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**A MOLECULAR ANALYSIS OF
THE B-CELL ANTIGEN RECEPTOR
COMPLEX**

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of Doctor of Philosophy

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CONTENTS

Declaration	06
Abstract	07
Acknowledgments	09
Abbreviations	10

1 INTRODUCTION

1.1 ANTIBODY STRUCTURE AND FUNCTION

Introduction	15
The Discovery of Antibody and its Basic Structure	16
Early Models of Diversity Generation	17
The Structure and Assembly of Antibody Genes	18
The Generation of Variable Region Diversity	19
The Production of Different Classes of Antibody	20
Membrane-Anchored Antibody	23
The Discovery of the Heterodimer	24
B-Cell Development: An Overview	26
The Involvement of Membrane Immunoglobulin	28
B-Cell Programmed Cell Death and Tolerance	30
Antigen Presentation by the B-Cell Antigen Receptor	32

1.2 SIGNAL TRANSDUCTION

Receptors that Do Contain Intrinsic Kinase Activity	36
Receptor Activation	36
Interaction with Cytoplasmic Signalling Machinery	37

CONTENTS

Declaration	06
Abstract	07
Acknowledgments	09
Abbreviations	10

1 INTRODUCTION

1.1 ANTIBODY STRUCTURE AND FUNCTION

Introduction	15
The Discovery of Antibody and its Basic Structure	16
Early Models of Diversity Generation	17
The Structure and Assembly of Antibody Genes	18
The Generation of Variable Region Diversity	19
The Production of Different Classes of Antibody	20
Membrane-Anchored Antibody	23
The Discovery of the Heterodimer	24
B-Cell Development: An Overview	26
The Involvement of Membrane Immunoglobulin	28
B-Cell Programmed Cell Death and Tolerance	30
Antigen Presentation by the B-Cell Antigen Receptor	32

1.2 SIGNAL TRANSDUCTION

Receptors that Do Contain Intrinsic Kinase Activity	36
Receptor Activation	36
Interaction with Cytoplasmic Signalling Machinery	37

The T-Cell Antigen Receptor	38
T-Cell Antigen Receptor Associated Kinases	40
T-Cell Antigen Receptor Accessory Transmembrane Proteins	42
Signal Transduction by the B-Cell Antigen Receptor	44
The α/β Sheath Can Mediate Intracellular Signalling	47
B-Cell Antigen Receptor Accessory Tyrosine Kinases	48
B-Cell Antigen Receptor Accessory Transmembrane Proteins	52

2 MATERIALS AND METHODS

Chemicals/Materials	58
Cells	59
Antisera	60
Standard Buffers	60
Methods	61

RESULTS

3 ASSOCIATION OF CD22 WITH THE B-CELL ANTIGEN RECEPTOR

Introduction	79
Results	79
Figures	88

4 TYROSINE PHOSPHORYLATION OF CD22 AFTER B-CELL ANTIGEN RECEPTOR STIMULATION

Introduction	104
Results	104
Figures	112

5 SIGNAL TRANSDUCTION BY THE B-CELL ANTIGEN RECEPTOR

Introduction	117
Results	117
Figures	123

6 DOWNREGULATION OF THE B-CELL ANTIGEN RECEPTOR

Introduction	130
Results	130
Figures	138

7 DISCUSSION

8 REFERENCES

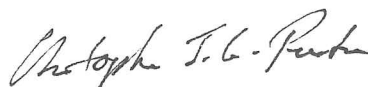
158

9 APPENDIX

Transfectants	185
Plasmids	186
Publications Arising from, or Featuring this Work	187

DECLARATION

I hereby declare that this dissertation/thesis entitled 'A Molecular Analysis of the B-Cell Antigen Receptor Complex' is not substantially the same as any that I have submitted for a degree or diploma or other qualification at any other University. I further state that no part of my dissertation/thesis has already been or is being concurrently submitted for any such degree, diploma or other qualification. All the work in this thesis was performed at the Medical Research Council, Laboratory of Molecular Biology from November 1991 to March 1995. All the experiments are the result of my own work with the exception of the FACS analyses on page 139 that were performed in collaboration with Gareth T. Williams.



