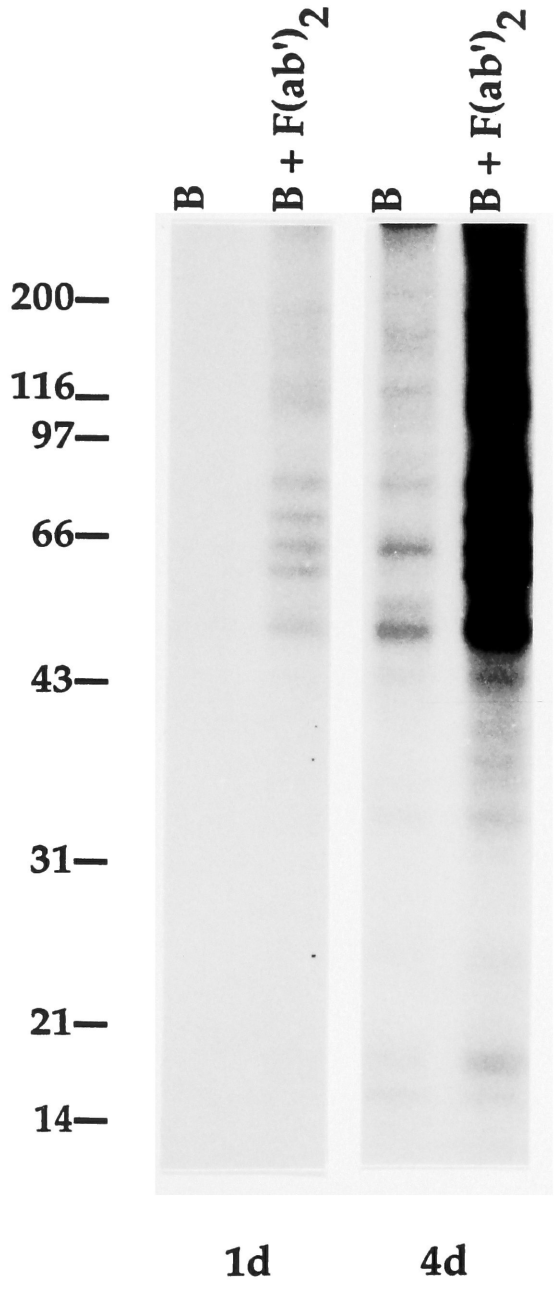
Figure 13. The optimal anti-Ig induced accumulation of tyrosine phosphorylated proteins requires receptor crosslinking. A. High density B cells were stimulated at 5 x 10^7/ml on ice for 1 hour with the indicated dose of F[ab']$_2$ or Fab' fragment of RαM Ig. The B cells were pelleted to remove unbound antibody and lysed. The lysates were resolved on a 10% SDS-PAGE, transferred to PVDF membranes, and analyzed by immunoblotting with the anti-phosphotyrosine antibody, PY69. B. High density B cells were induced by stimulation with 0.25 μM of intact RαM Ig, or F[ab']$_2$ or Fab' fragments of RαM Ig for 60 minutes on ice. Where indicated, the Fab' fragments were crosslinked with F[ab']$_2$ fragments of MαR Ig as secondary. Lysates were analyzed by immunoblotting as in A.

A
200 –
116 –
97 –
66 –
43 –
Control
0.01 μM
0.1 μM
0.25 μM
0.5 μM
F(ab')2
1.0 μM Fab'
B
200 –
116 –
97 –
66 –
43 –
Control
Fab'
F(ab')2
Intact
Fab' + MoRab
MoRab

reported by Campbell and Sefton (208). However, in both cases the B cells were stimulated with intact anti-Ig which does not induce B cell proliferation because of a dominant negative signal mediated by the $Fc_{\gamma}R_{II}$. In our hands, intact anti-Ig also induced a rapid accumulation of several tyrosine phosphorylated proteins [Figure 13B] suggesting that the $Fc_{\gamma}R_{II}$ mediated inhibition was not due to blocking the PTK pathway. The requirement of mIg crosslinking for tyrosine phosphorylation by either intact or $F[ab']_2$ fragments of anti-Ig was recently demonstrated by Ravetch, et al. (305).

Since many tyrosine kinases are inducibly phosphorylated, we also assayed for phosphotransferase [kinase] activity associated with immune complexes formed by precipitation of B cell lysates with anti-phosphotyrosine antibodies. A low and variable degree of phosphorylation of several endogenous substrates was detected in *in vitro* kinase assays of immune complexes from lysates of resting B cells [Figure 14]. This most likely represents basal levels of kinase activity in resting B cells, contamination of B cell preparations with activated B cells, or deregulation of kinase activity in these *in vitro* conditions. Phosphorylation of most of these proteins was markedly upregulated in immune complexes from lysates of B cells treated with $F[ab']_2$ fragments of anti-Ig [Figure 14]. In all experiments, over exposure revealed co-migration of most of the substrates of the basal and induced kinase activities [Figure 14]. Consistent with the *in situ*

Figure 14. Anti-phosphotyrosine immune complex associated phosphotransferase activity is stimulated by anti-Ig. A. One x 10^7 high density B cells were stimulated with 0.25 μM of F[ab']$_2$ fragments of RαM Ig on ice for 1 hour and lysed in 1% NP-40. Lysates were immunoprecipitated with Rα phosphotyrosine and the isolated immune complexes were assayed for *in vitro* phosphotransferase activity on endogenous substrates in the presence of ^{32}P-γATP. The immune complexes were resolved on a 5-12% SDS-polyacrylamide gel. The autoradiographs represent a 1 or 4 day exposure. Molecular weight standards are indicated in kd.


B
B + F(ab')2
B
B + F(ab')2
200
116
97
66
43
31
21
14
1d
4d

data above [Figure 13], monovalent fragments of anti-Ig alone failed to induce the kinase activity. However, crosslinking of the receptors by the addition of a secondary, affinity-purified, $F[ab']_2$ fragment of MαR Ig, induced the kinase activity [Figure 15]. Moreover, the array of phosphorylated proteins detected in the kinase assay overlapped with the tyrosine phorphorylated proteins induced *in situ* as detected by anti-phosphotyrosine immunoblotting [Figures 13-15].

A phosphorylated endogenous substrate with a molecular weight of approximately 72 kD was consistently one, if not the most abundant of the species detected in the kinase assay of lysates from anti-Ig treated B cells. However, unlike the other tyrosine phosphorylated species observed, this species was never detectable in the lysates from resting B cells. Thus the 72 kD species apparently had a more stringent requirement for receptor crosslinking. This also suggests that the small amount of phosphorylation observed in resting B cells was probably not due to contaminating activated lymphoblasts, but rather reflected basal levels of phosphorylation of particular substrates and/or activity of some of the inducible kinsaes.

Phosphorylation of the 72 kD species following stimulation with anti-Ig was evident in B cells at different stages of activation/differentiation. Treatment of αIg blasts or allo-B blasts with soluble anti-Ig resulted in a similar array of phosphorylated proteins in the *in vitro* kinase

Figure 15. The anti-Ig induced kinase activity in anti-phosphotyrosine immune complexes requires receptor crosslinking. One x 10^7 high density B cells were stimulated with 0.25 μM of intact, Fab', and F[ab']$_2$ fragments of RαM Ig on ice for 1 hour. Bound Fab' fragments were then crosslinked where indicated with 0.50 μM F[ab']$_2$ MαR Ig for 30 minutes on ice. B cells were lysed in 1% NP-40 and lysates were immunoprecipitated with anti-phosphotyrosine and assayed for *in vitro* kinase activity. The immune complexes were resolved on a 5-12% SDS-polyacrylamide gel. Molecular weight of standards are indicated in kD.

0
RAM intact
RAM Fab'2
RAM Fab' + MAR
RAM Fab'
MAR
200
116
97
66
43
31

activity as observed with resting B cells [Figure 16]. This is consistent with the data demonstrating that both αIg and allo-B blasts can be restimulated via their surface Ig [Chapter 1 and (306)].

B. The induction of tyrosine phosphorylated proteins and kinase activity is specifically mediated by mIgM and mIgD.

A requirement for receptor crosslinking of mIg for signal transduction is further supported by experiments in which resting B cells were treated with the monoclonal anti-IgM antibody, Bet2. Bet2 alone at concentrations up to 0.5 μM induced only low levels of tyrosine phosphorylation and kinase activity [Figure 17]. This correlated with the inefficient but detectable levels of DNA synthesis induced by the Bet2 antibody [Table 7]. However, crosslinking of the Bet2 bound to the B cell by the addition of MαRat Ig, induced proliferation [Table 7] and enhanced the induction of both tyrosine phosphorylation and the kinase activity [Figure 17]. It has been demonstrated that B cell activation via membrane IgM and IgD results in a similar induction of tyrosine phosphorylation (208,209). We found the kinase activity induced by crosslinked IgM [Bet2] and IgD [Hδ^a/1] specific MAb were also comparable [Figure 18].

Crosslinking of several other B cell surface proteins including CD45, CD44, or Ia, with mAbs followed by secondary

Figure 16. The *in vitro* kinase activity is induced in preactivated B lymphoblasts upon stimulation with RαM Ig. High density B cells, αIg blasts or allo-B blasts were stimulated with 0.25 μM $F[ab']_2$ RαM Ig on ice for 60 minutes and lysed in 1% NP-40. Equivalent amounts of total protein from each lysate were immunoprecipitated with anti-phosphotyrosine and the immune complexes assayed for phosphotransferase activity in the presence of ^{32}P-γATP. The immune complexes were resolved by SDS-PAGE and analyzed by autoradiography. Molecular weight standards are indicated in kD.

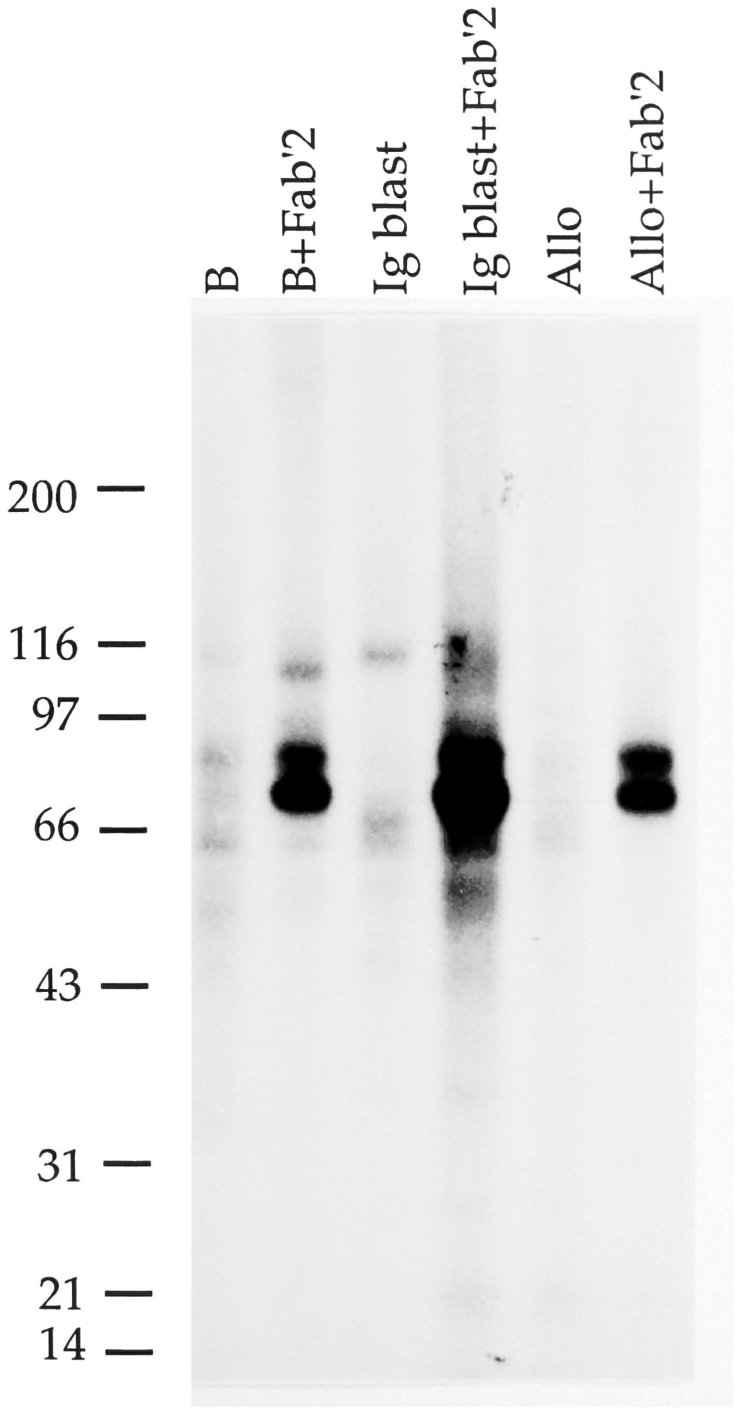

B
B+Fab'2
Ig blast
Ig blast+Fab'2
Allo
Allo+Fab'2
200
116
97
66
43
31
21
14

Figure 17. Induction of the phosphotyrosine proteins and the kinase activity is specific to mIg. High density B cells were stimulated with 0.25 μM of rat mAb to the indicated B cell surface antigens for 30 minutes on ice, either alone or followed by crosslinking with 0.5 μM F[ab']$_2$ fragments of MαRat Ig for 30 minutes on ice. Cells were lysed in 1% NP-40. A. Cell lysates were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotted with the anti-phosphotyrosine mAb, PY69. B. Cell lysates were immunoprecipitated with Rα phosphotyrosine and the immune complexes were assayed for kinase activity. The immune complexes were resolved by SDS-PAGE and analyzed by autoradiography. Molecular weight standards are indicated in kD.

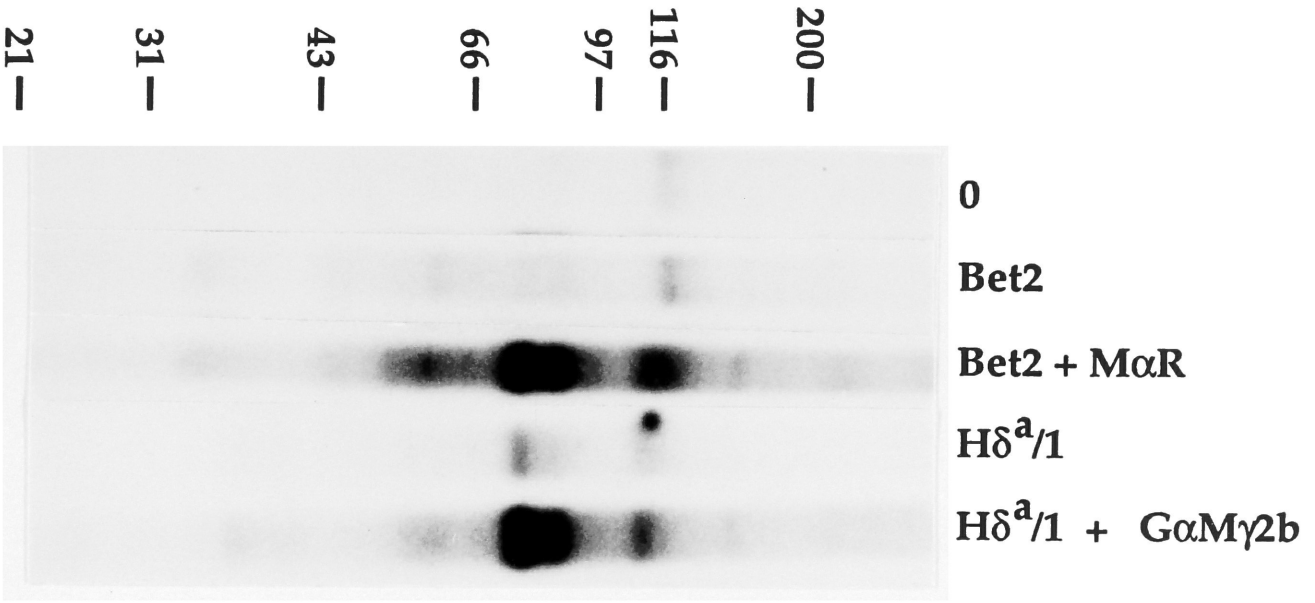
A
MαRat
Anti-μ
MoRat
Anti-μ
Anti-CD44
Anti-Ia
Anti-CD45
200
116
97
66
43
B
MαRat
Anti-μ
MoRat
Anti-μ
Anti-CD44
Anti-Ia
Anti-CD45
200
116
97
66
43
31

Table 7. Induction of B cell proliferation in Response to Soluble Anti-Ig

Stimuli	Dose [μM]	Response [cpm]
Control	-	705
Anti-μ	0.01	1,362
	0.10	1,920
	0.25	2,877
Anti-μ + MαRat Ig	0.01	322
	0.10	2,622
	0.25	29,674
MαRat Ig	0.50	1,880
Anti-CD45	0.25	854
Anti-CD45 + MαRat Ig	0.25	705

High density B cells were cultured at 5 x 10^5ml in 200 μl final volume with the mAb Bet2 [anti-μ] or M1/9.3.4 [anti-CD45] at the indicated dose for 48 hours. Where indicated, secondary MαRat Ig $F[ab']_2$ was added to 0.5 μM. After 36 hours, the cultures were pulsed with one μCi of ^{3}H-TdR for 12 hours and harvested.

Figure 18. Crosslinking mIgM or mIgD on B cells induces the kinase activity. One x 10^7 high density B cells were stimulated with 0.25 μM of monoclonal antibodies to IgM [Bet2] or IgD [Hδ^a/1] for 60 minutes on ice. Where indicated, Bet2 and Hδ^a/1 were crosslinked with 0.50 μM F[ab']$_2$ MαRat Ig and GαM IgG$_{2b}$, respectively, for 30 minutes on ice. Cells were lysed in 1% NP-40 and the lysates were immunoprecipitated with anti-phosphotyrosine. The immune complexes were assayed in the *in vitro* kinase assay, resolved by SDS-PAGE, and analyzed by autoradiography. Molecular weight standards are indicated in kD.


200
116
97
66
43
31
21
0
Bet2
Bet2 + MαR
Hδ^a/1
Hδ^a/1 + GαMγ2b

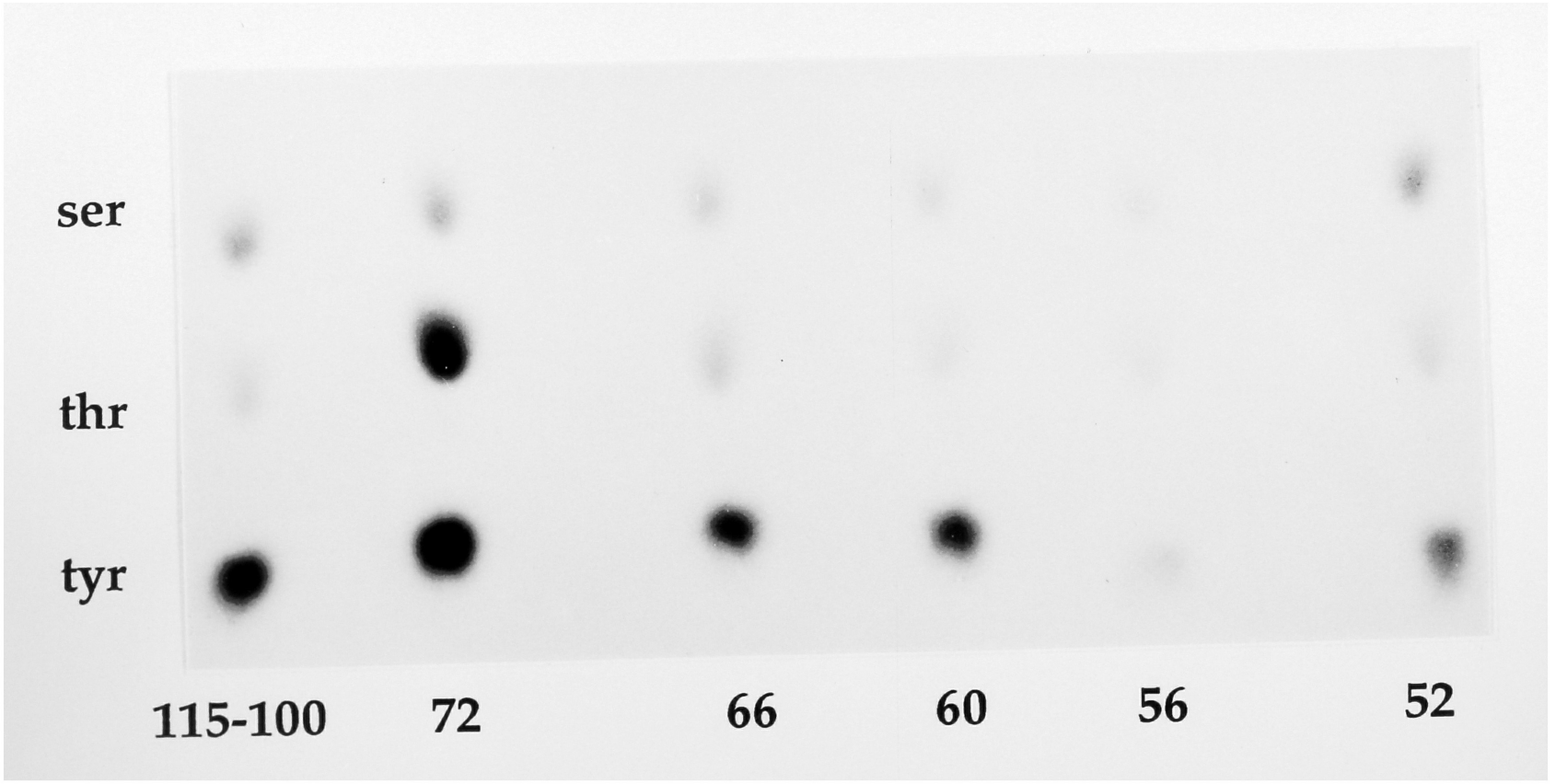
crosslinking antibodies had no effect on *in situ* tyrosine phosphorylation [Figure 17A], *in vitro* kinase activity [Figure 17B], or on DNA synthesis [Table 7]. An exception occured with some anti-CD45 mAb that weakly induced the kinase activity [Figure 17B]. Ledbetter and colleagues have demonstrated a similar effect of anti-CD45 mAb in T cells in which ligation of CD45 induced tyrosine phosphorylation of several endogenous substrates (307).