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 April 1995

ABSTRACT

The antigen receptor on B lymphocytes mediates the response of B cells to antigen and is composed of a membrane-anchored form of antibody sheathed by an α/β heterodimer. This study demonstrates that the B-cell antigen receptor associates with the B-cell specific, transmembrane protein CD22. Although first characterised in a human Burkitt lymphoma cell line, the interaction itself, is also present in primary cells. Furthermore, it appears to be evident with multiple receptor isotypes (IgM, IgD and IgG). Analysis of the interaction shows that, whilst it is specific and stable, it is of a relatively low stoichiometry *in vitro* with 0.2-2% of membrane immunoglobulin associated with CD22. These results intimate that CD22 acts as a co-receptor for the antigen receptor complex during antigen mediated B-cell activation.

Evidence that CD22 functions during transmembrane signalling comes from the findings that CD22 is rapidly tyrosine phosphorylated after antigen receptor crosslinking in mouse and human cells and contains within its tail a sequence with a high degree of homology to a motif previously shown to mediate lymphocyte activation. It is therefore tempting to speculate that CD22 recruits SH2-domain containing proteins. Use of cell lines expressing mutant antigen receptors shows that firstly, the ability of the antigen receptor to mediate the phosphorylation of CD22 can be ascribed to the α/β sheath and secondly, also implicates tyrosines within the sheath as important during signal generation. Thus the sheath may also recruit SH2-domain containing proteins during CD22 phosphorylation.

Investigation into how surface expression of the antigen receptor is regulated, reveals that treatment of J558L cell transfectants or WEHI-231 cells with phorbol ester; a potent activator of protein kinase C (PKC), appears to rapidly decrease surface mIgM expression. In addition PMA stimulates an increase in an intracellular pool of mIgM in WEHI-231. These data suggest that a PKC-regulated mechanism modulates the

internalisation and intracellular pool size of the receptor. This may be important for the ability of the antigen receptor to mediate the internalisation of antigen for its subsequent processing and presentation to T cells.

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ABBREVIATIONS

α	<i>mb-1</i> gene product <i>or</i> alpha heavy chain
2D	two dimensional
ATP	adenosine triphosphate
β	<i>B29</i> gene product
BCR	B-cell antigen receptor
BH	Bolton Hunter
BSA	bovine serum albumin
C	constant region
Ci	curie
cm	centimetre
δ	delta heavy chain
DAG	diacylglycerol
DMEM	Dulbrecco's modified eagle's medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ϵ	epsilon heavy chain
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetracetic acid (disodium salt)
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
FCS	foetal calf serum
γ	gamma heavy chain

GAP	GTPase activating protein
GTP	guanosine triphosphate
H	heavy chain
HIGMI	X-linked immunodeficiency with hyper-IgM
HRP	horseradish peroxidase
Ig	immunoglobulin
IgM (A, D, E, G)	immunoglobulin M (other isotypes)
IL-2	interleukin 2
IL-4	interleukin 4
IP3	inositol 1,4,5-trisphosphate
IR	insulin receptor
i.e.	that is
J	joining region <i>or</i> joining
κ	kappa light chain
kD	kilodaltons
λ	lambda light chain
l	litre
L	leader region <i>or</i> light chain
M	molar
m	milli
mA	milliampere
mAb	monoclonal antibody
mBq	millibequerel
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
MHC	major histocompatibility complex

mIg	membrane immunoglobulin
mIgM (A/D/E/G)	membrane immunoglobulin M (other isotypes)
μ	micro <i>or</i> mu heavy chain
μ M	micromolar
n	nano
NGF	neuronal growth factor
NGFR	neuronal growth factor receptor
NHS-biotin	N-hydroxysuccinimidobiotin
NHS-SS-biotin	sulfosuccinimidyl 2-(biotinamido) ethyl- 1,3-dithiopropionate
NIP	5-iodo-4-hydroxy-3-nitrophenylacetyl
NIPcap-OH	5-iodo-4-hydroxy-3-nitrophenylacetyl aminocaproate
NIPcap-OSu	5-iodo-4-hydroxy-3-nitrophenylacetyl aminocaproic succinate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PDGFR	platelet-derived growth factor receptor
PI3-kinase	phosphatidylinositol 3-kinase
PI4-kinase	phosphatidylinositol 4-kinase
PtdInsP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PLC- γ 1	phospholipase C isoform