To define the nature of the kinase activities further, we excised the major bands induced by receptor crosslinking and subjected each to phosphoamino acid analysis [Figure 19]. Each species exhibited some degree of phosphorylation on serine and threonine, however the majority of the phosphorylation occurred on tyrosine in almost all of the substrates. The predominant species that migrated at 72 kD was the only protein that was highly phosphorylated on threonine in addition to tyrosine.

C. IL-4 synergizes with anti-Ig in inducing phosphorylation of the 72 kD species.

Stimulation of B cells with several cytokines, including IL-2, IL-5, IL-6, IL-7, IL-10, and IFNγ enhanced the basal level and anti-Ig-induced phosphorylation of some of the substrates. These effects, however, were variable both in the species and the extent to which they were effected. The 72 kD

Figure 19. Phosphoamino acid analysis of the endogenous substrates of the anti-phosphotyrosine immune complex-associated kinase activity. One x 10^7 high density B cells were stimulated with 0.25 μM F[ab']$_2$ fragments of RαM Ig on ice for 60 minutes. Cells were lysed in 1% NP-40 and immunoprecipitated with anti-phosphotyrosine. The immune complexes were assayed for kinase activity and resolved by SDS-PAGE. The bands migrating at 115-110, 72, 66, 60, 56, and 52 kD were excised from the gel and subjected to phosphoamino acid analysis as described. Phosphoamino acid standards were visualized by ninhydrin staining. The TLC plates were exposed to film for 1 week.

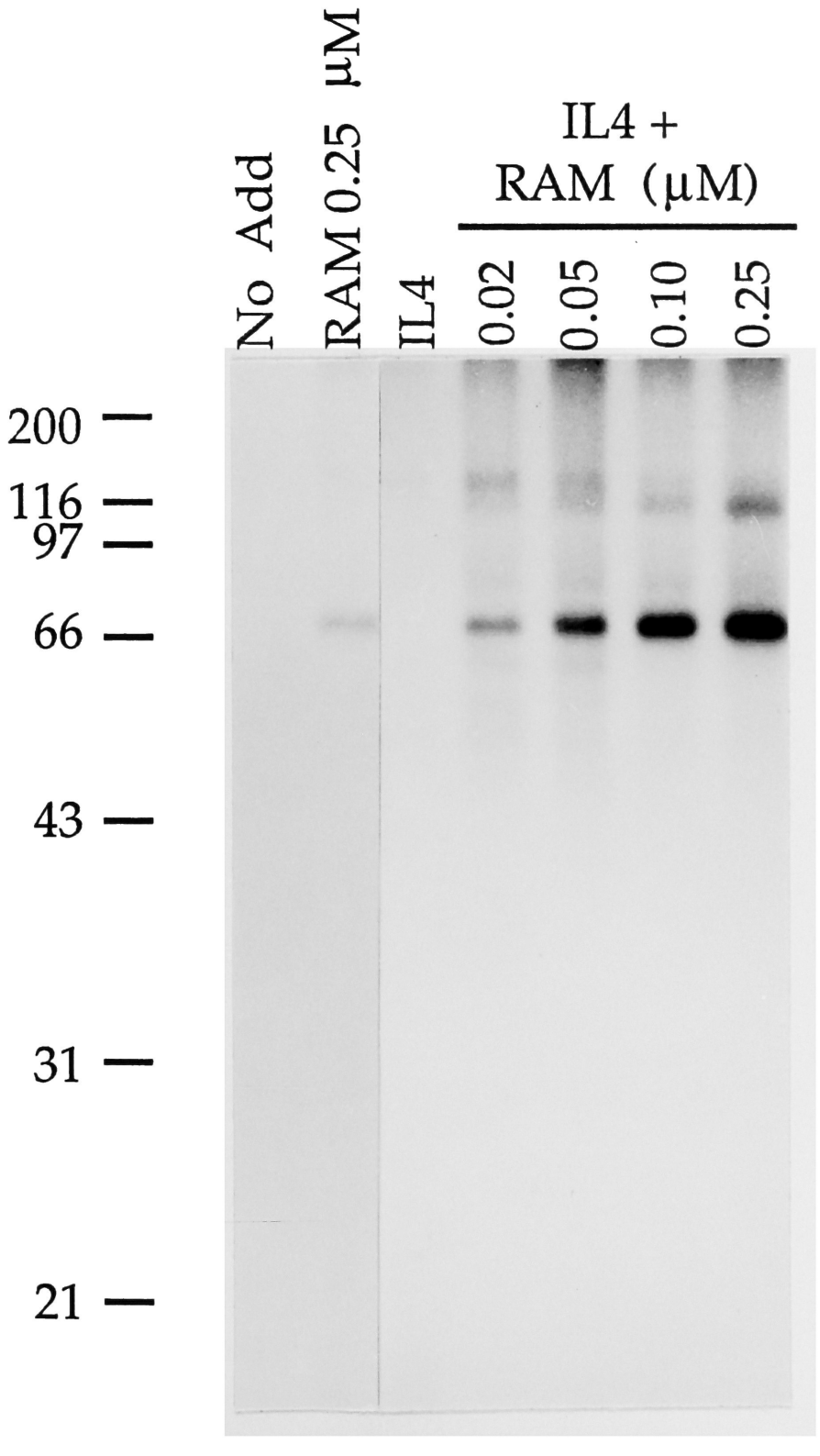

ser
thr
tyr
115-100
72
66
60
56
52

substrate was never detected in the kinase assay using cells treated with any of these cytokines. In contrast, although IL-4 had no effect on basal levels of tyrosine phosphorylation, it consistently synergized with anti-Ig in inducing phosphorylation of the 72 kD substrate [Figure 20]. Thus, IL-4 alone at concentrations up to 100 U/ml variably stimulated low levels of phosphorylation of some of the substrates in the kinase assay in some experiments. However, in the presence of IL-4, a 10 fold lower dose of anti-Ig was required to optimally induce phosphorylation of the 72 kD species [Figure 20]. The synergy between IL-4 and anti-Ig appeared to be preferential for the 72 kD species, since other bands were not consistently enhanced. This finding is of particular interest because IL-4 along with anti-Ig is known to co-stimulate entry of B cells into the cell cycle (108).

D. Identification of the dominant 72 kD species as PTK72.

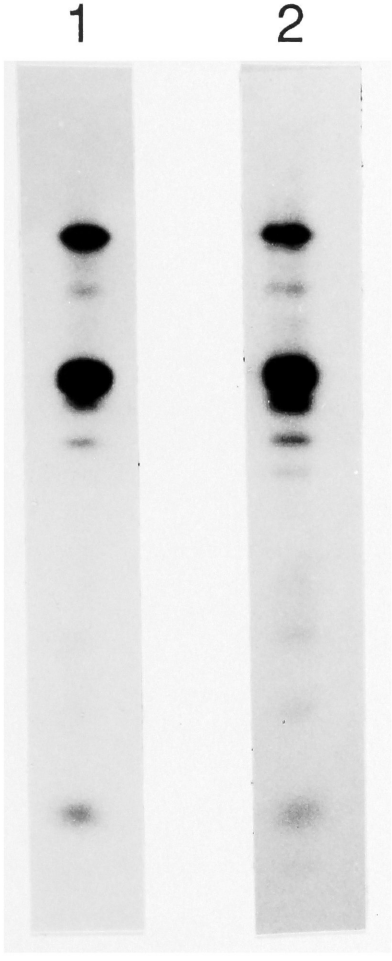
At the time we were performing these experiments, Dr Gaehlen and colleagues were independently studying a PTK of 72 kD, called PTK72. He originally purifed this enzyme as a catalytically active 40 kD protein from bovine thymus (238). He subsequently demonstrated that this kinase was a proteolytic fragment of a 72 kD molecule (239). To determine the relationship between the prominent 72 kD endogenous substrate in the anti-phosphotyrosine immune complex kinase

Figure 20. IL-4 synergizes with anti-Ig in the induction of the 72 kD band detected in the *in vitro* kinase assay. One x 10^7 high density B cells were stimulated on ice for 60 minutes with 0.25 μM F[ab']$_2$ RαM Ig, 100 U/ml IL-4 or 100 U/ml IL-4 plus the indicated amount [in μM] of F[ab']$_2$ fragments of RαM Ig. Cells were lysed in 1% NP-40 and immunoprecipitated with Rα phosphotyrosine. The immune complexes were assayed for kinase activity, resolved by SDS-PAGE, and analyzed by autoradiography. Molecular weight standards are indicated in kD.


No Add
RAM 0.25 μM
IL4
IL4 +
RAM (μM)
0.02
0.05
0.10
0.25
200
116
97
66
43
31
21

assay and PTK72, we immunoprecipitated lysates of anti-Ig activated B cells with anti-PTK72 [obtained from Dr. Gaehlen] or anti-phosphotyrosine. Phosphotransferase assays were performed on each of the resulting immune complexes and the products resolved by SDS-PAGE. The 72 kD species detected in each of these were recovered from the gel and digested with *Staph* V8 protease. The resulting peptides were resolved by SDS-PAGE [Figure 21]. Each of the phosphopeptides in the two digests co-migrated indicating that the 72 kD species detected in the anti-phosphotyrosine immune complex phosphotransferase assay was PTK72. This result is consistent with findings from several B cell lines including, L10A6.2, A20, BCL-1 and WEHI 231 that the 72 kD species that is tyrosine phosphorylated upon mIg ligation is PTK72 (237).

Figure 21. Identity of the 72 kD substrate as PTK72. High density B cells were stimulated with 0.25 μM of F[ab']$_2$ RαM Ig on ice for 1 hour. Cell were lysed in 1% NP-40 and lysates were immunoprecipitated with anti-PTK72 [lane 1] or anti-phosphotyrosine [lane 2]. The immune complexes were assayed for phosphotransferase activity and resolved by SDS-PAGE. The 72 kD species detected in each was recovered and digested with *Staph aureus* V8 protease. The resulting peptides were resolved on a 15% SDS-polyacrylamide gel and exposed to film.


1
2

CHAPTER 3

THE CLONING AND SEQUENCING OF HUMAN SYK

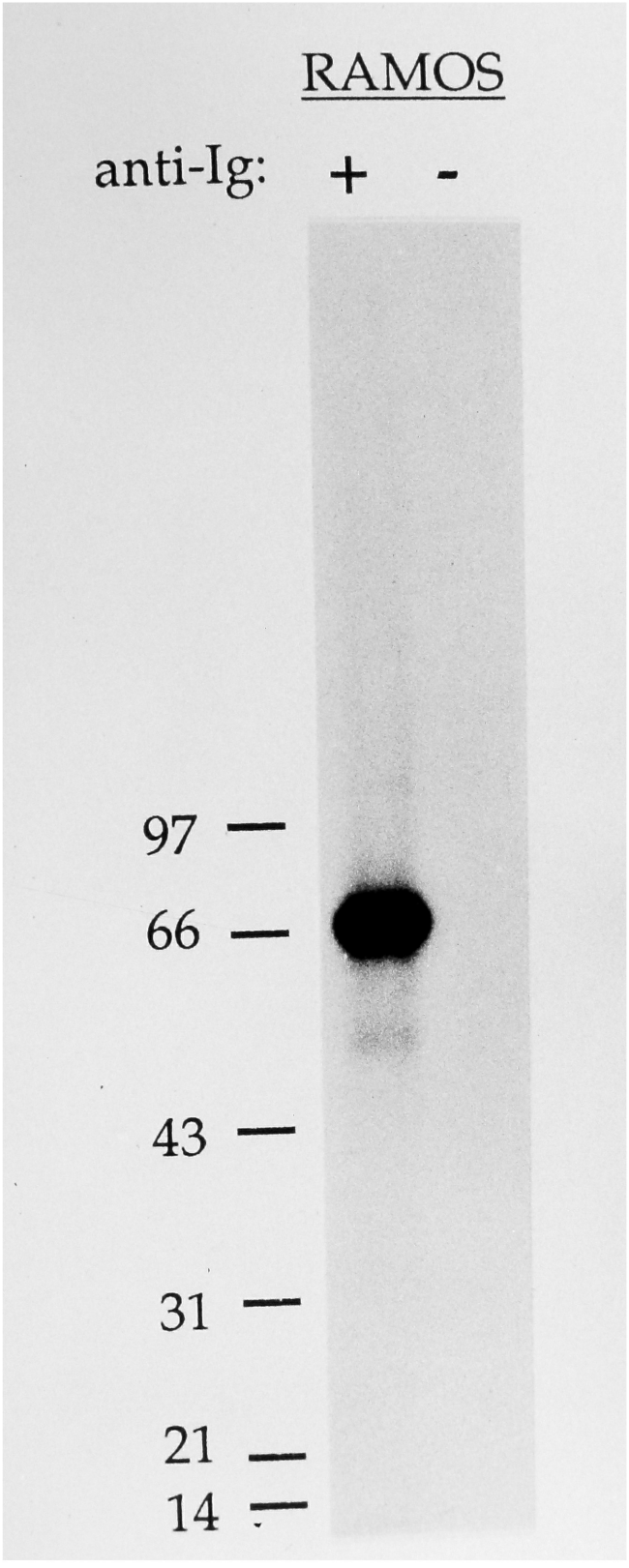
A. Identification of cell lines expressing high levels of PTK72.

The identification of PTK72 as a major substrate in the anti-phosphotyrosine immune complex kinase assay prompted us to isolate a cDNA clone of the gene that encodes for PTK72. Several human and mouse B cell lines were screened for high levels of phosphorylated PTK72 in the *in vitro* kinase assay. The human EBV^{neg} B cell lymphoma RAMOS was selected based on the high level of phosphorylation of the 72 kD species induced upon BCR crosslinking [Figure 22]. Using poly A+ RNA isolated from the RAMOS cell line, we constructed a cDNA library in the prokaryotic expression vector pCDNAII.

B. The cloning and sequencing of human *syk*

In an independent series of studies, Yamamura and colleagues had identified a 40 kD cytosolic PTK from porcine spleen (308). A cDNA encoding this protein was isolated based on partial amino acid sequence. The cDNA encoded a predicted 72 kD protein that resembled PTK72 based on at least three criteria, including apparent molecular weight, tissue

Figure 22. Anti-Ig induces high levels of phosphorylation of PTK72 in the *in vitro* kinase assay of the EBV^{neg} human B lymphoma cell line RAMOS. Five x 10^6 cells were stimulated with 0.25 μM of F[ab']$_2$ fragments of GαH Ig on ice for one hour. Cells were lysed in 1% NP-40 and lysates were immunoprecipitated with Rα phosphotyrosine. The immune complexes were assayed for kinase activity, resolved by SDS-PAGE and analyzed by autoradiography. Molecular weight standards are indicated in kD.


RAMOS
anti-Ig: + -
97
66
43
31
21
14

distribution, and susceptibility to cleavage, which yielded a 40 kD catalytically active fragment (245). This PTK was named *syk* for Spleen tYrosine Kinase. Gaehlen generated an anti-peptide antibody specific for the deduced amino acid sequence of *syk*. Using this antibody, he demonstrated that murine B cell PTK72 and porcine *syk* were homologous [personal communication].

To clone human *syk*/PTK72, we isolated a cDNA fragment of porcine *syk*. First strand cDNA was generated from pig spleen poly A+ RNA using reverse transcriptase. A fragment corresponding to nucleotides 934-1884 of porcine *syk* was specifically amplified using *syk*-specific primers in the polymerase chain reaction. This PCR product was cloned and used to screen the RAMOS library [cloning strategy summarized in Figure 23]. Several partial cDNA clones were isolated representing human *syk*. The identity of the clones was determined by sequencing and comparison with the published sequence of porcine *syk* (245). The longest clone corresponded to nucleotides 1188-2508 of porcine *syk*. The 5′ end of this clone [Mbo II-Xmn I fragment] was used to screen a human tonsillar library, λTon, and several more partial cDNA clones were isolated, the longest corresponding to nucleotides, 634-2508.

To clone the 5′ end of the gene, we utilized a procedure called 5′ RACE. Three nested anti-sense primers were generated corresponding to nucleotides 864-884, 895-915, and

Figure 23. Strategy for cloning human *syk*.

Cloning of Human syk

RT-PCR amplification of porcine syk
nucleotides 934-1884

↓

Screened a human Ramos B cell plasmid library

↓

Isolated cDNA clones of human syk
nucleotides 1188-2508

↓

Screened human tonsillar λ phage library

↓

Isolated cDNA clones of human syk
nucleotides 634-2508

↓

Utilizing 5' RACE, isolated a PCR product from Ramos mRNA
nucleotides -99-+915

↓

Screened a human tonsillar plasmid library

↓

Isolated cDNA clone of human syk
nucleotides -99-+1828

↓

Clones representing the 5' and 3' ends spliced together
BglII site

1132-1152. Primer 1132-1152 was utilized with reverse transcriptase to generate cDNA specific for the anti-sense strand of the 5′ half of *syk*. A poly dC tail was added to the 3′ of this cDNA using Tdt. Utilizing the *syk*-specific nested primers [895-915 and 864-884] as well as an anchor primer specific for the poly-dC tail, we generated a cDNA fragment of human *syk* in a PCR that corresponded to nucleotides -99-915. The 5′ end of this partial clone [Eco RI-Apa I] was used to screen the other human tonsillar library, paTon, and an overlapping cDNA fragment was isolated representing nucleotides -99 -1828. The overlapping 5′ [nucleotides -99-1828] and 3′ [nucleotides 634-2508] portions of the gene were spliced together at the Bgl II restriction enzyme site [nucleotide 1464] to generate a full length cDNA of human *syk*.

All of the cDNA clones isolated were sequenced on both the sense and antisense strand and a summary of the sequencing strategy is presented in Figure 24. The sequence revealed a clone of 2607 base pairs in length with an open reading frame of 1890 nucleotides [Figure 25]. The open reading frame, starting at the second ATG codon at the position designated +1 through 3 and terminating with a TAA codon at position 1891-1893, encoded a 630 amino acid polypeptide with a predicted molecular weight of 71,632 [Figure 26]. Human *syk* has a remarkable sequence homology with porcine *syk* with over 87% nucleotide identity and 94% amino acid identity [Figure 27]. The N- and C-terminal SH2 domains [see below] bear 92% and 95%

Figure 24. Strategy for sequencing human *syk*. All sequence reactions were carried out using the dideoxy sequencing method. [_____] reaction initiated using plasmid-specific oligonulceotide primers; [_____] reaction initiated using *syk*-specific oligonucleotide primers.

Sequencing Strategy of Human Syk

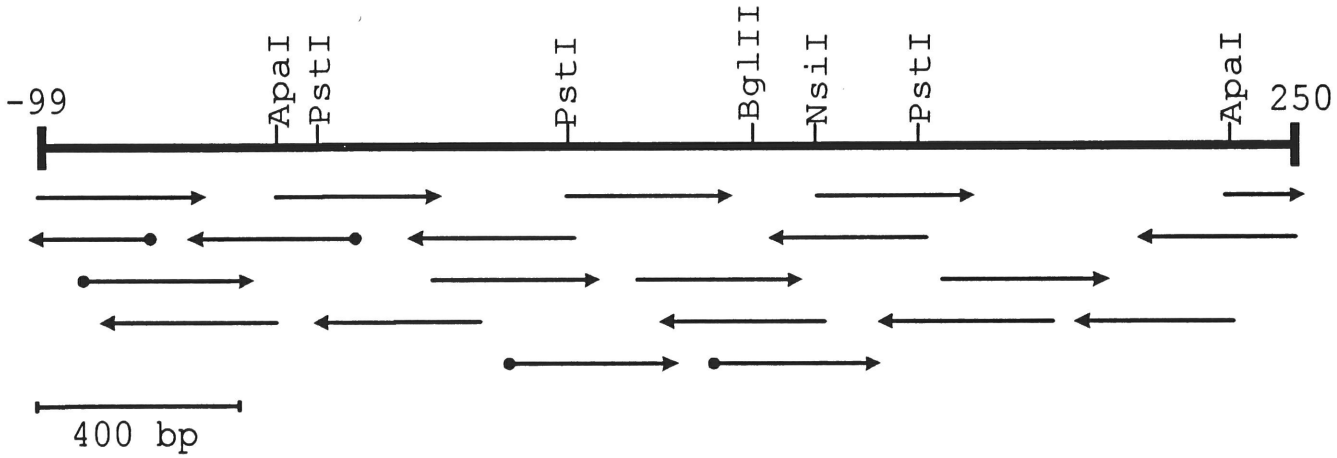


Figure 25. Nucleotide and deduced amino acid sequence of the full length human *syk* cDNA clone. The cloning and sequencing of the human *syk* cDNA clone were performed as described in the materials and methods and summarized in Figures 23 and 24. Predicted amino acids are numbered on the right and nucleotides on the left. The initiation codon is at position 1 and @ represents the stop codon. The 5′ untranslated region are indicated as negative numbers.

Nucleotide and Deduced Amino Acid Sequence of Human Syk

```
-99     CTAAAGGCG GGTGGCCCCG CTGCCCCGCC CTCGCCTCAC CTGGCGCAGG TGGACACATG GTGTGTGCCC TCCGGCCCCT GAAGCATGGC CAGCAGCGGC

  1 ATG GCT GAC AGC GCC AAC CAC CTG CCC TTC TTT TTC GGC AAC ATC ACC CGG GAG GAG GCA GAA GAT TAC CTG GTC CAG GGG GGC ATG
    M  A  D  S  A  N  H  L  P  F  F  F  G  N  I  T  R  E  E  A  E  D  Y  L  V  Q  G  G  M  29

 88 AGT GAT GGG CTT TAT TTG CTG CGC CAG AGC CGC AAC TAC CTG GGT GGC TTC GCC CTG TCC GTG GCC CAC GGG AGG AAG GCA CAC CAC
    S  D  G  L  Y  L  L  R  Q  S  R  N  Y  L  G  G  F  A  L  S  V  A  H  G  R  K  A  H  H  58

175 TAC ACC ATC GAG CGG GAG CTG AAT GGC ACC TAC GCC ATC GCC GGT GGC AGG ACC CAT GCC AGC CCC GCC GAC CTC TGC CAC TAC CAC
    Y  T  I  E  R  E  L  N  G  T  Y  A  I  A  G  G  R  T  H  A  S  P  A  D  L  C  H  Y  H  87

262 TCC CAG GAG TCT GAT GGC CTG GTC TGC CTC CTC AAG AAG CCC TTC AAC CGG CCC CAA GGG GTG CAG CCC AAG ACT GGG CCC TTT GAG
    S  Q  E  S  D  G  L  V  C  L  L  K  K  P  F  N  R  P  Q  G  V  Q  P  K  T  G  P  F  E  116

349 GAT TTG AAG GAA AAC CTC ATC AGG GAA TAT GTG AAG CAG ACA TGG AAC CTG CAG GGT CAG GCT CTG GAG CAG GCC ATC ATC AGT CAG
    D  L  K  E  N  L  I  R  E  Y  V  K  Q  T  W  N  L  Q  G  Q  A  L  E  Q  A  I  I  S  Q  145

436 AAG CCT CAG CTG GAG AAG CTG ATC GCT ACC ACA GCC CAT GAA AAA ATG CCT TGG TTC CAT GGA AAA ATC TCT CGG GAA GAA TCT GAG
    K  P  Q  L  E  K  L  I  A  T  T  A  H  E  K  M  P  W  F  H  G  K  I  S  R  E  E  S  E  174

523 CAA ATT GTC CTG ATA GGA TCA AAG ACA AAT GGA AAG TTC CTG ATC CGA GCC AGA GAC AAC AAC GGC TCC TAC GCC CTG TGC CTG CTG
    Q  I  V  L  I  G  S  K  T  N  G  K  F  L  I  R  A  R  D  N  N  G  S  Y  A  L  C  L  L  203

610 CAC GAA GGG AAG GTG CTG CAC TAT CGC ATC GAC AAA GAC AAG ACA GGG AAG CTC TCC ATC CCC GAG GGA AAG AAG TTC GAC ACG CTC
    H  E  G  K  V  L  H  Y  R  I  D  K  D  K  T  G  K  L  S  I  P  E  G  K  K  F  D  T  L  232

697 TGG CAG CTA GTC GAG CAT TAT TCT TAT AAA GCA GAT GGT TTG ITA AGA GTT CTT ACT GTC CCA TGT CAA AAA ATC GGC ACA CAG GGA
    W  Q  L  V  E  H  Y  S  Y  K  A  D  G  L  L  R  V  L  T  V  P  C  Q  K  I  G  T  Q  G  261

784 AAT GTT AAT TTT GGA GGC CGT CCA CAA CTT CCA GGT TCC CAT CCT GCG ACT TGG TCA GCG GGT GGA ATA ATC TCA AGA ATC AAA TCA
    N  V  N  F  G  G  R  P  Q  L  P  G  S  H  P  A  T  W  S  A  G  G  I  I  S  R  I  K  S  290

871 TAC TCC TTC CCA AAG CCT GGC CAC AGA AAG TCC TCC CCT GCC CAA GGG AAC CGG CAA GAG AGT ACT GTG TCA TTC AAT CCG TAT GAG
    Y  S  F  P  K  P  G  H  R  K  S  S  P  A  Q  G  N  R  Q  E  S  T  V  S  F  N  P  Y  E  319

958 CCA GAA CTT GCA CCC TGG GCT GCA GAC AAA GGC CCC CAG AGA GAA GCC CTA CCC ATG GAC ACA GAG GTG TAC GAG AGC CCC TAC GCG
    P  E  L  A  P  W  A  A  D  K  G  P  Q  R  E  A  L  P  M  D  T  E  V  Y  E  S  P  Y  A  348

1045 GAC CCC GAG GAG ATC AGG CCC AAG GAG GTT TAC CTG GAC CGA AAG CTG CTG ACG CTG GAA GAC AAA GAA CTG GGC TCT GGT AAT TTT
     D  P  E  E  I  R  P  K  E  V  Y  L  D  R  K  L  L  T  L  E  D  K  E  L  G  S  G  N  F  377

1132 GGA ACT GTG AAA AAG GGC TAC TAC CAA ATG AAA AAA GTT GTG AAA ACC GTG GCT GTG AAA ATA CTG AAA AAC GAG GCC AAT GAC CCC
     G  T  V  K  K  G  Y  Y  Q  M  K  K  V  V  K  T  V  A  V  K  I  L  K  N  E  A  N  D  P  406

1219 GCT CTT AAA GAT GAG TTA TTA GCA GAA GCA AAT GTC ATG CAG CAG CTG GAC AAC CCG TAC ATC GTG CGG ATG ATC GGG ATA TGC GAG
     A  L  K  D  E  L  L  A  E  A  N  V  M  Q  Q  L  D  N  P  Y  I  V  R  M  I  G  I  C  E  435

1306 GCC GAG TCC TGG ATG CTG GTT ATG GAG ATG GCA GAA CTT GGT CCC CTC AAT AAG TAT TTG CAG CAG AAC AGA CAT GTC AAG GAT AAG
     A  E  S  W  M  L  V  M  E  M  A  E  L  G  P  L  N  K  Y  L  Q  Q  N  R  H  V  K  D  K  464

1393 AAC ATC ATA GAA CTG GTT CAT CAG GTT TCC ATG GGC ATG AAG TAC TTG GAG GAG AGC AAT TTT GTG CAC AGA GAT CTG GCT GCA AGA
     N  I  I  E  L  V  H  Q  V  S  M  G  M  K  Y  L  E  E  S  N  F  V  H  R  D  L  A  A  R  493

1480 AAT GTG TTG CTA GTT ACC CAA CAT TAC GCC AAG ATC AGT GAT TTC GGA CTT TCC AAA GCA CTG CGT GCT GAT GAA AAC TAC TAC AAG
     N  V  L  L  V  T  Q  H  Y  A  K  I  S  D  F  G  L  S  K  A  L  R  A  D  E  N  Y  Y  K  522

1567 GCC CAG ACC CAT GGA AAG TGG CCT GTC AAG TGG TAC GCT CCG GAA TGC ATC AAC TAC TAC AAG TTC TCC AGC AAA AGC GAT GTC TGG
     A  Q  T  H  G  K  W  P  V  K  W  Y  A  P  E  C  I  N  Y  Y  K  F  S  S  K  S  D  V  W  551

1654 AGC TTT GGA GTG TTG ATG TGG GAA GCA TTC TCC TAT GGG CAG AAG CCA TAT CGA GGG ATG AAA GGA AGT GAA GTC ACC GCT ATG TTA
     S  F  G  V  L  M  W  E  A  F  S  Y  G  Q  K  P  Y  R  G  M  K  G  S  E  V  T  A  M  L  580

1741 GAG AAA GGA GAG CGG ATG GGG TGC CCT GCA GGG TGT CCA AGA GAG ATG TAC GAT CTC ATG AAT CTG TGC TGG ACA TAC GAT GTG GAA
     E  K  G  E  R  M  G  C  P  A  G  C  P  R  E  M  Y  D  L  M  N  L  C  W  T  Y  D  V  E  609

1828 AAC AGG CCC GGA TTC GCA GCA GTG GAA CTG CGG CTG CGC AAT TAC TAC TAT GAC GTG GTG AAC TAA
     N  R  P  G  F  A  A  V  E  L  R  L  R  N  Y  Y  Y  D  V  V  N  @                    630

1894    CCGCTCCCGC ACCTGTCGGT GGCTGCCTTT GATCACAGGA GCAATCACAG GAAAATGTAT CCAGAGGAAT TGATTGTCAG CCACCTCCCT CTGCCAGTCG

1994    GGAGAGCCAG GCTTGGATGG AACATGCCCA CAACTTGTCA CCCAAAGCCT GTCCCAGGAC TCACCCTCCA CAAAGCAAAG GCAGTCCCGG GAGAAAAGAC

2094    GGATGGCAGG ATCCAAGGGG CTAGCTGGAT TTGTTTGTTT TCTTGTCTGT GTGATTTTCA TACAGGTTAT TTTTACGATC TGTTTCCAAA TCCCTTTCAT

2194    GTCTTTCCAC TTCTCTGGGT CCCGGGGTGC ATTTGTTACT CATCGGGCCC AGGGACATTG CAGAGTGGCC TAGAGCACTC TCACCCCAAG CGGCCTTTTC

2294    CAAATGCCCA AGGATGCCTT AGCATGTGAC TCCTGAAGGG AAGGCAAAGG CAGAGGAATT TGGCTGCTTC TACGGCCATG AGACTGATCC CTGGCCACTG

2394    AAAAGCTTTC CTGACAATAA AAATGTTTTG AGGCTTTAAA AAGAAAATCA AGTTTGACCA GTGCAGTTTC TAAGCATGTA GCCAGTTAAG GAAAGAAAGA

2494    AAAAAAAAAA AAAAA
```

Figure 26. Deduced amino acid sequence of human syk. The predicted amino acid sequence was analyzed using the program "Prosite" [PC/Gene]. Protein sequence motifs are as indicated.

MADSANHLPFFFGNITREEAEDYLVQGGMS
DGLYLLRQSRNYLGGFALSVAHGRKAHHYT
IERELNGTYAIAGGRTHASPADLCHYHSQE
SDGLVCLLKKPFNRPQGVQPKTGPFEDLKE
NLIREYVKQTWNLQGQALEQAIISQKPQLE
KLIATTAHEKMPWFHGKISREESEQIVLIG
SKTNGKFLIRARDNNGSYALCLLHEGKVLH
YRIDKDKTGKLSIPEGKKFDTLWQLVEHYS
YKADGLLRVLTVPCQKIGTQGNVNFGGRPQ
LPGSHPATWSAGGIISRIKSYSFPKPGHRK
SSPAQGNRQESTVSFNPYEPELAPWAADKG
PQREALPMDTEVYESPYADPEEIRPKEVYL
DRKLLTLEDKELGSGNFGTVKKGYYQMKKV
VKTVAVKILKNEANDPALKDELLAEANVMQ
QLDNPYIVRMIGICEAESWMLVMEMAELGP
LNKYLQQNRHVKDKNIIELVHQVSMGMKYL
EESNFVHRDLAARNVLLVTQHYAKISDFGL
SKALRADENYYKAQTHGKWPVKWYAPECIN
YYKFSSKSDVWSFGVLMWEAFSYGQKPYRG
MKGSEVTAMLEKGERMGCPAGCPREMYDLM
NLCWTYDVENRPGFAAVELRLRNYYYDVVN

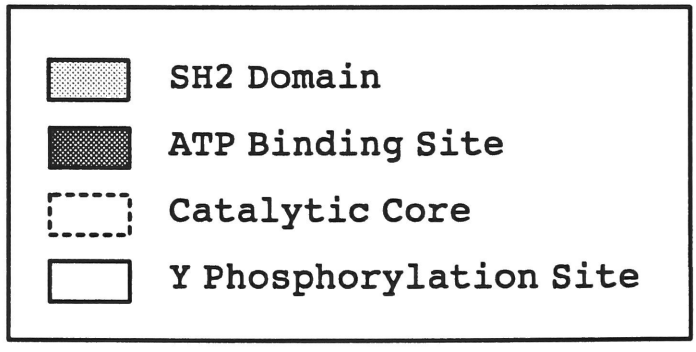
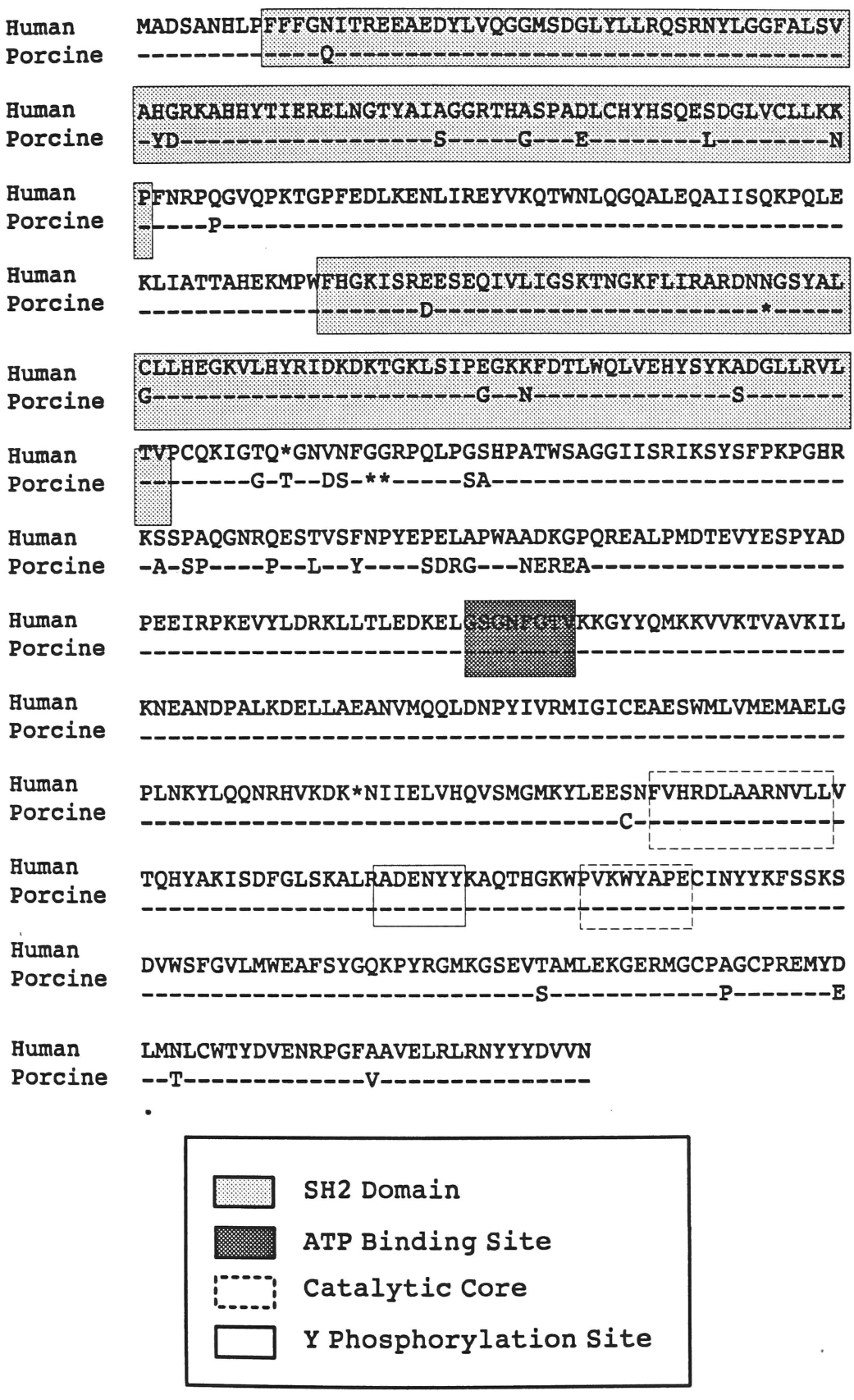


Figure 27. Comparison of the deduced amino acid sequences of human and porcine syk. The predicted amino acid sequence of human and porcine syk were aligned using the program "Pile-up" [GCG Sequence Analysis Software Package]. Protein sequence motifs and domains are as indicated. Dashes indicate sequence identity and * indicate gaps introduced into the sequence to maximize alignment.


Human
Porcine
MADSANHLPFFFGNITREEAEDYLVQGGMSDGLYLLRQSRNYLGGFALSV
Q
AHGRKAHHYTIERELNGTYAIAGGRTHASPADLCHYHSQESDGLVCLLKK
YD
S
G
E
L
N
PFNRPQGVQPKTGPFEDLKENLIREYVKQTWNLQGQALEQAIISQKPQLE
P
KLIATTAHEKMPWFHGKISREESEQIVLIGSKTNGKFLIRARDNNGSYAL
D
*
CLLHEGKVLHYRIDKDKTGKLSIPEGKKFDTLWQLVEHYSYKADGLLRVL
G
G
N
S
TVPCQKIGTQ*GNVNFGGRPQLPGSHPATWSAGGIISRIKSYSFPKPGHR
G-T--DS-**
SA
KSSPAQGNRQESTVSFNPYEPELAPWAADKGPQREALPMDTEVYESPYAD
-A-SP
P--L--Y
SDRG
NEREA
PEEIRPKEVYLDRKLLTLEDKELGSGNFGTVKKGYYQMKKVVKTVAVKIL
KNEANDPALKDELLAEANVMQQLDNPYIVRMIGICEAESWMLVMEMAELG
PLNKYLQQNRHVKDK*NIIELVHQVSMGMKYLEESNFVHRDLAARNVLLV
C
TQHYAKISDFGLSKALRADENYYKAQTHGKWPVKWYAPECINYYKFSSKS
DVWSFGVLMWEAFSYGQKPYRGMKGSEVTAMLEKGERMGCPAGCPREMYD
S
P
E
LMNLCWTYDVENRPGFAAVELRLRNYYYDVVN
T
V
SH2 Domain
ATP Binding Site
Catalytic Core
Y Phosphorylation Site

identity, respectively, while the kinase domain [see below] is almost 98% identical.