PLC- γ 2	phospholipase C isoform
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethanesulphonyl fluoride
PNGF	endoglycosidase F/poly-N-glycosidase F
Poly A	poly-adenylation
Pre-B	precursor B cell
Pro-B	progenitor B cell
RT	room temperature
rpm	revolutions per minute
S	switch region
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
SH2	<i>src</i> homology 2
SH3	<i>src</i> homology 3
sulfo-SHPP	sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate
sulfo-NHS-biotin	sulfosuccinimidobiotin
TEMED	N,N,N',N'- tetramethylethylenediamine
TCR	T-cell antigen receptor
Tris	N-tris-(hydroxymethyl)-methyl-2- aminoethanesulphonic acid
UV	ultraviolet
v/v	volume per volume
V	variable region
w/v	weight per volume
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinaemia

1 INTRODUCTION

1.1 ANTIBODY STRUCTURE AND FUNCTION

Introduction

One of the ways by which the immune system fights disease is by producing large numbers of proteins with different binding specificities called antibodies that bind to foreign proteins or antigens. How this is achieved has been studied for many years.

Several models of antibody formation were proposed in the 1930s and 1940s (Breinl and Haurowitz, 1930; Mudd, 1932; Alexander, 1932; Pauling, 1940). They have been categorised as *instructive* theories because they generally hypothesise that structural elements of antigen act as a template which then determines antibody specificity. In this respect they differ fundamentally from the earlier ideas of Paul Ehrlich who, in 1900 attempted to explain how the body fights disease by suggesting that foreign toxin (antigen) binds to reactive cell surface side-chains and results in cells being stimulated to produce serum antitoxins (antibodies) (Ehrlich, 1900). This model became known as a *selective* theory of antibody formation because he hypothesized that foreign proteins select which antitoxins are produced from a large, pre-existing pool. An important implication of Ehrlich's theory is that for animals to mount effective antibody responses, they need to contain a sufficient variety of side-chains to cope with the diversity of toxins. However, in the 1940s Landsteiner demonstrated that animals could produce antibodies to synthetic haptens as well as to natural antigens (Landsteiner, 1945). Ehrlich's selective theory therefore seemed unrealistic because, at this time, it appeared unlikely that animals could contain a sufficiently large enough variety of pre-existing side chains to cope with the enormous repertoire of antigens that, from Landsteiner's work, would be predicted to exist. Nevertheless, this did not deter Jerne from proposing in 1955 that antibodies against all antigenic determinants may exist in the individual before encounter with antigen (Jerne, 1955) or subsequently, Burnet from extending Ehrlich's and Jerne's ideas with his clonal selection theory (Burnet, 1957). He suggested that prior to exposure to antigen, each cell expresses a

single specificity of receptor and furthermore, there is a pool of cells which may all possess different binding specificities so that upon exposure to antigen (which productively binds only to cells of the appropriate specificity) antigen reactive clones can be induced to proliferate and produce specific antibody.

Nowadays it is believed that antibody production occurs predominantly via selective mechanisms. The instructive theories have been largely discounted as it has become increasingly appreciated that the determinants of antibody structure and the mechanisms that generate diversity are contained largely within the genome. Implications of Ehrlich's, Jerne's and Burnet's theories include firstly, that there should be cells that express receptors and produce antibody; a job which is performed by B cells, and secondly, that there must be a mechanism to ensure antibody variability. To accomplish this, the structure of antibody genes is manipulated to allow the production of a diverse antibody repertoire. These issues will be amongst those reviewed in more detail in the remainder of this introduction. Except where noted, this introduction will concentrate on the murine and human systems.