C. Domain structure of syk

An analysis of the deduced amino acid sequence with the program "Prosite" [PC/Gene], as well as a comparison of the cDNA sequence with the GenEMBL data base, revealed a marked homology with other known PTK. The deduced amino acid sequence contained two putative SH2 domains (175) and a C-terminal kinase domain [see Figure 26]. The amino acid sequence contained a conserved, glycine-rich motif G^{373}SGNFGTV that represents the putative ATP binding site (309,310). In addition, a downstream K^{397} probably functions in the phosphotransferase reaction (311). Syk also contains a pair of tyrosines within a predicted phosphorylation consensus sequence, RADENY^{520}Y^{521}, consistent with the highly conserved autophosphorylation target site[s] found in the src family PTK (312,313). There are also specific motifs within kinase domains that distinguish PTK from serine/threonine kinases (314). The deduced amino acid sequence of the kinase domain of syk contained motifs within its catalytic core [F^{485}VHRDLAARNVLL and P^{530}VKWYAPE] that were consistent with other tyrosine kinases.

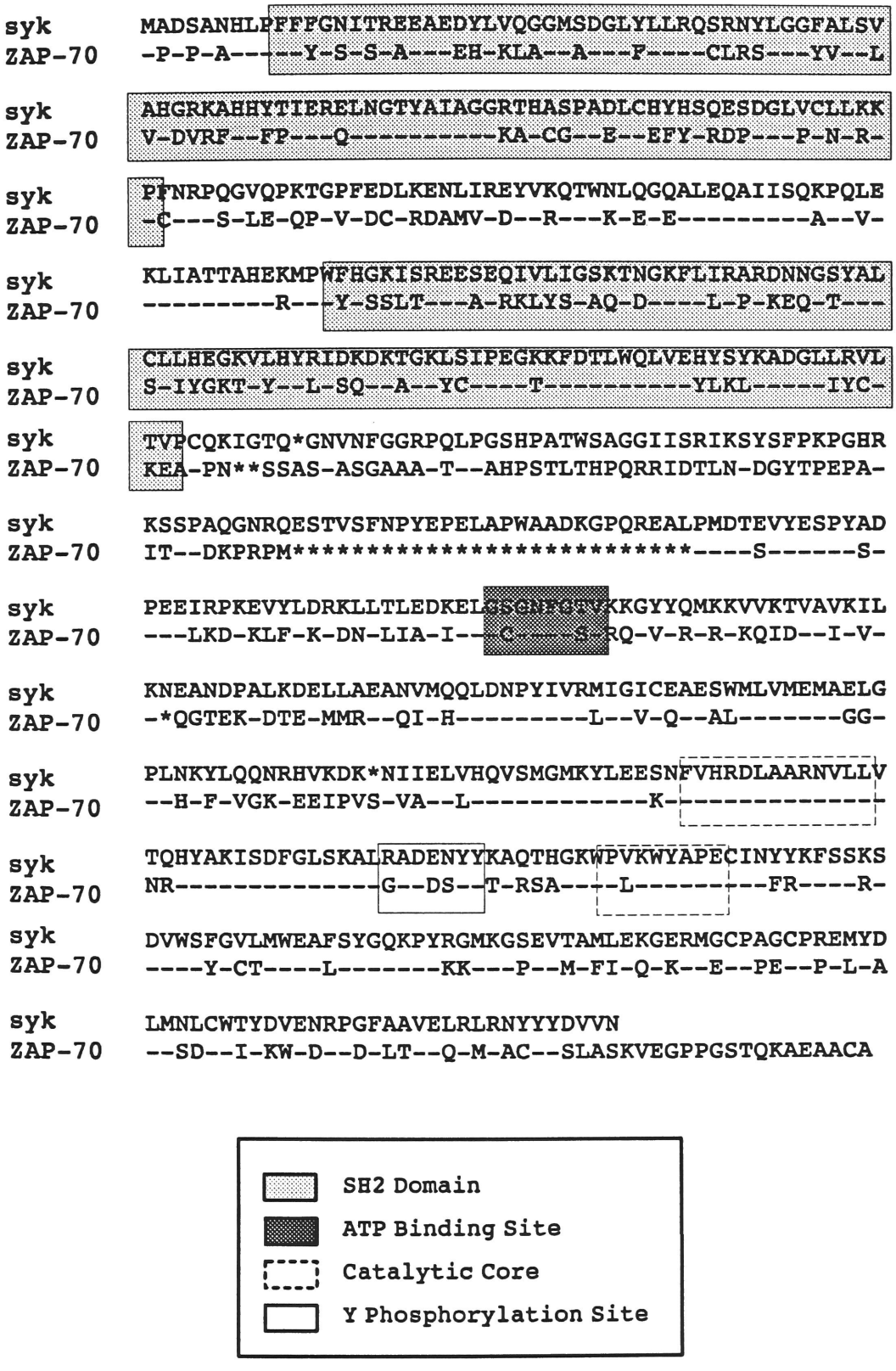
D. Syk and ZAP-70 define a new family of protein tyrosine

kinases.

Porcine and human *syk* have the greatest homology to the recently cloned, TCR-associated PTK, ZAP-70 (246) [Figure 28]. There is an overall amino acid identity of 53%, and 57%, 52%, and 62% amino acid identity in the N-terminal SH2, C-terminal SH2 and kinase domains, respectively. Syk and ZAP-70 differ significantly in two regions. The first is the protease sensitive region of syk between the C-terminal SH2 domain and the kinase domain where syk has a 26 amino acid insertion not found in ZAP-70. The second is a 17 amino acid C-terminal tail in ZAP-70. These regions may mediate specific interactions of the enzymes. However, the region between the C-terminal domain and the kinase domain of syk has the least homology between human and pig [Figure 27]. This lack of sequence conservation argues against a syk-specific role for this portion of the enzyme.

Syk and ZAP-70 are distinct from other previously defined families of receptor complex-associated PTK, including the src family. They lack both an N-myristoylation consensus motif as well as an SH3 domain, while possessing a second SH2 domain. In addition, all src family PTK have the sequence DLRAAD within the kinase catalytic core and this sequence varied in syk [D^{489}LAARN] and ZAP-70 [D^{461}LAARN] (314). The kinase domain sequences of syk and ZAP-70 were assessed for their "relatedness" to each other as well as other PTK, using

Figure 28. Comparison of the deduced amino acid sequence of human syk and human ZAP-70. The predicted amino acid sequence of human syk and ZAP-70 were aligned using the program "Pile-up" [GCG Sequence Analysis Software Package]. Protein sequence motifs and domains are as indicated. Dashes represent sequence identity and * indicate gaps introduced into the sequence to maximize alignment.


syk MADSANHLPFFFGNITREEAEDYLVQGGMSDGLYLLRQSRNYLGGFALSV
ZAP-70 -P-P-A-----Y-S-S-A---EH-KLA--A---F----CLRS---YV--L
syk AHGRKAHHYTIERELNGTYAIAGGRTHASPADLCHYHSQESDGLVCLLKK
ZAP-70 V-DVRF--FP---Q----------KA-CG--E--EFY-RDP---P-N-R-
syk PFNRPQGVQPKTGPFEDLKENLIREYVKQTWNLQGQALEQAIISQKPQLE
ZAP-70 -C---S-LE-QP-V-DC-RDAMV-D--R---K-E-E---------A--V-
syk KLIATTAHEKMPWFHGKISREESEQIVLIGSKTNGKFLIRARDNNGSYAL
ZAP-70 ---------R---Y-SSLT---A-RKLYS-AQ-D----L-P-KEQ-T---
syk CLLHEGKVLHYRIDKDKTGKLSIPEGKKFDTLWQLVEHYSYKADGLLRVL
ZAP-70 S-IYGKT-Y--L-SQ--A--YC----T----------YLKL-----IYC-
syk TVPCQKIGTQ*GNVNFGGRPQLPGSHPATWSAGGIISRIKSYSFPKPGHR
ZAP-70 KEA-PN**SSAS-ASGAAA-T--AHPSTLTHPQRRIDTLN-DGYTPEPA-
syk KSSPAQGNRQESTVSFNPYEPELAPWAADKGPQREALPMDTEVYESPYAD
ZAP-70 IT--DKPRPM****************************----S------S-
syk PEEIRPKEVYLDRKLLTLEDKELGSGNFGTVKKGYYQMKKVVKTVAVKIL
ZAP-70 ---LKD-KLF-K-DN-LIA-I---C----S-RQ-V-R-R-KQID--I-V-
syk KNEANDPALKDELLAEANVMQQLDNPYIVRMIGICEAESWMLVMEMAELG
ZAP-70 -*QGTEK-DTE-MMR--QI-H---------L--V-Q--AL-------GG-
syk PLNKYLQQNRHVKDK*NIIELVHQVSMGMKYLEESNFVHRDLAARNVLLV
ZAP-70 --H-F-VGK-EEIPVS-VA--L----------K-----------
syk TQHYAKISDFGLSKALRADENYYKAQTHGKWPVKWYAPECINYYKFSSKS
ZAP-70 NR----------G--DS--T-RSA---L-------FR----R-
syk DVWSFGVLMWEAFSYGQKPYRGMKGSEVTAMLEKGERMGCPAGCPREMYD
ZAP-70 ----Y-CT----L-------KK---P--M-FI-Q-K--E--PE--P-L-A
syk LMNLCWTYDVENRPGFAAVELRLRNYYYDVVN
ZAP-70 --SD--I-KW-D--D-LT--Q-M-AC--SLASKVEGPPGSTQKAEAACA
SH2 Domain
ATP Binding Site
Catalytic Core
Y Phosphorylation Site

the program "Pile-up" [GCG Sequence Analysis Software Package] [Figures 29 and 30]. According to the algorithms of this program, syk and ZAP-70 are more closely related to each other than to other PTK. Based on all these criteria, syk and ZAP-70 define a novel family of PTK distinct from both the src family and abl.

Both of the SH2 domains of syk contain the invariant arginines and histidine present in all SH2 domains that are required for the phosphotyrosine binding site [Figure 31] (175,315). The two SH2 domains have only 33% identity with each other. This contrasts with the greater than 50% homology that the N- and C-terminal SH2 domains of syk share with the respective N- and C-terminal domains of ZAP-70. However, the two SH2 domains are still more related to each other than to the SH2 domains of other PTK as well as vav [Figures 31 and 32].

However, syk and ZAP-70 have two features in common with src family PTK that are essential for the regulation of the latter. The first is a pair of tyrosines [$Y^{520}Y^{521}$, syk; $Y^{492}Y^{493}$, ZAP-70] within a predicted tyrosine phosphorylation consensus sequence which aligns with a tyrosine in src family PTK that upon phosphorylation upregulates kinase activity [Figure 29] (316). In addition, although not within a known tyrosine phosphorylation consensus sequence, syk and ZAP-70 contain tyrosine residues in tandem near the C-terminus [syk $Y^{624}Y^{625}Y^{626}$; and ZAP-70 $Y^{597}Y^{598}$].

Figure 29. Alignment of the catalytic domain of syk with the catalytic domain of other PTK. The kinase domains of the indicated molecules were aligned using the program "Pile-up" [GCG Sequence Analysis Software Package]. The protein sequence motifs were determined using the program Prosite [PC/Gene] and are as indicated. Dots indicate gaps introduced into the sequence to maximize alignment.

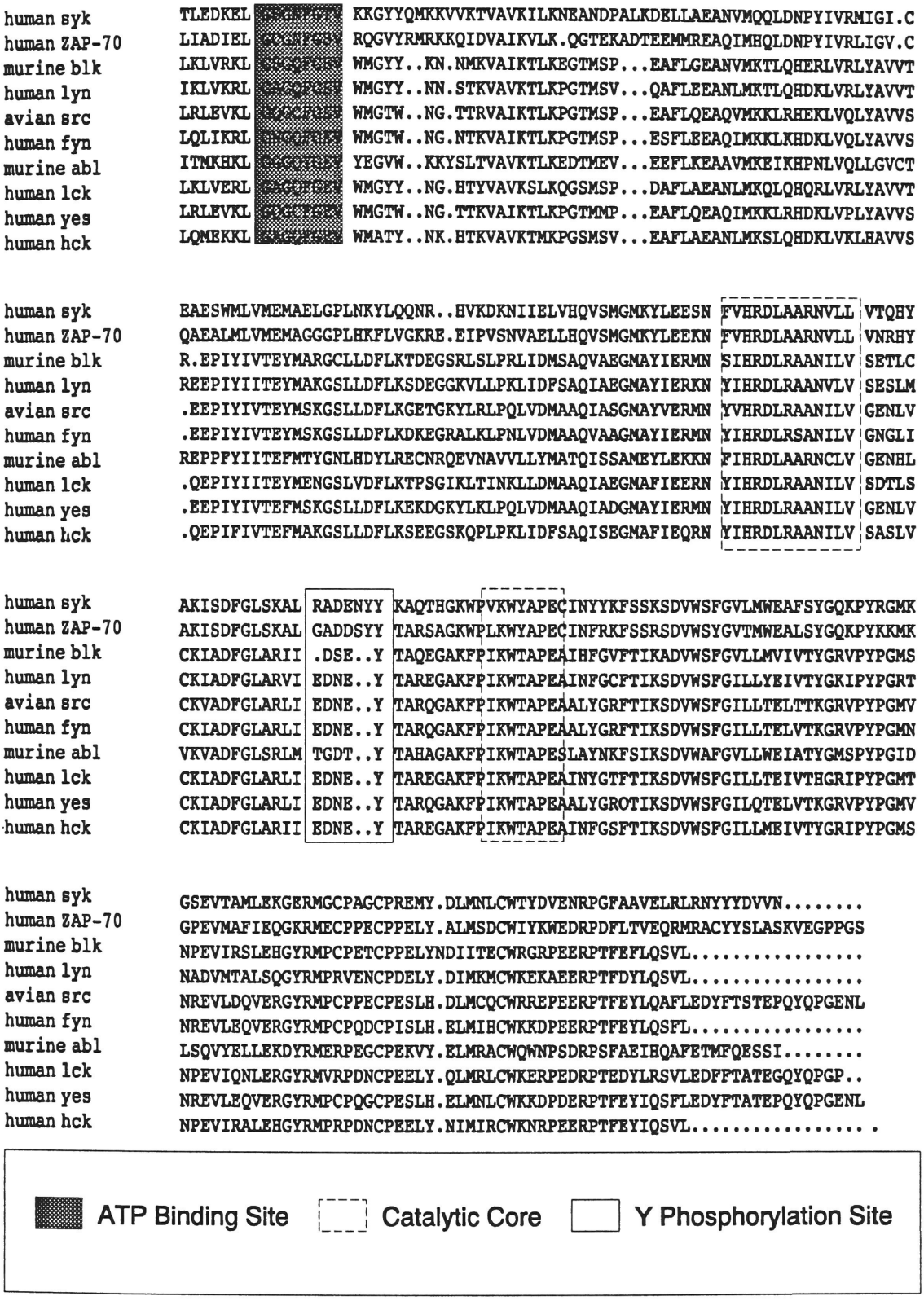

human syk
human ZAP-70
murine blk
human lyn
avian src
human fyn
murine abl
human lck
human yes
human hck
ATP Binding Site
Catalytic Core
Y Phosphorylation Site

Figure 30. Syk and ZAP-70 define a new family of PTK based on signature motifs in the kinase domains. Relatedness of kinase domains was determined using the program "Pile-up" [GCG Seqeunce Analysis Software Package] and displayed in tree format.

Kinase Domains

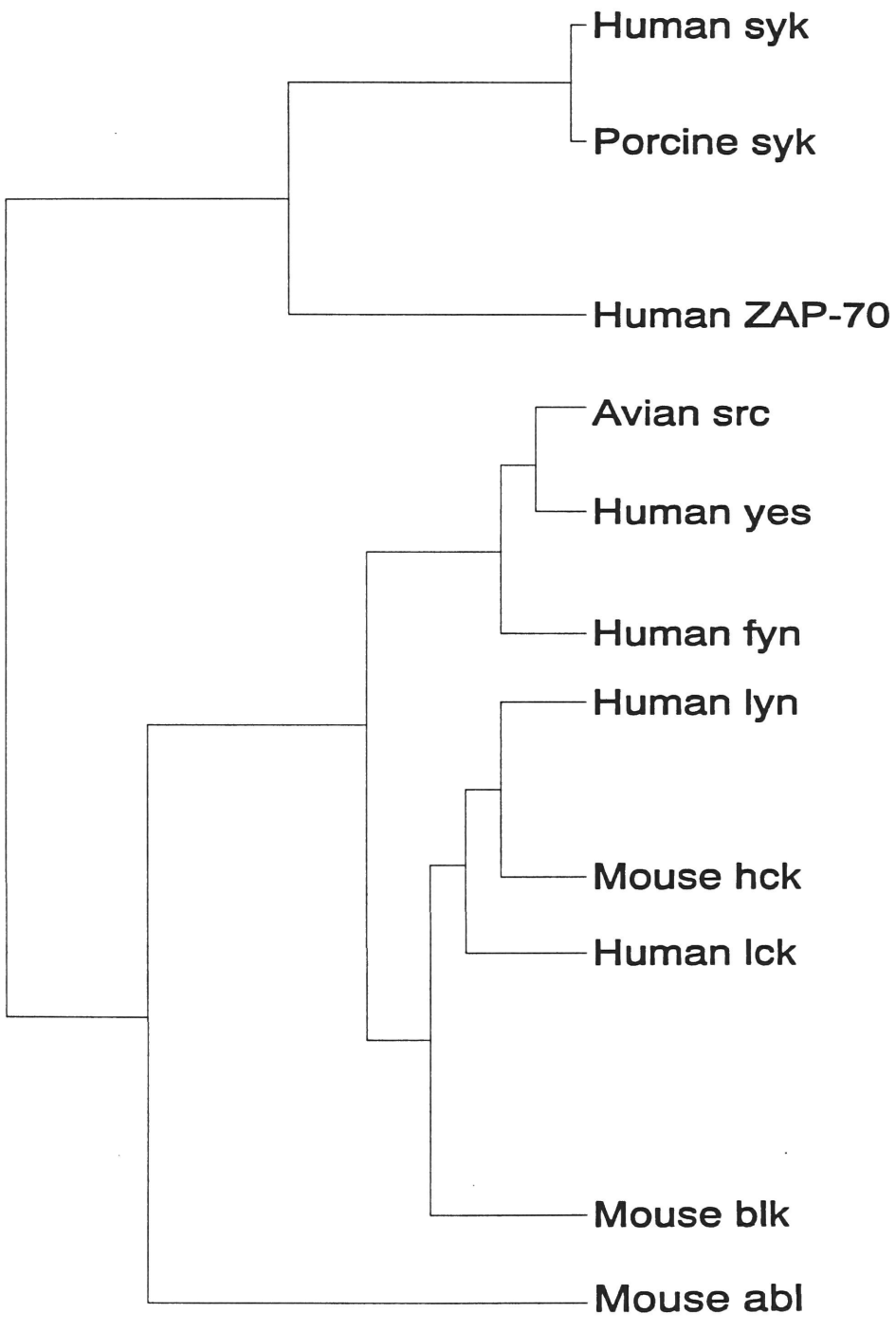
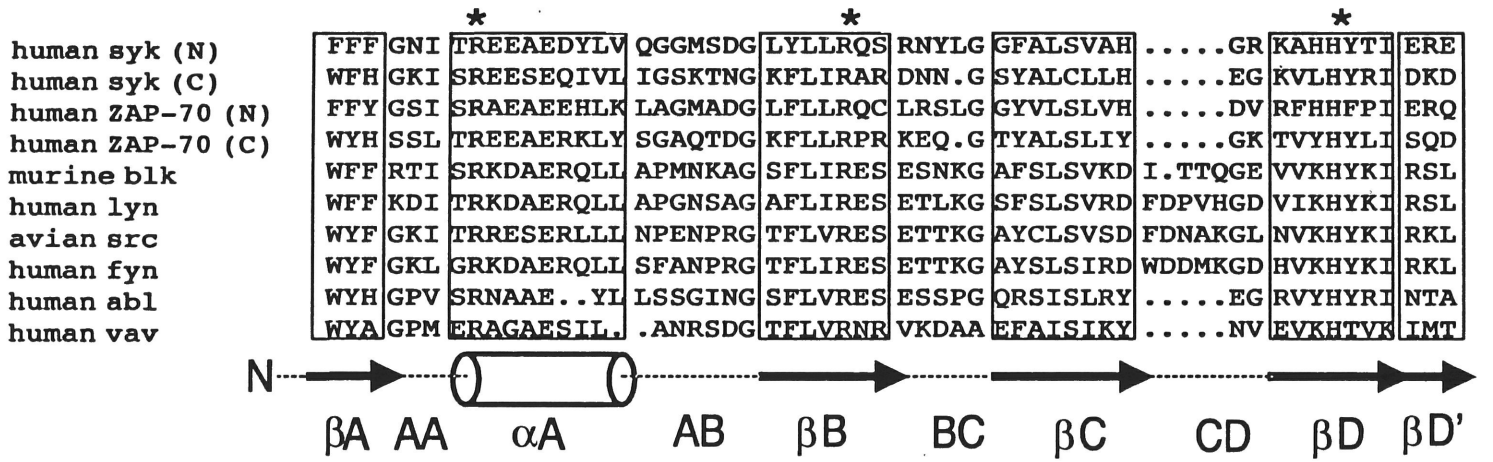


Figure 31. Alignment of the SH2 domains of syk with the SH2 domains of other PTK and vav. The SH2 domains of the indicated molecules were aligned using the program "Pile-up" [GCG Sequence Analysis Software Package]. The boundaries of the secondary structural regions as defined by Waksman, et al. (317) are as indicated, and the notation for these elements is shown schematically below. * represents conserved residues and dots indicate gaps introduced into the sequence to maximize alignment.


human syk (N)
human syk (C)
human ZAP-70 (N)
human ZAP-70 (C)
murine blk
human lyn
avian src
human fyn
human abl
human vav
FFF GNI TREEAEDYLV QGGMSDG LYLLRQS RNYLG GFALSVAH .....GR KAHHYTI ERE
WFH GKI SREESEQIVL IGSKTNG KFLIRAR DNN.G SYALCLLH .....EG KVLHYRI DKD
FFY GSI SRAEAEEHLK LAGMADG LFLLRQC LRSLG GYVLSLVH .....DV RFHHFPI ERQ
WYH SSL TREEAERKLY SGAQTDG KFLLRPR KEQ.G TYALSLIY .....GK TVYHYLI SQD
WFF RTI SRKDAERQLL APMNKAG SFLIRES ESNKG AFSLSVKD I.TTQGE VVKHYKI RSL
WFF KDI TRKDAERQLL APGNSAG AFLIRES ETLKG SFSLSVRD FDPVHGD VIKHYKI RSL
WYF GKI TRRESERLLL NPENPRG TFLVRES ETTKG AYCLSVSD FDNAKGL NVKHYKI RKL
WYF GKL GRKDAERQLL SFANPRG TFLIRES ETTKG AYSLSIRD WDDMKGD HVKHYKI RKL
WYH GPV SRNAAE..YL LSSGING SFLVRES ESSPG QRSISLRY .....EG RVYHYRI NTA
WYA GPM ERAGAESIL. .ANRSDG TFLVRNR VKDAA EFAISIKY .....NV EVKHTVK IMT
N
βA AA αA AB βB BC βC CD βD βD'

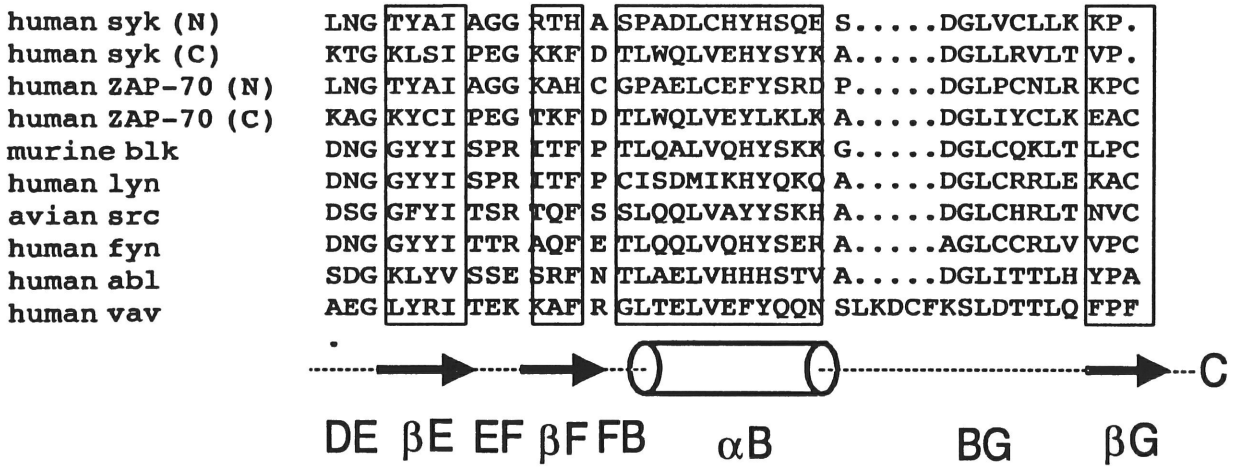
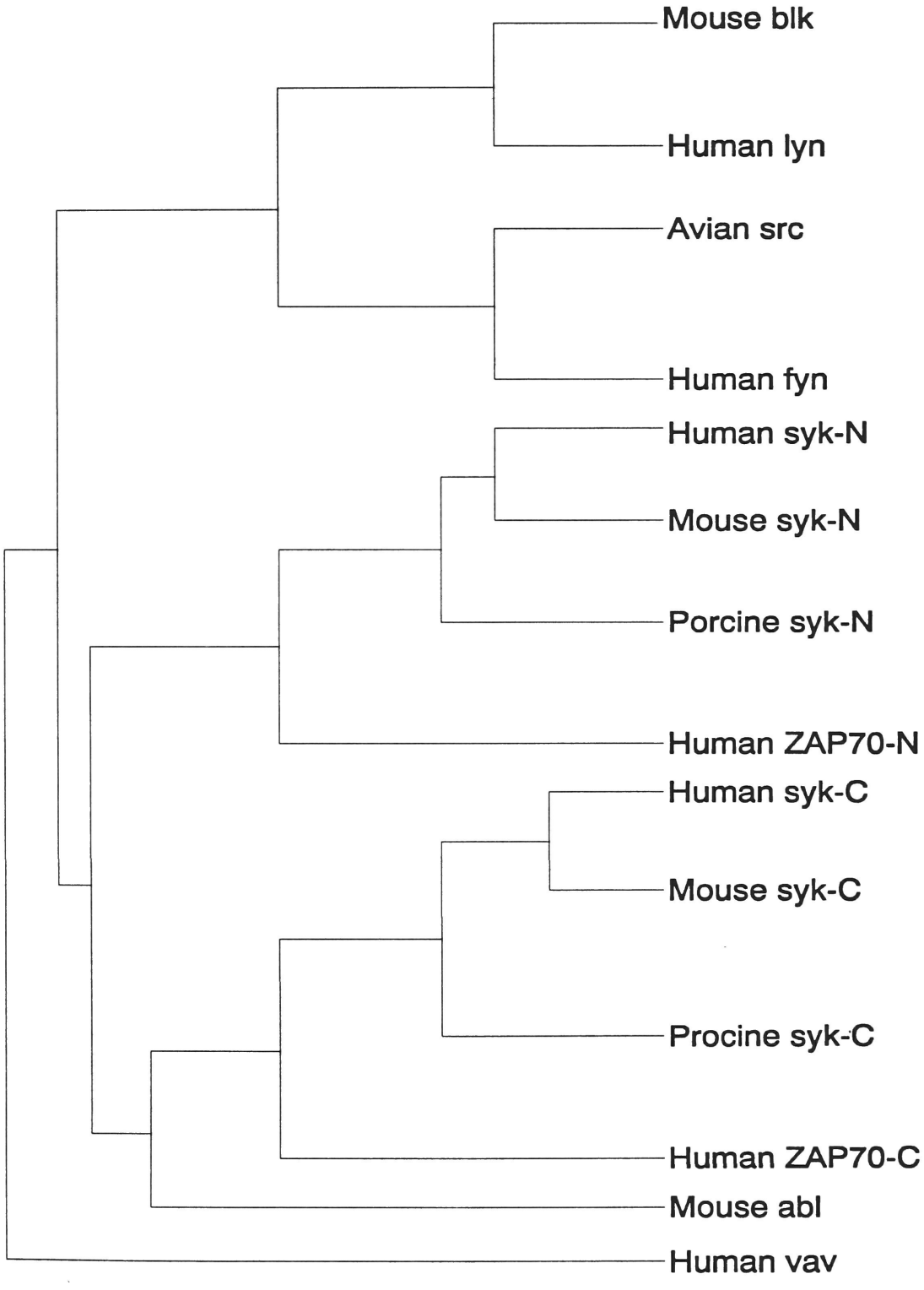

human syk (N)
human syk (C)
human ZAP-70 (N)
human ZAP-70 (C)
murine blk
human lyn
avian src
human fyn
human abl
human vav
LNG TYAI AGG RTH A SPADLCHYHSQE S.....DGLVCLLK KP.
KTG KLSI PEG KKF D TLWQLVEHYSYK A.....DGLLRVLT VP.
LNG TYAI AGG KAH C GPAELCEFYSRD P.....DGLPCNLR KPC
KAG KYCI PEG TKF D TLWQLVEYLKLK A.....DGLIYCLK EAC
DNG GYYI SPR ITF P TLQALVQHYSKK G.....DGLCQKLT LPC
DNG GYYI SPR ITF P CISDMIKHYQKQ A.....DGLCRRLE KAC
DSG GFYI TSR TQF S SLQQLVAYYSKH A.....DGLCHRLT NVC
DNG GYYI TTR AQF E TLQQLVQHYSER A.....AGLCCRLV VPC
SDG KLYV SSE SRF N TLAELVHHHSTV A.....DGLITTLH YPA
AEG LYRI TEK KAF R GLTELVEFYQQN SLKDCFKSLDTTLQ FPF
C
DE βE EF βF FB αB BG βG

Figure 32. Syk and ZAP-70 define a new family of PTK based on homologies of SH2 domains. Relatedness of SH2 domains was determined using the program "Pile-up" [GCG Sequence Analysis Software Package] and displayed in tree format.

SH2 Domains


Mouse blk
Human lyn
Avian src
Human fyn
Human syk-N
Mouse syk-N
Porcine syk-N
Human ZAP70-N
Human syk-C
Mouse syk-C
Procine syk-C
Human ZAP70-C
Mouse abl
Human vav

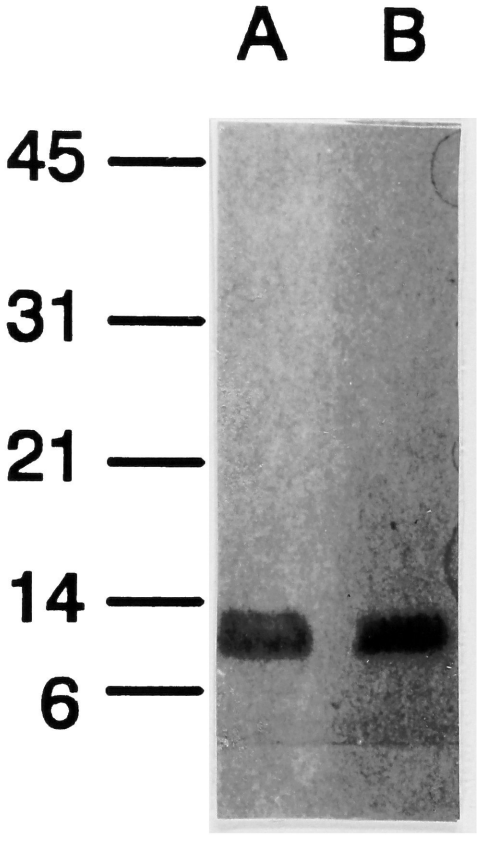
We have generated several mutations that are designed to elucidate the function and regulation of the enzymatic activity of syk. The first is a point mutation of K^{397}->R. Based on analogy to other PTK, this mutation should ablate the enzymatic activity of syk. To test our hypothesis regarding the regulation of syk, we mutated the putative positive and negative regulatory tyrosines of *syk* [$Y^{520}Y^{521}$->FF and Δ623] to phenylalanine. Finally, we engineered a N-myristoylation consensus sequence onto the N-terminus of syk in order to determine if membrane localization alters its cellular function.

E. Generation of syk-specific polyclonal antibodies.

Further characterization of syk required the availability of anti-syk antibodies. A fragment of the cDNA corresponding to the C-terminal SH2 domain of *syk* was subcloned in frame into the bacterial expression vector pD10 immediately following the codons for $[His]_6$ and transfected into the *E. Coli* strain, S9. Protein expression was induced with IPTG and the bacteria were lysed. The his-syk SH2C fusion protein was affinity purified on a nickel agarose column and dialyzed into PD/N_3. Protein purity was assessed by Coomassie staining following resolution by SDS-PAGE [Figure 33].

Rabbits were immunized subcutaneously with the fusion protein emulsified in CFA and boosted at one month intervals

Figure 33. Preparation of affinity purified his-syk fusion protein. S9 bacteria were transfected with the vector, pD10, containing cDNA encoding for the C-terminal SH2 domain of *syk*. Expression of the his-syk fusion protein was induced with IPTG for 3 hours. The bacteria were lysed in 6 M guanidine-HCl and the fusion protein was affinity purifed on a Ni-NTA-column [Qiagen]. The protein was subsequently dialyzed into PD/N_3 and 1 μg of protein assessed for purity by Coomassie staining of a 15% SDS-polyacrylamide gel. A and B represent two different preparations of the fusion protein.


A
B
45
31
21
14
6

with protein emulsified in IFA. Sera were tested for reactivity with the immunizing fusion protein by ELISA or immunoblot. The IgG fraction was purified by 50% ammonium sulfate precipitation followed by protein G affinity chromatography. The anti-syk antibody was affinity purified on a his-syk agarose column. The antibody was subsequently tested for reactivity with the fusion protein in an ELISA [data not shown] or with intact protein in an immunoblot against L10A6.2 lysates [Figure 34]. The cell line L10A6.2 is a murine B cell lymphoma and thus, the anti-human-syk antibody cross-reacts with murine syk.

Utilizing the affinity purified antibodies, we are currently studying the subcellular localization of syk in B cell lines during mIg patching, capping, and internalization. In addition, we are examining the effect of over expression of wild type and mutant syk [described above] in transiently and stably transfected cell lines. To date, we have been able to use this antibody to demonstrate the expression of enzymatically active recombinant wild type syk in Cos transfectants [Figure 35].

Figure 34. Anti-human syk SH2[C] antibody immunoblots murine syk. Five x 10^5 L10A6.2 cells were lysed in 1% NP-40. Lysates were resolved by SDS-PAGE, transferred to PVDF membranes and analyzed by immunoblotting with affinity-purified anti-syk antibody or a negative control [affinity-purified salmonella typhimurium O antigen-specific antibody]. Molecular weight standards are indicated in kD.

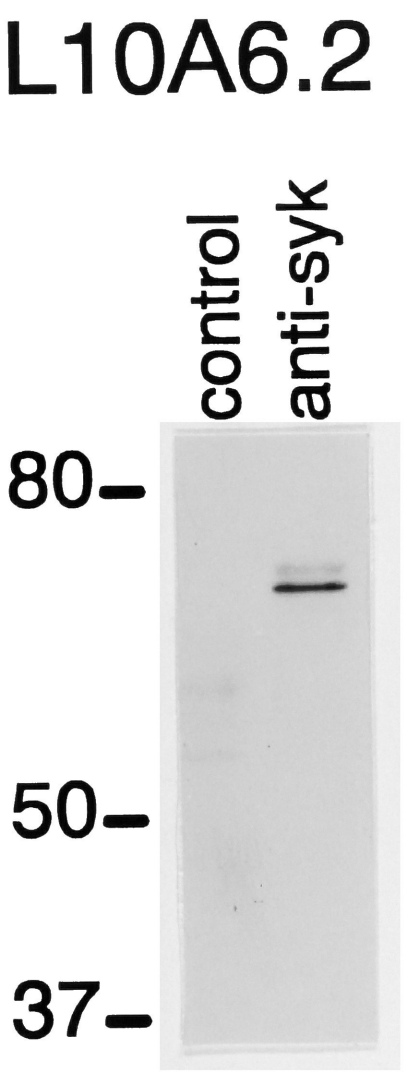
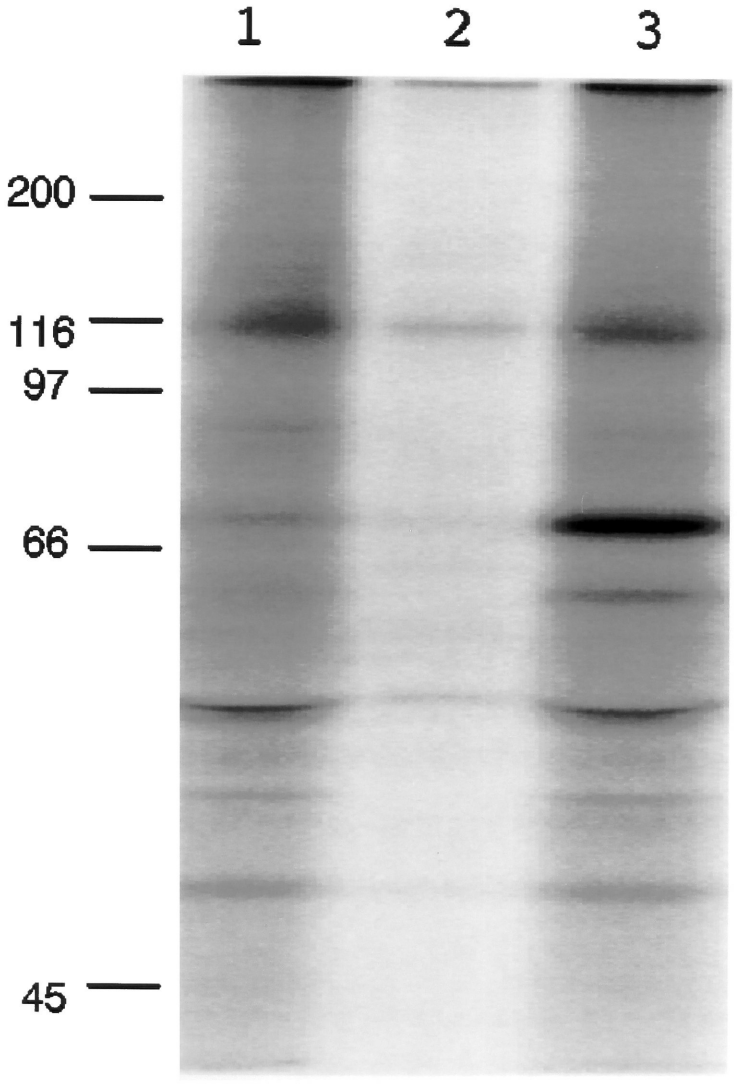

L10A6.2
control
anti-syk
80
50
37

Figure 35. Phosphotransferase assay of anti-syk precipitates of lysates from transiently transfected Cos-7 cells. Cos-7 cells were transiently transfected with pRC-CMV alone [lane 1] or containing insert encoding wild-type *syk* [lanes 2 and 3]. After 72 hours, the cells were harvested and lysed in 1% NP-40. Lysates were immunoprecipitated with either normal rabbit IgG [lane 2] or anti-syk SH2[C] [lane 1 and 3] antibody and the immune complexes were assayed for kinase activity. The immune complexes were resolved by SDS-PAGE and analyzed on a phosphoimager. Molecular weight standards are indicated in kD.


1
2
3
200
116
97
66
45

CHAPTER 4

A MODEL FOR THE THREE DIMENSIONAL STRUCTURE OF THE SH2 DOMAINS OF HUMAN SYK

SH2 domains mediate protein-protein interactions by virtue of their affinity for phosphorylated tyrosine residues by direct interaction with conserved amino acids (317,318). Specificity of these interactions is conferred by unique interactions with residues flanking the phosphorylated tyrosine (315). We set out to design a rational mutation analysis of the predicted phosphotyrosine binding site as well as to identify the amino acids that confer substrate specificity of each of the SH2 domains of syk. We therefore constructed a model of the three dimensional structure of these domains based on the known structure of the src SH2 domain (284).