The Discovery of Antibody and its Basic Structure

Early experiments that involved immunising rabbits with different antigens demonstrated that serum components have heterogeneous binding characteristics (Heidelberger and Kendall, 1935; Landsteiner and van der Scheer, 1936). Fractionation of rabbit serum indicated that this binding activity was present in a relatively homogeneous fraction called the γ globulins (Tiselius and Kabat, 1939). Consistent with this homogeneity, later studies revealed that there are common elements to γ globulin structure. Enzymatic digestion generated 2 major fragments; one that retained antigen binding properties (Fab fragment) and another, called the Fc fragment, which was relatively easy to crystallise (Porter, 1959). Additionally, analysis after denaturation showed that γ antibody consists of four disulphide-linked polypeptides;

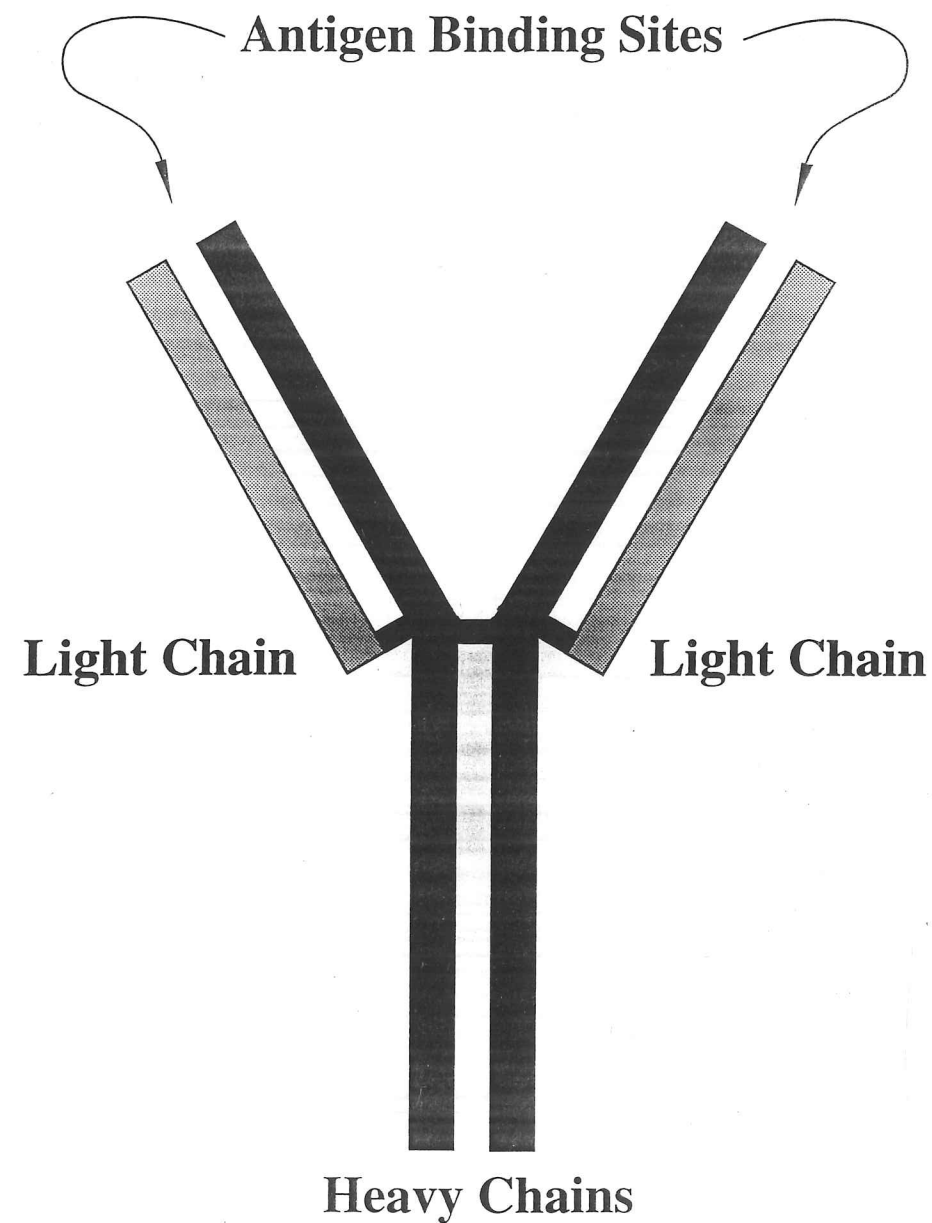


Figure 1

Schematic Diagram of the Basic Structure of Antibody

The basic unit of antibody structure consists of two heavy chains of the same isotype (α , δ , ϵ , γ , or μ) and two light chains of the same species (κ or λ) in a $(HL)_2$ structure linked together by disulphide bonds.

two heavy chains and two light chains (Edelman and Poulik, 1961). It is now known that the light chains are present in two serologically distinct types named κ and λ (Nisonoff et al., 1975). Antibody heavy chains are more diverse and are classified into five major species; α , δ , ϵ , γ and μ . There are also five major classes of antibody; namely IgA, IgD, IgE, IgG and IgM because each class consists of a single species of heavy chain (α , δ , ϵ , γ and μ respectively) paired with either κ or λ light chains (Figure 1). The different classes of antibody are specialised to perform different functions and it is, primarily, the heavy chain constant regions that mediate these effects. There are several subclasses of some of these types of antibody, for example there are at least four IgG subclasses in the mouse (IgG1, IgG2a, IgG2b and IgG3). The subclasses generally differ in their effector functions and each contain a different variant of heavy chain.