The syk SH2 domains were aligned with the src SH2 domain using the program "Pile-up" [GCG Sequence Analysis Software Package] [Figure 31]. Using the known coordinates for the src SH2 domain, the amino acids were changed to those of syk, and "dummy" coordinates were entered where there were differenes. The new coordinates were subjected to energy minimization and molecular dynamics using the program "XPLOR" (285). Finally, the coordinates were tested for their probability using the program "Profile" (286,287). The N and C-terminal SH2 domains

had a total score [length dependent] of 45.99 and 36.25, and a Z score [length independent] of 30.10 and 28.78. A Z score of greater than 7 indicates a high probability that the coordinates are correct. In addition, the length dependent score was within the predicted range for proteins of its size. The profile window plots [Figure 36] also strongly supported a high probability that the coordinates were correct because the 3D-1D score, which represents the statistical preference for an amino acid at a give position, was consistently greater then 0.2. The 3D-1D score of the first few residues of the C-terminal domain dropped just below 0.2 indicating that the coordinates of these residues had a lower probability. This was not entirely unexpected because without the flanking sequences there were less constraints on the structure. However, at all times, the score remained greater than 0.

The carbon backbone of each of the SH2 domains of syk was compared to that of src [Figure 37, bottom panels]. The structures were remarkable for their conservation with only minor differences throughout the backbone. The one exception was in the "CD" loop which is present in the SH2 domains of the src family of PTKs (317). The functional consequences of this loop are unknown since it does not appear to interact with tyrosine phosphorylated peptides in the known structures.

The surfaces of the modeled SH2 domains were distinct from each other as well as from src [Figure 38]. In each of the SH2 domains there was a distinct pocket or groove which

Figure 36. Profile window plots of the predicted coordinates of the SH2 domains of human syk. The coordinates of the SH2 domains of human syk were modeled based on the known structure of src (284) as described in the Materials and Methods. The coordinates were tested for their probability using the program profile (286,287). Displayed are the profile window plots for each SH2 domain. The vertical axis gives the average 3D-1D score for residues in a 21-amino acid sliding window, the center of which is at the sequence position indicated by the horizontal axis. The 3D-1D score represents the statistical preference for the amino acid at a given position.

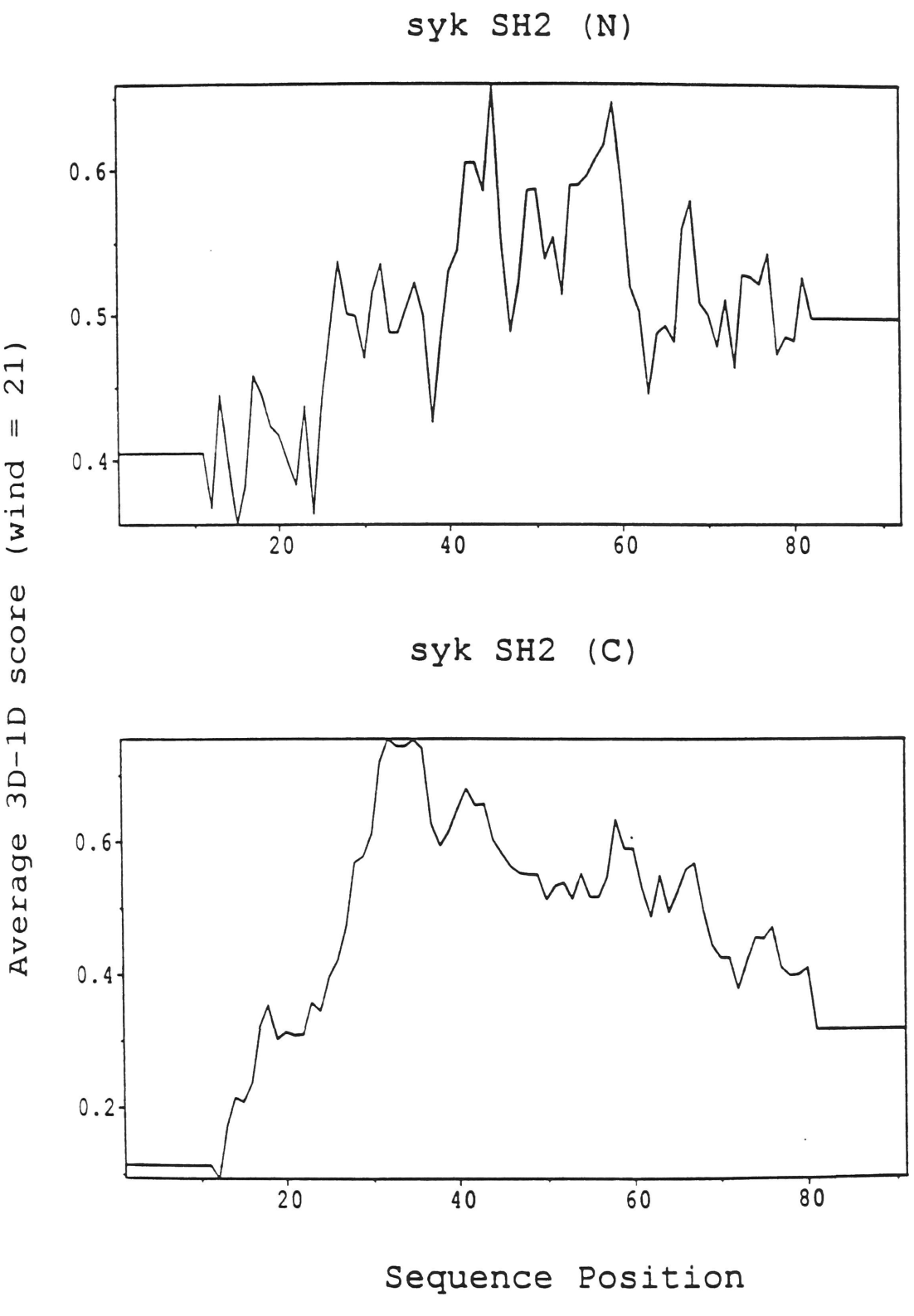

syk SH2 (N)
0.6
0.5
0.4
20
40
60
80
syk SH2 (C)
0.6
0.4
0.2
20
40
60
80
Average 3D-1D score (wind = 21)
Sequence Position

Figure 37. Carbon backbones of the predicted 3-dimensional structure of the SH2 domains of human syk. The predicted coordinates of SH2 domains were displayed using the program "Grasp" (288). Top Row. The carbon backbones of the N-terminal [left,white] and C-terminal [right,blue] SH2 domains. The residues predicted to interact with phosphotyrosine bound peptide are shown in color as follows: green - specificity binding pocket; yellow - phosphotyrosine binding pocket; red - conserved residues that are being mutated to assess function [i.e. αA2 [R^{17},R^{170}] and βD5 [Y^{59},Y^{211}]] or residues that may distinguish specificity of the SH2 domain of syk from that of src [i.e. βD3 [H^{57},L^{209}] and βD'1[E^{61},D^{213}]]. Bottom Row. The carbon backbones of the N-terminal [left,white] and C-terminal [right, blue] SH2 domains of syk are compared to the carbon backbone of src [red]. The CD loop that is specific to src family PTK is evident at the top of the figures.

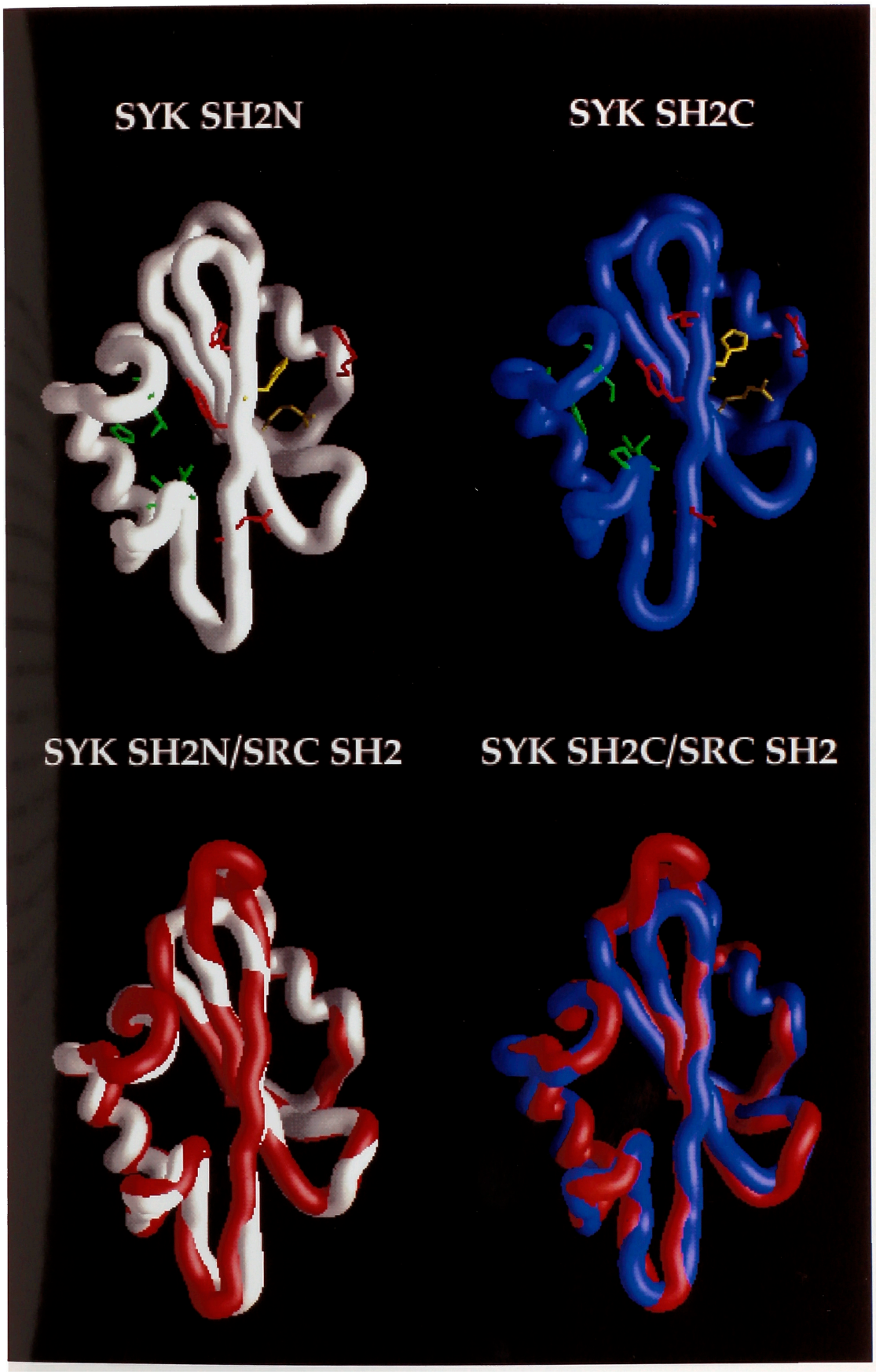
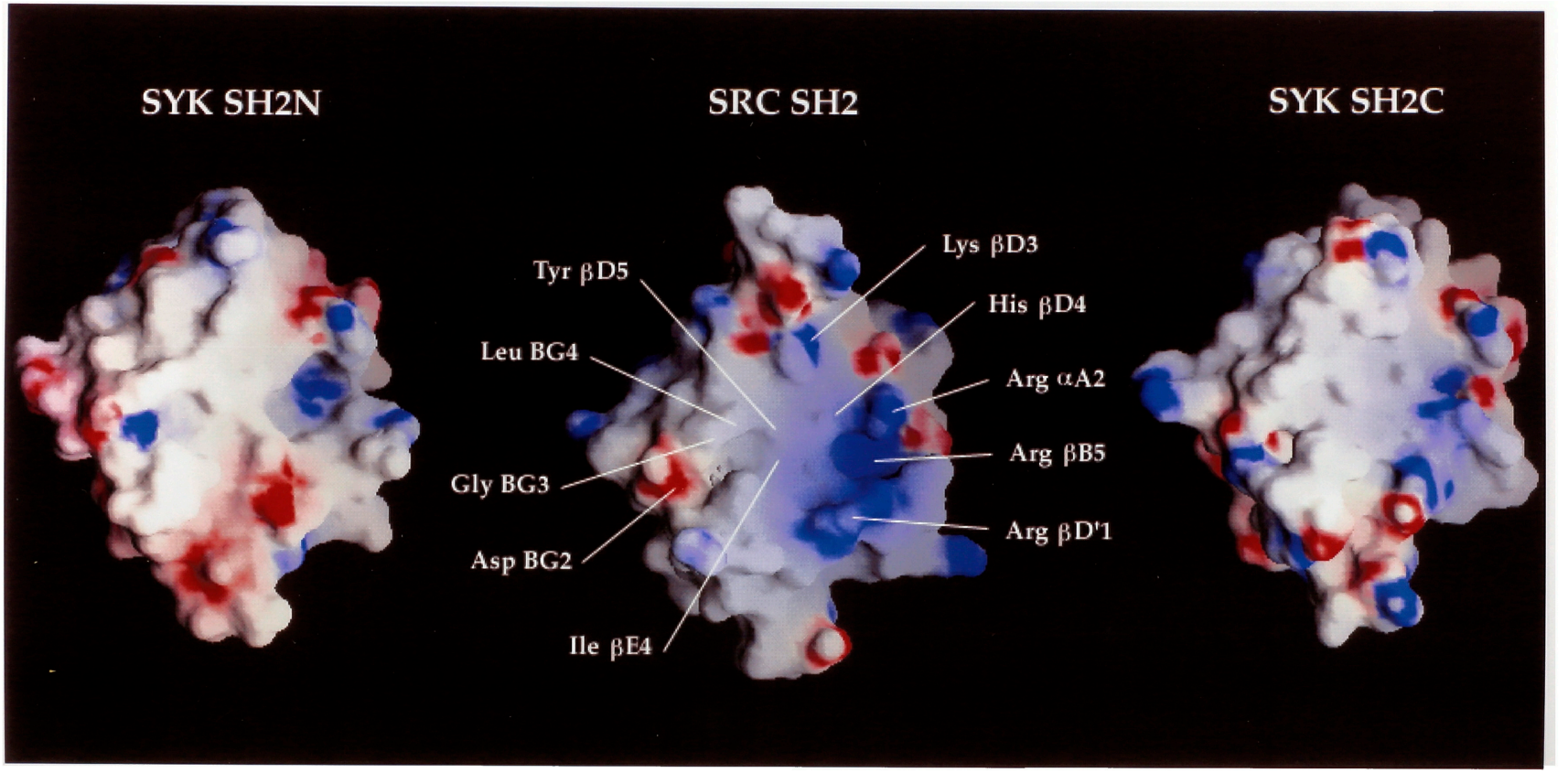

SYK SH2N
SYK SH2C
SYK SH2N/SRC SH2
SYK SH2C/SRC SH2

Figure 38. Surfaces of the predicted SH2 domains of syk. The predicted SH2 domains of the N-terminal [left] and C-terminal [right] SH2 domains of syk and the src SH2 domain [center] (284) are displayed using the program Grasp (288). Acidic residues are depicted in red and basic residues are depicted in blue. The indicated amino acids on the src structure are those known to interact with bound tyrosine phosphorylated peptide (317).


SYK SH2N
SRC SH2
SYK SH2C
Tyr βD5
Leu BG4
Gly BG3
Asp BG2
Ile βE4
Lys βD3
His βD4
Arg αA2
Arg βB5
Arg βD'1

was lined by the conserved basic residues [arginine αA2, arginine βB5, and histidine βD4] of the predicted phosphotyrosine binding domain. However, the regions of the domain felt to impart specificity appeared quite distinct [Figure 37 and 38; residues highlighted in green or red]. In the src SH2 domain, the residue βD'1 is the basic amino acid arginine [highly conserved within the src family of PTK], and this residue is important in the interaction with a glutamic acid residue immediately following the phosphotyrosine residue [Y^{PO4} + 1] (317). In the N and C-terminal SH2 domains of syk there was an acidic amino acid residue at this position [glutamic acid and aspartic acid, respectively] which dramatically altered the surface charge of the molecule. It was predicted that SH2 domains of syk would bind a similar tyrosine phosphorylated protein as src (315) but based on at least this one difference, this seem unlikely. In addition, in src, the lysine at position βD3 [also conserved in the src family of PTK] interacts with a glutamic acid at position Y^{PO4} + 2. In syk, the βD3 residues were histidine and leucine [N- and C-terminal SH2, respectively] and these were non-conservative substitutions which would likely have different interactions from src as well as each other.

The third amino acid following the phosphorylated tyrosine residue is also important for determining the SH2 domains with which a particular substrate will interact. Src has specificity for isoleucine at this amino acid while PI-3

kinase prefers methionine (315). In both cases, this specificity is mediated by a hydrophobic groove adjacent to the phosphotyrosine binding pocket [(284,317-320) and Figure 38]. This hydrophobic pocket is conserved in the SH2 domains of src and syk with mostly identical amino acids constituting the binding pocket [Figure 37, highlighted amino acids]. However, the groove was not as well formed in the binding pockets of the syk SH2 domains. This may reflect a difference in amino acid specificity of the domain [i.e. a shorter side chain], or conversely, may simply represent limitations of the model.

Based on this data, we are mutating several amino acids that we predict may impart specificity of the SH2 domains using site-directed mutagenesis. The conserved arginine αA2 in both SH2 domains is being mutated to glutamine. By removing the positively charged amino acid from the phosphotyrosine binding pocket, the ability to bind to tyrosine phosphorylated proteins will probably be ablated. In addition, we are mutating the tyrosine ßD5 to glutamine. In each case, the mutation should either block substrate binding and/or alternatively, alter the specificity of the pocket. Finally, the residues that we predict determine the specificity of the SH2 domains of syk relative to src are being mutated [SH2-N, ßD3 H->K and ßD'1 E->K; SH2-C, ßD3 L->S and ßD'1 D->K]. These mutations should also significantly alter or ablate the specificity of the SH2 domains. By

blocking the function or altering substrate specificity we should gain insight into the role of syk in B cell function.

DISCUSSION

During the activation and differentiation of B cells, there are critical stages of regulation that determine the fate of the cell. A resting B cell is initially stimulated by antigen or T cells through antigen-specific cell surface receptors [BCR and class II MHC, respectively]. Depending on the context, as well as timing, of this signal, the B cell is activated, becomes tolerant, or is induced to undergo programmed cell death [i.e. apoptosis]. The various fates of the B cell are probably related to differences in the receptor-associated signaling pathways that are activated.

If the initial signal productively activates the B cell, it undergoes blast transformation. If the B lymphoblast does not receive a subsequent signal, it will probably die. However, during this time the B lymphoblast has acquired enhanced responsiveness to antigen independent signals, i.e. cytokines. The array of cytokines with which the B cell interacts, as well as further T cell contact-dependent stimulation, will determine one of several fates including proliferation, or differentiation into antibody secreting cells [plasma cells] or memory cells. The Ig genes of B cells at this blast stage are a target of somatic mutation and cytokine directed isotype switching. We studied several aspects of each of these critical steps.

A. Tyrosine phosphorylation and mIg-mediated signaling.

Membrane Ig mediated stimulation of B cell growth and Ig secretion requires receptor crosslinking (302-304). The initial event observed following ligation of mIg is increased tyrosine phosphorylation of numerous proteins (208-210,321). We have established that receptor crosslinking is required for the accumulation of tyrosine phosphorylated proteins. Receptor crosslinking is also required for the induced activity of at least one kinase in anti-phosphotyrosine immune complexes that phosphorylates several endogenous substrates. This increase in kinase activity probably represents precipitation of greater quantities of tyrosine phosphorylated proteins with phosphotransferase activity as well as an increase in the precipitated amount of kinase substrate. The increase in kinase activity may also represent an increase in the activity of the precipitated kinase[s].

Ligation of mIg induces the phosphorylation and kinase activity of several src family PTK (223-225). Moreover, anti-Ig induces these PTK to associate with the BCR complex (223-225). These include lyn (227) and blk (226), which are preferentially expressed in B cells, as well as fyn and lck. In addition, the PTK syk is phosphorylated and associates with the BCR upon mIg ligation (240,241). We demonstrated that syk is one of the major endogenous substrates of the anti-Ig induced kinase activity, and this activity is absolutely

dependent on crosslinking of the receptor.

Syk is related to a cytosolic PTK preferentially expressed in T cells, ZAP-70 (246) which associates with members of the CD3 complex of the TCR (322). These two enzymes define a novel family of cytosolic PTK with several features that distinguish them from members of the src family of PTK [Figure 39] . Syk and ZAP-70 each have two SH2 domains and no SH3 domains. In addition, they do not have an N-myristoylation consensus sequence at the N-terminus (289). Lastly, the sequence of the kinase catalytic domain motifs are distinct from that of src family members (314).

The means by which PTK associate with receptor complexes without intrinsic enzymatic activity are beginning to be elucidated. The BCR (156-160) and TCR (323), as well as $Fc_{\epsilon}R_{I}$ (324) and $Fc_{\gamma}R_{III}$ (325) are each associated with molecules which contain the ARH-1 motif (170) [Figure 40]. Upon tyrosine phosphorylation, this motif is capable of binding to SH2 domains including those in PTK (179,228). This motif is also sufficient to couple chimeric receptors to downstream signaling events (172-174). Interestingly, this motif was usurped by two viruses, bovine leukemia virus and Epstein-Barr virus, that are capable of transforming B cells (171).

Numerous substrates of the induced PTK have been identified, although the specific enzymes that mediate the phosphorylation of these substrates are unknown. The Ig-associated molecules Ig_{α} and Ig_{β} are rapidly phosphorylated

Figure 39. Comparison of the domain structures of the syk/ZAP-70 and the src families of PTK.

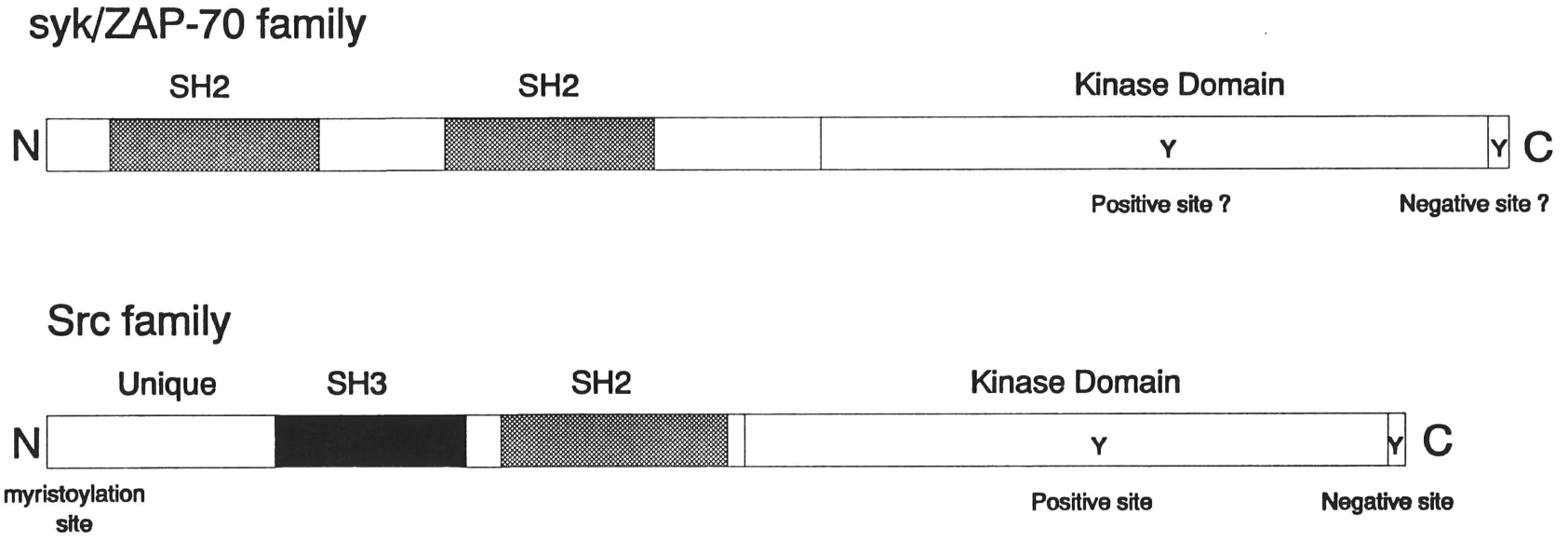
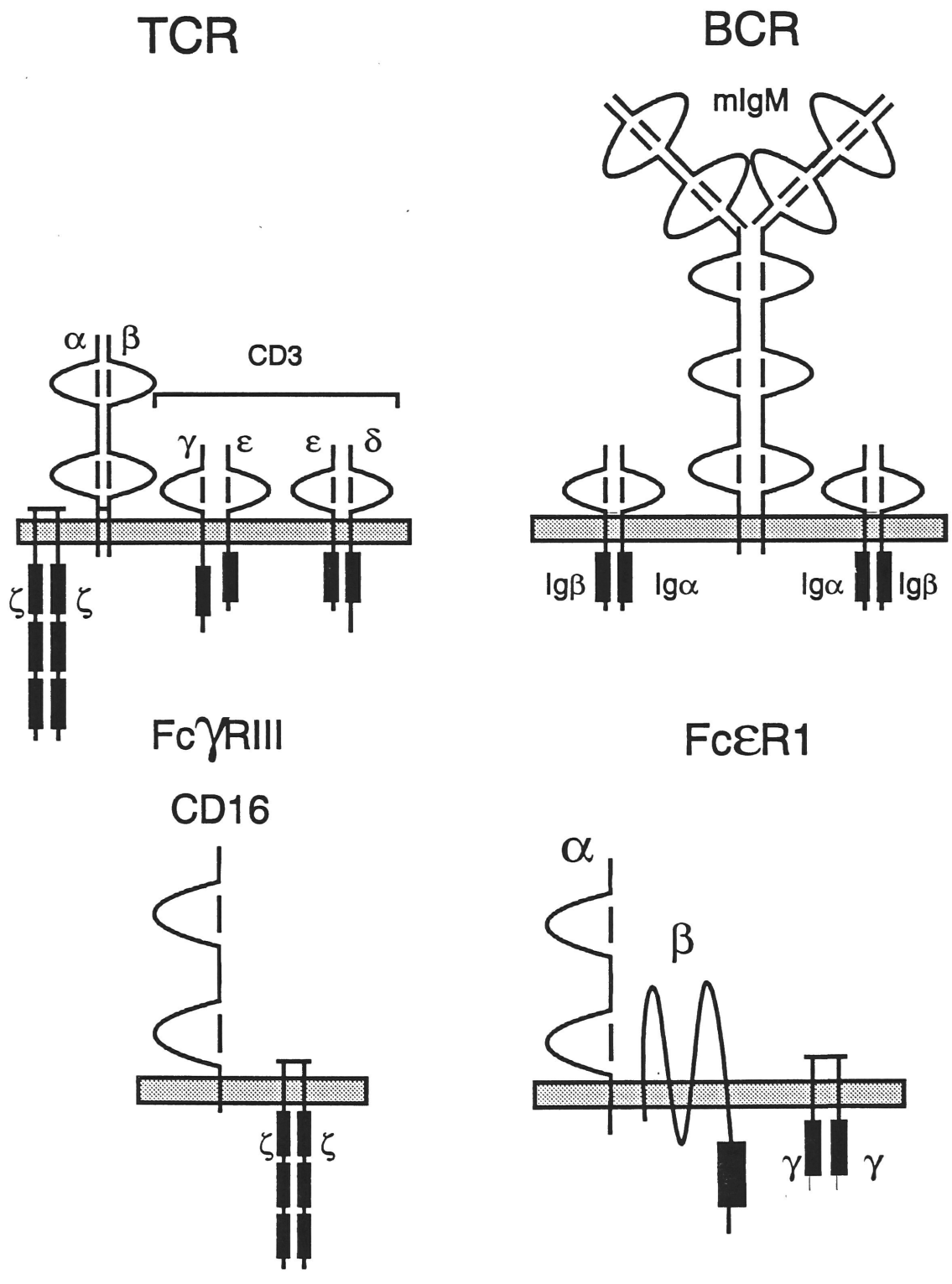

syk/ZAP-70 family
SH2
SH2
Kinase Domain
N
Y
Y
C
Positive site ?
Negative site ?
Src family
Unique
SH3
SH2
Kinase Domain
N
Y
Y
C
myristoylation site
Positive site
Negative site

Figure 40. Hematopoietic cell antigen receptor complexes. ARH-1 motifs are shown as black rectangles.

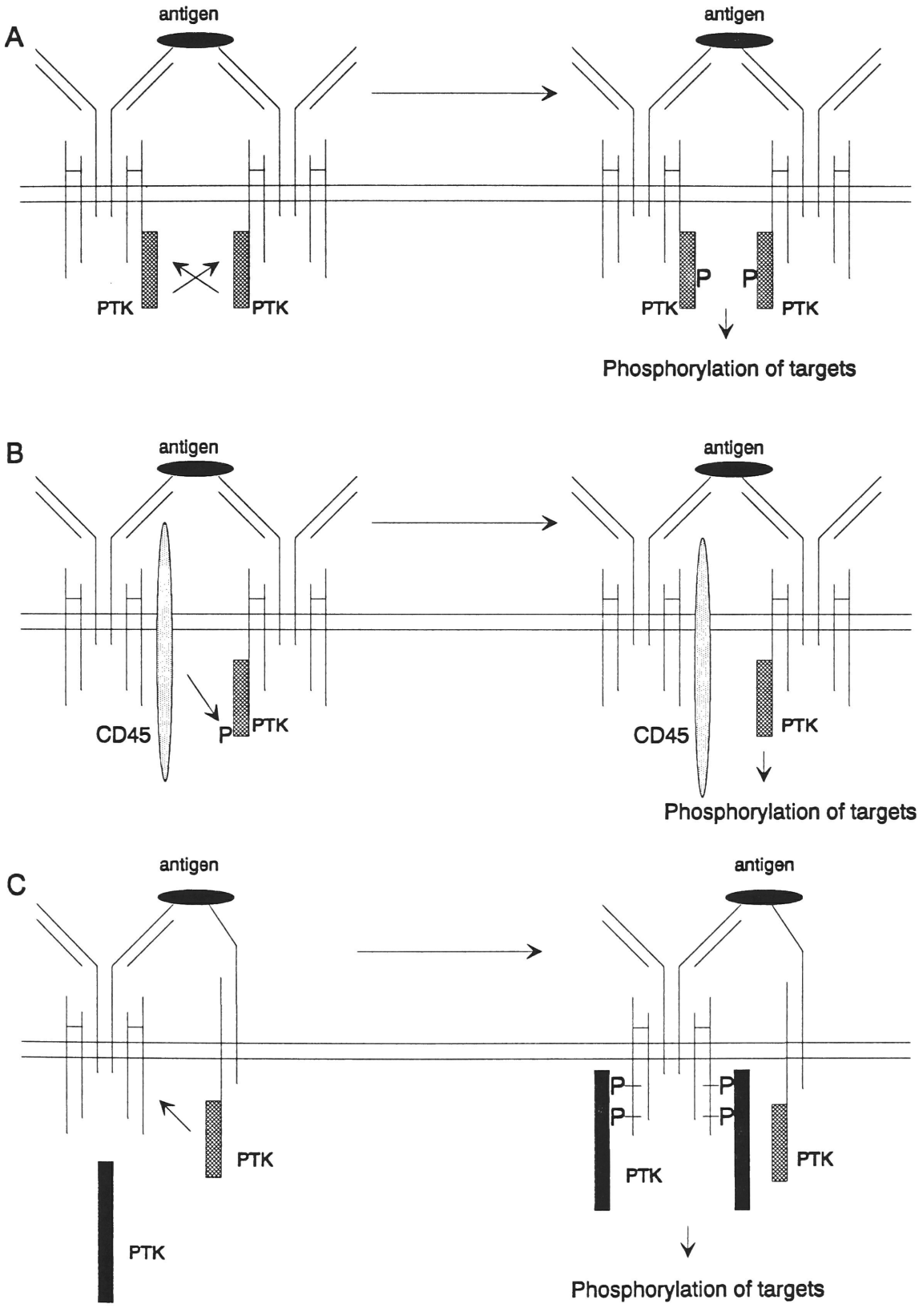

TCR
α
β
CD3
γ
ε
ε
δ
ζ
ζ
BCR
mIgM
Igβ
Igα
Igα
Igβ
FcγRIII
CD16
ζ
ζ
FcεR1
α
β
γ
γ

upon stimulation of the BCR (160,213). The associated components of the BCR, CD19 and CD22, are also phosphorylated (91,92,218). In addition, numerous downstream effector molecules, including the enzymes MAP-2 kinase (219), PI-3 kinase (220), $PLC_{\gamma 1}$ (214,215) and $PLC_{\gamma 2}$ (214,216,217), as well as the regulatory molecules vav (221), GAP and the GAP-associated molecules p62 and p190 (222) are phosphorylated.

B. The mechanism of BCR-mediated PTK activation.

The mechanism by which crosslinking of the BCR leads to PTK activation remains unknown. Receptor dimerization is required for signal transduction by several growth factor receptors with intrinsic kinase activity, for example, the epidermal growth factor receptor [EGFR] (326). Upon ligand-induced dimerization, the EGFR is auto-trans-phosphorylated on tyrosine residues which upregulates the intrinsic receptor activity. Moreover, this phosphorylation facilitates coupling of the receptor to the downstream signaling apparatus. In an analogous manner, mIg crosslinking might bring together BCR-associated PTK. The PTK could phosphorylate each other on the positive regulatory tyrosines characteristic of the src family (316) or the potentially analogous sites in syk [Figure 41A]. The activated PTK could phosphorylate the Ig-associated molecules which might couple the receptor to downstream signaling.

Figure 41. Models for the mIg-mediated activation of PTK. A. Antigen crosslinking of two or more mIg molecules brings together two PTK which phosphorylate each other on positive regulatory sites providing activation of the kinases leading to phosphorylation of other substrates. B. Antigen crosslinking brings together a tyrosine phosphatase [i.e. CD45] with a mIg-associated PTK which dephosphorylates a negative regulatory site providing activation of the kinase leading to phosphorylation of other substrates. C. Antigen bridging between mIg and a coreceptor brings a coreceptor-associated PTK into proximity of the Ig-associated molecules. Phosphorylation of the tyrosines with the ARH-1 motif by this PTK allows a different PTK to associate with the Ig-associated molecules through its SH2 domain[s] which allows phosphorylation of other substrates.


A
antigen
antigen
PTK
PTK
P
P
PTK
PTK
Phosphorylation of targets
B
antigen
antigen
CD45
P
PTK
CD45
PTK
Phosphorylation of targets
C
antigen
antigen
PTK
PTK
P
P
P
P
PTK
PTK
Phosphorylation of targets

Alternatively, crosslinking of mIg might induce tyrosine phosphatase activity. Dephosphorylation of the negative regulatory site of src family PTK (327,328) or potentially analogous sites in syk would activate these kinases, thus allowing for phosphorylation of downstream targets [Figure 41B]. A likely candidate for this activity is CD45, which can associate with both the BCR and TCR complexes (233,329,330). *In vitro*, CD45 can dephosphorylate the negative regulatory tyrosine residues on the TCR-associated, lck and fyn, resulting in their activation (331). T cells expressing enzymatically inactive CD45 are not signaled efficiently by mAb specific for the TCR (235,236). In these cells, the basal level of phosphorylation of lck and fyn is enhanced on the negative regulatory tyrosine residue (332-334). In T cells, the activity of lck and fyn is also regulated by csk (335). Csk phosphorylates the negative regulatory site of both lck and fyn, and as a result, overexpression of csk induces T cell hybridomas to be refractory to stimulation (335). Thus, like src, the phosphorylated negative regulatory site of fyn or lck may bind to its own SH2 domain thus blocking the enzymatic activity (336). By dephosphorylating these tyrosines, CD45 activates these PTK which can subsequently activate [or deactivate] specific molecules of the downstream signaling apparatus.

A third model is dependent on co-receptor function analogous to the TCR CD4 and CD8 molecules [Figure 41C]. Both

of these molecules bind conserved domains of MHC class II and class I, respectively (56-58,337) on their extracellular domain and lck on their cytoplasmic domain (59,60). These interactions are essential for TCR-mediated signal transduction especially with low antigen concentration (338,339). By interacting with the TCR/MHC complex, CD4 or CD8 brings lck into close proximity with the CD3 and zeta chains of the TCR. Phosphorylation of these molecules on tyrosine residues within the ARH-1 motif allows other molecules to associate with the complex via their SH2 domains (340). Likely candidates in the T cell are ZAP-70 and syk. This model is consistent with data showing that sequestration of CD4 prior to TCR stimulation inhibits T cell activation (341). Furthermore, mutation of ZAP-70 results in defective TCR-mediated signaling as well as thymic development (342). This model is also consistent with data obtained using fusion proteins of various PTK with CD16. In this system, crosslinking of CD16 fused to src family PTK was not as efficient in signaling as fusion proteins containing either syk or ZAP-70 (343). One interpretation is that syk and ZAP-70 function downstream in the signaling pathway and must be clustered with the receptor to efficiently amplify the signal. The CD16/src family PTK fusion proteins lack this ability to cluster syk and/or ZAP-70.

Candidates for coreceptor function in the B cell include CD19 and CD22. Ligation of CD22 augments anti-Ig induced

proliferation and calcium release (344,345). Co-modulaton of mIg and CD19, which associates with CD21, TAPA-1, and Leu-13 (184,185), lowers the threshold for mIg mediated activation (183). Conversely, independent ligation of CD19 inhibits subsequent activation via the BCR probably by sequestration of the coreceptor complex. The CD19/CD21/TAPA-1/Leu-13 complex could be targeted on the same antigen as mIg by CD21 which has affinity for the C3 fragments of complement (186,187) [Figure 2]. In addition, CD21 could mediate the association of mIg and CD19 by its affinity for CD23 (188,189) expressed on FDC (194) which concentrate native antigen on their cell surface in the form of immune complexes (18-20).

By association with mIg, the coreceptor would bring the CD19-associated kinases, lyn and PI-3 kinase, to the BCR complex (218,229). Lyn could potentially phosphorylate either the Ig-associated proteins, Ig_{α} and Ig_{β}, or CD22. CD22, like Ig_{α} and Ig_{β}, is associated with mIg (91) and contains tyrosines within an ARH-1 motif (346,347). Thus, like the TCR, phosphorylation of the tyrosine residues within the ARH-1 motif might allow association with downstream effector molecules. None of these models are mutually exclusive and all three of these mechanisms may play a role in BCR activation.

CD19 may also play a role in mIg internalization by virtue of its association with PI-3 kinase (182,218). In two distinct systems, PI-3 kinase or a related molecule are

required for protein trafficking within membrane-bound organelles. In one instance, internalization of platelet-derived growth factor receptor is ablated if the interaction of PI-3 kinase with the phosphorylated receptor is blocked (348). In a second system, a yeast protein, Vps34, with homology to PI-3 kinase, is essential for protein sorting to lysosome-like vesicles (349,350). In B cells, the p85 subunit of PI-3 kinase associates with tyrosine phosphorylated CD19 through its two SH2 domains (218). In addition, mIg internalization is blocked by PTK inhibitors (212). By analogy to the two systems described above, inhibition of tyrosine phosphorylation may block the association of CD19 with PI-3 kinase which could be required for receptor internalization.

C. IL-4 mediated signaling.

IL-4 has been characterized for its activity as a co-stimulant of B cell entry into the cell cycle (108,351). IL-4 has also been reported to stimulate other early activation events in B cells including RNA synthesis, CD23 expression and IL-6 production (352). However, the basis of IL-4-induced early signal transduction events are unclear. IL-4 promotes anti-Ig induced translocation of PKC from the cytosol to the membrane and induces an increase in PKC activity (353). It does not, however, modify signal transduction via the PI

pathway induced by anti-Ig (353). Thus, it was proposed that IL-4 receptor mediated signaling increased and/or prolonged mIg induced PKC activity. In this study, we demonstrate that IL-4 also synergizes with anti-Ig to induce the phosphorylation of syk in anti-phosphotyrosine immune complexes by the associated phosphotransferase activity. The induction of tyrosine phosphorylation as well as the activity of PTK like syk precedes PI hydrolysis or activation of PKC, and thus, may contribute to the regulation of these downstream signaling events. This is consistent with data that IL-4 rapidly stimulates tyrosine phosphorylation on numerous substrates in hematopoietic and myeloid cell lines (354-356) including the IL-4R and a molecule of unknown identity, p170 (355,356). IL-4 also induces the association of PI-3 kinase with the IL-4R (355). If phosphorylation of the IL-4R is blocked, IL-4-mediated signal transduction is ablated probably by blocking the association with downstream signaling molecules like PI-3 kinase. Moreover, IL-2Rγ is a second component of the IL-4R (357,358). As disucssed below, IL-2Rγ is coupled to both PTK activity and the induction of fos and jun (359). We are currently investigating the mechanisms by which IL-4 and anti-Ig synergize in the induction of the activity of syk.