The discovery of common components to antibody structure did not reveal how serum antibody can display such promiscuous binding. An explanation came following the analysis of protein sequences obtained from purified immunoglobulin. This was made possible by the development of techniques that allowed the large scale purification of immunoglobulin light chain (in a form called Bence-Jones fragments) from the urine of patients with myeloma in yields and of purities suitable for amino acid sequencing (Kunkel, 1965). Comparisons between the protein sequences of κ light chain showed that there were areas of variability located towards the amino terminus (Hood and Talmadge, 1970). Analogous variable or V regions have subsequently been shown to exist in both heavy and light chains and it is now known that these regions of heavy and light chain determine antibody binding specificity.

Early Models of Diversity Generation

By the early 1970s, although it was appreciated that the heterogeneous binding properties of immunoglobulin could be explained by the structural heterogeneity of

antibody itself, it was not clear how the variability arose. Not surprisingly, there were several models that sought to explain how immunoglobulin diversity is generated. Generally, the germline theories suggested that cells contain a massive array of immunoglobulin genes and, consistent with Burnet's clonal selection theory, each cell expresses only one (or a limited number) of these genes. The somatic theories, in contrast, proposed that mutation during development, from a relatively limited number of germline genes, could generate a sufficiently extensive spectrum of antibody specificities to account for the observed heterogeneity of binding. Dreyer and Bennett proposed a third model that introduced some novel concepts (Dreyer and Bennett, 1965). They suggested that the constant and variable regions of antibody are expressed as distinct gene segments. To allow for the production of genes capable of encoding whole antibody molecules, constant regions would be shunted together with variable regions.

It is now realised that the generation of immunoglobulin diversity involves aspects of all of these models. A summary of what is understood about antibody gene structure and diversity generation now follows.

The Structure and Assembly of Antibody Genes

In the 1970s, studies of the structure of the λ light chain gene showed that light chain segments are separate in the germ line, but are in contrast, apparently present as an uninterrupted sequence in the mRNA (Hozumi and Tonegawa, 1976). This finding was the first evidence for the existence of immunoglobulin gene rearrangement; a process that had been predicted by Dreyer and Bennett. Subsequent analysis has revealed that immunoglobulin chains are produced from genes that are assembled from multiple DNA segments. λ and κ light chain genes are assembled from V, J and C gene segments. Heavy chain gene assembly involves an additional component; a D region, and thus heavy chain genes are composed of V, D, J and C regions (Brack et al., 1978;

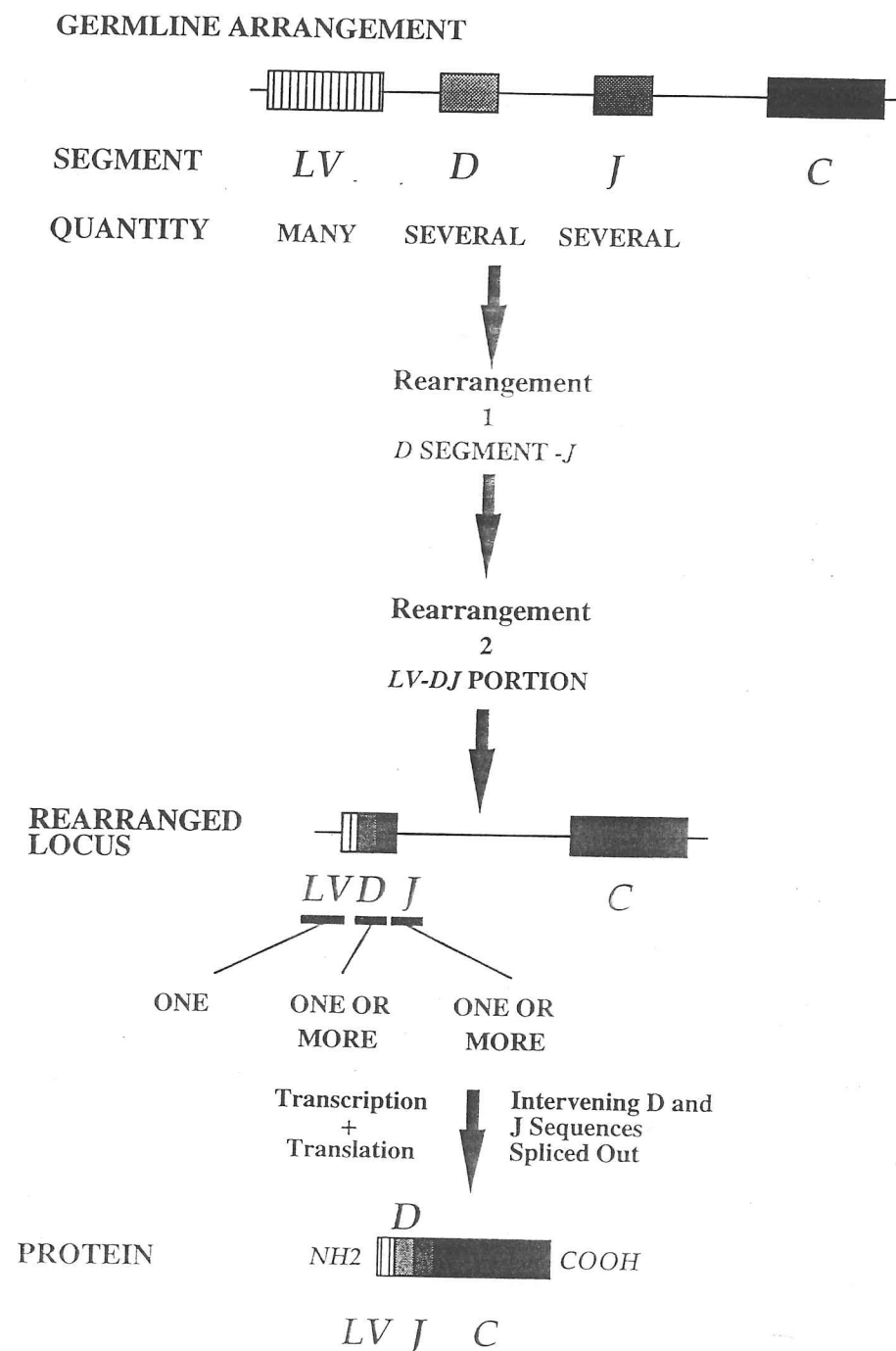


Figure 2

Schematic Diagram of Antibody Heavy Chain Gene Assembly

(L encodes a hydrophobic leader that is cleaved off, see text for further details)