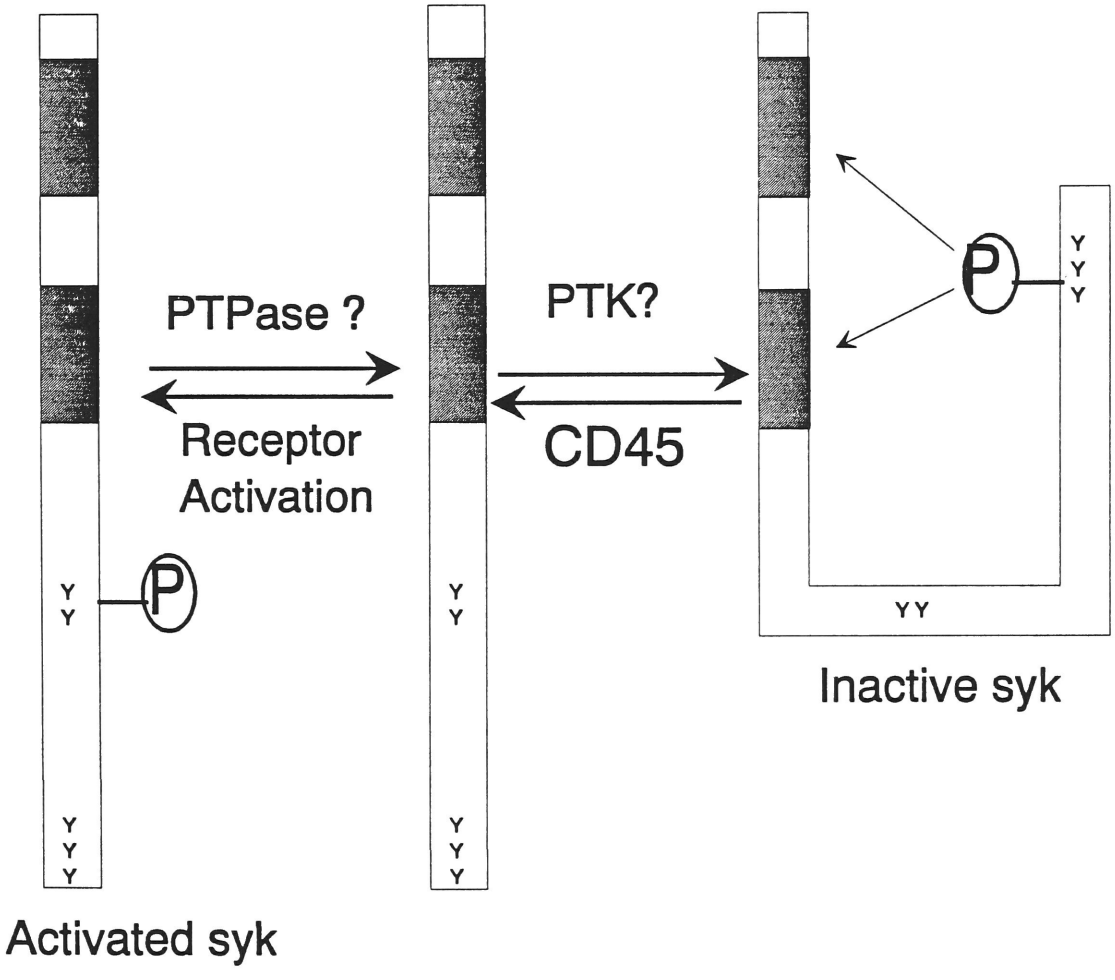
D. Regulation of syk.

In contrast to src family PTK, virtually nothing is known about the regulation of syk. One possible model was described above [Figure 41C]. In this model, based on the TCR complex, phosphorylation of the tyrosines within the ARH-1 motif of the Ig-associated molecules regulates whether or not syk is associated with the BCR complex and thus has access to potential substrates (340). In support of this model is the fact that syk has no N-terminal myristoylation or other acylation sites which can mediate the association of cytosolic proteins with membrane-associated receptor complexes (360). In addition, syk has two tandem SH2 domains which may have affinity for the two tyrosines contained in the ARH-1 sequence as has been proposed for ZAP-70 and the TCR (340). Other PTK known to associate with the BCR only have one SH2 domain.

This model does not account for any intrinsic regulation of syk. We are currently testing a hypothesis based on the regulation of src family PTK [Figure 42]. The deduced amino acid sequence of syk has a tyrosine residue within a phosphorylation consensus sequence that aligns with the positive regulatory site of src (316). Moreover, there are three consecutive tyrosine residues at the C-terminus which could potentially function as a negative regulatory site. It is not known whether any of these sites are phosphorylated, but phosphorylation of the tyrosines at the C-terminus might serve as a binding target for one of the SH2 domains, thus blocking interactions with potential substrates [Figure 42].

Figure 42. A model of the regulation of syk. Phosphorylation and dephosphorylation of the putative positive and negative regulatory tyrosine residues regulate the state of activation of syk.

Regulation of syk


PTPase ?
Receptor
Activation
PTK?
CD45
P
Y
Y
Y

These potential regulatory sites are also apparent in ZAP-70and thus there may have been evolutionary pressure for conservation of these tyrosine residues.

Syk is expressed in all hematopoietic cells and is associated with at least two other receptor complexes, $Fc_{\epsilon}R_I$ and $Fc_{\gamma}R$, types I and II (242-244). The mechanism coupling these receptor complexes to tyrosine phosphorylation cascades is probably similar to that of the TCR and BCR in that they are associated with ARH-1-containing proteins (170). Thus, syk may provide a common mechanism for amplifying receptor complex mediated signals.

The substrate specificity of the phosphotransferase activity of syk is for tyrosines flanked by acidic residues (361), although no specific endogenous substrates have been identified. The specificity of the SH2 domains of syk are also being investigated. The primary structure of the SH2 domains of several proteins, including src (284,317), abl (319), lck (318), and the p85α subunit of PI-3 kinase (320) have been determined. All of these have a similar overall structure including amino acids that form a basic phosphotyrosine binding pocket and amino acids that determine the SH2 domain specificity (362). Songyang, et al. determined the specificity of several SH2 domains using phosphopeptide binding assays (315). Using this data, they predicted that syk and ZAP-70 would bind phosphorylated tyrosine with similar surrounding sequences as src family PTK [YEEI], consistent

with most of the ARH-1 motifs. However, based on our proposed models of the SH2 domains described above, syk will probably have quite distinct specificity from the YEEI motif of src family PTK, especially in the first position immediately following the tyrosine. In these models, both the N- and C-terminal SH2 domains have an acidic residue at position βD'1 [glutamic acid and aspartic acid, repectively]. In src, this residue is arginine and interacts with a glutamic acid in the tyrosine phosphorylated peptide. A similar interaction is unlikely for either glutamic acid or aspartic acid.

E. Downstream pathways activated by BCR-induced tyrosine phosphorylation [Figure 3].

As discussed previously, numerous proteins are phosphorylated upon crosslinking of mIg. In the case of several growth factor receptors, phosphorylation of $PLC_{\gamma 1}$ is required for PI hydrolysis (363). In an analogous manner, tyrosine phosphorylation of both $PLC_{\gamma 1}$ and $PLC_{\gamma 2}$ may regulate their activity in B cells. PI hydrolysis is also regulated by G proteins (203-205), although the specific molecules involved are unclear. The PLC-mediated release of DAG and IP_3 cause activation of PKC as well as calcium mobilization, respectively (202). In T cells, calcium binds to and activates the phosphatase, calcineurin, which directly activates a subunit of the transcription factor, NF-AT

(364-367). In B cells, the effects of increased intracellular calcium are largely unknown, although it probably activates calmodulin and calmodulin-dependent protein kinases. Targets of PKC include MARCKS (201) and the MAP kinases (219,368) which may play a role in association with actin and fos/jun activation, respectively (369,370).

Crosslinking mIg induces ras activity as measured by an increase in the ratio of the ras^{GTP} to ras^{GDP} (206). This is accompanied by tyrosine phosphorylation of several proteins, including vav, GAP [GTPase activating protein] and the GAP-associated proteins, p62 and p190 (221,222). Vav and GAP regulate the activity of ras by promoting the GTP or GDP-bound form, respectively (206,371). The effect of phosphorylation of GAP is unclear but it may allow the interaction between GAP and p190, thus preventing its association with ras (222). Vav may regulate ras by its guanine nucleotide exhange factor activity (371). In addition, its two SH3 domains and single SH2 domain (372,373) may allow it to function as an adaptor molecule linking the BCR to ras, homologous to the Grb2 adaptor which associates with the guanine nucleotide exchange factor, SOS [see Figure 3] (374-378). These interactions may be associated with the receptor complex via another "linker" molecule, Shc (379,380). Consistent with this model is data which shows co-capping of ras and mIg (207). This association of ras and mIg is probably mediated by some of the "linker" molecules described above.

Some of the downstream effects of ras were recently identified in nonlymphoid cells. Ras directly associates with raf-1, a serine/threonine kinase (381,382). The association either directly activates raf-1 or brings it into proximity to potential sustrates. Raf-1 phosphorylates the kinase, mek, which in turn phosphorylates MAP-kinase (383). In T cells, ras indirectly activates MAP-kinases (370) which induce the transcription of fos and jun (384). A complex of fos/jun constitutes the calcium-independent component of NF-AT, which together with the calcinerin-activated subunit of NF-AT, promote IL-2 transcription (384). Whether or not this ras-dependent pathway is active in B cells has yet to be determined.

F. Priming of B cells to proliferate and secrete Ig.

Activation of resting B cells with anti-Ig primes them for entry into S phase as well as for Ig secretion in response to restimulation with T cell-derived lymphokines (31,33,34) and anti-Ig (306). We found that activating B cells in the presence of T cell help further enhanced this priming and rendered the blasts responsive to IL-2. The magnitude of the proliferative response and the quantity and quality of the antibody response suggest that the B lymphoblasts generated in the presence of T cell help represent either a further differentiated state than those lymphoblasts generated under

T-independent conditions, or the induction of a distinct differentiation pathway. It could also be due to the stimulation of a different subset of B cells, but we think this is unlikely because of the large percentage of B cells that blast transform under each of the conditions.

The B cell-T cell interaction not only activates the B cell, but also restimulates the T cell through the TCR. The T cell blasts are induced to produce B cell-stimulating factors including at least IL-2, IL-4, IL-5 and IL-10 [Table 6 and (290)]. Thus, the allo-T blasts provide lymphokines which act on B cells but may also provide a direct signal to the B cells via cell surface Ia. Ia-mediated signaling may be supported by production of IL-4 by the T blasts, since it has been shown that IL-4 induces MHC class II expression (113). The generation of allo-B blasts presumably occurs independently of the Ig-mediated pathway of B cell activation; we can not eliminate the possibility that some of the B cells in the culture also receive a mIg-mediated signal, for example, in the form of antigens present in the FCS. However, the large percentage of cells that were induced to blast transform makes it less likely that a specific Ig-mediated signal is required.

G. MHC class II-mediated signaling.

Cell signaling which occurs during T cell-B cell

interaction is bi-directional. Signaling in the T cell is mediated by the TCR and facilitated by numerous accessory molecules [see Figure 1] (177). On the other hand, signaling in B cells through MHC class II via bivalent antibody or ligands leads to a number of biological responses. Crosslinking of MHC class II induces proliferation of human tonsillar B cells as well as Ig secretion by B lymphoma cell lines (385,386). Furthermore, murine B cells, primed with either anti-Ig plus IL-4 or anti-CD40, proliferate in response to crosslinking anti-Ia antibodies (387,388). Homotypic cell adhesion is also induced by engagement of MHC class II (389-393). This adhesion is mediated, in part, by LFA-1/ICAM-1 interactions (391-393) although other, as yet undetermined, mechanisms play a role (389-392). The LFA-1/ICAM-1 interaction are dependent on tyrosine phosphorylation and/or PKC as it was inhibited by both herbimycin and sphingosine (392,393). Finally, cell lines expressing truncated MHC class II molecules are defective in their ability to present antigen to T cells (394) due to a failure to express CD80 (395). This defect can be overcome by the addition of cyclic AMP [cAMP] analogs to the B cell which restore CD80 expression (395,396). The effects of cAMP may be mediated by cAMP-dependent protein kinase because the R_{II} subunit of the kinase is induced to associate with $I\text{-}A^k$ in a murine B lymphoma cell line (397).

A second, cAMP-independent, pathway is induced by crosslinking class II MHC. Resting and preactivated B cells

mobilize calcium after class II crosslinking (385,398,399). The increase in cytoplasmic calcium is probabaly the result of Ia-induced PI turnover and tyrosine phosphorylation (385,399). This pathway may be mediated by the PTK, lyn, which is activated immediately after Ia crosslinking (388).

In vitro, the activation of B cells by T cells, or conversely, T cells by B cells, requires that the activating cell population be previously activated (46,290-292). *In vivo*, the initial activating event might be engagement of mIg on the B cell by native antigen, TCR engagement by a "professional" antigen presenting cell such as the DC (400), or both. During an immune response, the B cell specifically internalizes antigen by mIg, and the antigen is subsequently processed and presented to T cells in the context of MHC class II. A class II-mediated signal induces expression of CD80, (395,401) which can subsequently bind its ligand CD28 (77). The ligation of both CD28 and the TCR induces T cell activation leading to cytokine production and gp39 expression (65,66,75,76). This in turn restimulates the B cell via both CD40 and cytokine receptors.

In a similar manner, DC also activate T cells and induce gp39 expression. The activated T cells can subsequently interact with B cells through CD40. Ligation of CD40 induces CD80 expression (402), thus further facilitating interaction with CD28 on the T cell. Thus, the B cells and T cells can efficiently "talk" to each other, while in the absence of

antigen-specific lymphocyte activation T cell-B cell interaction is reduced.

H. IL-2 is the predominant stimulatory factor for T-dependent B lymphoblasts.

While resting B cells or B lymphoblast generated in the absence of T cell help do not proliferate in response to individual cytokines, IL-2 is the predominant stimulatory factor for T-dependent B lymphoblasts. Preactivation of the B cells with anti-Ig alone was not sufficient to induce IL-2 responsiveness to IL-2 despite upregulation of CD25 [IL-2Rα] and expression of high affinity binding sites for IL-2. The addition of IL-5 during the initial phase of activation was able to confer partial responsiveness to IL-2 despite a minimal change in the number of high affinity binding sites.

The high affinity IL-2R is formed by a heterotrimeric complex consisting of IL-2Rα, IL-2Rß [p70; CD122] and IL-2Rγ (279,403). The IL-2Rß and IL-2Rγ chains are members of the cytokine receptor family characterized by four conserved cysteines and the sequence WSXWS [the WS motif] (404). While the IL-2Rß chain appears to be specific to the IL-2R, the IL-2Rγ chain is a component of several receptors, including IL-4 (357,358) and IL-7 (405), and is defective in X-linked severe combined immune deficiency (406). The IL-2Rß couples the receptor to the signaling apparatus by virtue of its

association with the PTK lck in T cells (407) and lyn and fyn in B cells (408,409). The IL-2Rγ also links the receptor to PTK activity, but, in addition, couples the receptor to a distinct signaling pathway that induces expression of the transcription factors, c-fos and c-jun (359). The T cell-derived cytokines, IL-4, IL-5, and IL-10 induce expression of components of the IL-2R as well as IL-2 responsiveness in B cells [Figure 12 and (134,135)]. Thus, the presence of IL-5 or IL-10 during the initial phase of B cell activation may induce the coupling of the receptor to the signaling apparatus.

I. Priming for production of Ig utilizing downstream heavy chain constant region genes.

Relative to other types of B lymphoblasts, the allo-B blasts secrete increased levels of Ig that are encoded for by downstream heavy chain constant region genes. This phenomenon reflects either a selective expansion of B cells that are already committed to isotype switching or induction of isotype switching dependent on the production of specific cytokines by activated T cells. In earlier work, the enhanced production of isotypes utilizing downstream heavy chains involved co-culture with LPS (109,138,144,410). Activation of the B cell through ligation of mIg or CD40 plus specific cytokines also promote secretion of Ig utilizing downstream heavy chain

constant region genes [data not shown] and (63,136,411). Thus, it appears that isotype switching requires a minimum of two signals. The first is an activation signal that promotes DNA synthesis (412,413). Kenter and Watson have suggested that the DNA rearrangement required for isotype switching occurs during S phase (413). In the case of the allo-B blasts, the activating signal is probably mediated by both Ia and CD40. Ligation of either of these molecules induces B cell proliferation (62,69,385,387).

A second, isotype-specific signal, is provided by cytokines which promote the germline transcription of constant region genes which precedes the production of Ig utilizing the downstream heavy chains (414-416). Thus, the difference in Ig secretion between the allo-B blasts and the αIg/EL-4 blasts is probably due to the difference in cytokines present in EL-4 sn and those secreted by the allo-T blasts rather than the presence of cell-cell contact. This notion is supported by the fact that B cells activated with anti-Ig plus cytokines derived from the allo-B/T co-culture, demonstrate a similar profile of Ig secretion as B cells primed by allo-reactive T cell contact. The allo-B/T supernatant has high levels of IL-4 and IL-5 which promote IgG_1 and IgA. respectively (109,145,146).

J. Allo-B blasts resemble germinal center B cells.

Several features of allo-stimulated B lymphoblasts resemble those of germinal center B cells. First, generation of germinal centers is T cell dependent (14,15). In addition, germinal center B cells are IgD^{low} (299) and PNA^{hi} (297). T cell help was required for maximal downregulation of mIgD. In allo-B blasts, this decrease in mIgD occurred in a time-dependent fashion, evidently without a requirement for crosslinking of mIgD. In addition, the down-modulation of mIgD was specific in that there was no change in the expression of mIgM. Most likely, mIgD was specifically modulated off the cell surface; alternatively, the activation of B cells may have blocked synthesis of IgD.

Allo-B blasts also exhibited enhanced reactivity with PNA. PNA reacts with the penultimate N-acetyl glucosamine residues of glycoproteins exposed by removal of the N-terminal sialic acid residues (297). Thus, allo-T blast-induced activation of B cells may induce neuraminidase activity on the surface of T cells or B cells (417,418). Alternatively, newly synthesized proteins not bearing terminal sialic residues may be expressed.

In one respect, the phenotype of allo-B blasts differs from germinal center B cells. The mAb 23G2, dependent on the terminal sialic acid residues for reactivity with $CD45R_B$, does not react with germinal center B cells (265,298). However, 23G2 reactivity with allo-B blasts is not reduced relative to resting B lymphocytes.

Allo-B blasts also resemble germinal center B cells based on functional properties. The allo-B blasts proliferate rapidly in response to restimulation with anti-Ig, similar to the finding that mIg can provide a signal that maintains the viability of human tonsillar germinal center B cells (22). Allo-B blasts appear to undergo isotype switching at an enhanced rate or frequency, which is also a property of germinal center B cells (12). The differentiation induced by the allo-T blasts is probably mediated in part by CD40. The ligand for CD40, gp39, is defective in individuals with hyper-IgM syndrome (71-73). In individuals with this disease, germinal centers do not form in response to foreign antigen. B cells from these people are capable of isotype switching *in vitro* if CD40 is ligated with either mAb or cell-associated gp39, but *in vivo*, little or no isotype switching is observed. In conclusion, these data suggest that allo-B blasts may reflect a stage of differentiation more closely related to germinal center B cells than B cells activated in the absence of T cell contact-dependent activation.

K. Future directions.

The allo-B blasts represents a good system to study the molecular basis of T cell-dependent processes such as isotype switching. Furthermore, it will be interesting to determine if somatic mutation can be induced *in vitro* by the allo-

activation of B cells. This could be accomplished by isolating the transcribed variable regions of well-defined Ig gene families.

There is obviously a great deal that remains to fully understand the role of syk in B cell function and our work will focus on several aspects. The first is the regulation of syk and the role of tyrosine phosphorylation in regulating the activity of syk. Secondly, how syk interacts with receptor complexes and potential substrates will shed light on the intracellular pathways that syk regulates. Finally, mIg receptor internalization is dependent on tyrosine phosphorylation (212) and tyrosine phosphorylated proteins localize underneath mIg patches and caps (211). By effecting the function of syk we can determine the role, if any, that syk has in this process.

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