Seidman et al., 1979; Early et al., 1980; Sakano et al., 1980) (Figure 2). Once productive rearrangement has occurred and a B cell expresses one light chain locus and one heavy chain locus, other immunoglobulin loci are prevented from being expressed. This process of allelic exclusion ensures each B cell normally expresses a single specificity of antibody. Although there is normally only a single functional heavy chain locus, all five classes of heavy chain can be produced because it contains multiple constant (C) regions, one for each class and subclass and the heavy chain isotype is determined by which constant domain is transcribed into the mRNA. μ is produced early in development because rearrangement usually initially places the VDJ region proximal to the constant region encoding μ heavy chain sequence (C_μ). How other isotypes can be efficiently produced will be reviewed later.

Generation of Variable Region Diversity

Extensive antibody diversity can be generated during somatic rearrangement because any one of several J/D sequences and any one of many variable regions can be combined with a constant region. Further variability is generated during VJ (for light chain) and VDJ (for heavy chain) joining because these processes are imprecise thus leading to the addition or deletion of nucleotides at the junctions (Kurosawa and Tonegawa, 1982; Alt et al., 1984).

In the 1970s and early 1980s, analyses of immunoglobulin sequences pointed to the existence of antibody gene mutation distinct from that generated during rearrangement and segment joining. Sequence comparisons between the variable regions of antibodies from transformed antibody secreting cells and their presumed germ line prototypes revealed that 30-50% of the analysed sequences appeared to have arisen by non-junctional mutation of germline V regions (Weigert et al., 1970; Brack and Tonegawa, 1977; Gearhart et al., 1981; Crews et al., 1981; Kim et al., 1981). This form of antibody mutation has been shown to display unique characteristics that

distinguish it from other types of mutation. Estimations of frequency suggest that it has a rate 10^4 -fold greater than the rate of normal eukaryotic spontaneous mutation (Stablitzky et al., 1985; Adetugbo et al., 1977; McKean et al., 1984); findings that have led to it being called somatic hypermutation. Additionally, the detectable mutations are distributed in a non-random manner and are targeted mostly to the variable regions of the heavy and light chain loci (Gearhart and Bogenhagen, 1983; Lebecque and Gearhart, 1990; Both et al., 1990; Weber et al., 1991; Steele et al., 1992; Rothenfluh et al., 1993). The large number of mutated sequences which have been analysed, and the identification of some that increase the affinity of binding to antigen and consistently appear in response to certain antigens have contributed to the current view that somatic hypermutation mutation contributes significantly to antibody diversity. It appears to be particularly important for the production of high affinity antibodies from naive repertoires (reviewed Betz et al., 1993; references therein). Relatively little is known about how somatic hypermutation occurs, however it does appear that DNA elements direct the process because analyses of transgenic mice expressing various κ light chain immunoglobulin transgenes have shown that transgene hypermutation is drastically retarded if stretches of DNA containing enhancer sequences that are normally located in an intron and 3' to the coding region are not present in the transgene (Betz et al., 1994). Interestingly, in this study whilst a role for the endogenous immunoglobulin in mediating mutation cannot be discounted, the importance of these elements is not entirely due to their ability to potentiate protein expression because a transgene that is expressed but does not encode functional κ protein also mutates (Betz et al., 1994).

The Production of Different Classes of Antibody

In the mid 1970s, it was found that greater than 90% of B cells each express significant quantities of both IgM and IgD. (Abney et al., 1976; Goodman et al., 1975). This seems to be a consequence of B cells being able to produce IgM and IgD simultaneously; a property attributable to differential splicing so that either μ or δ

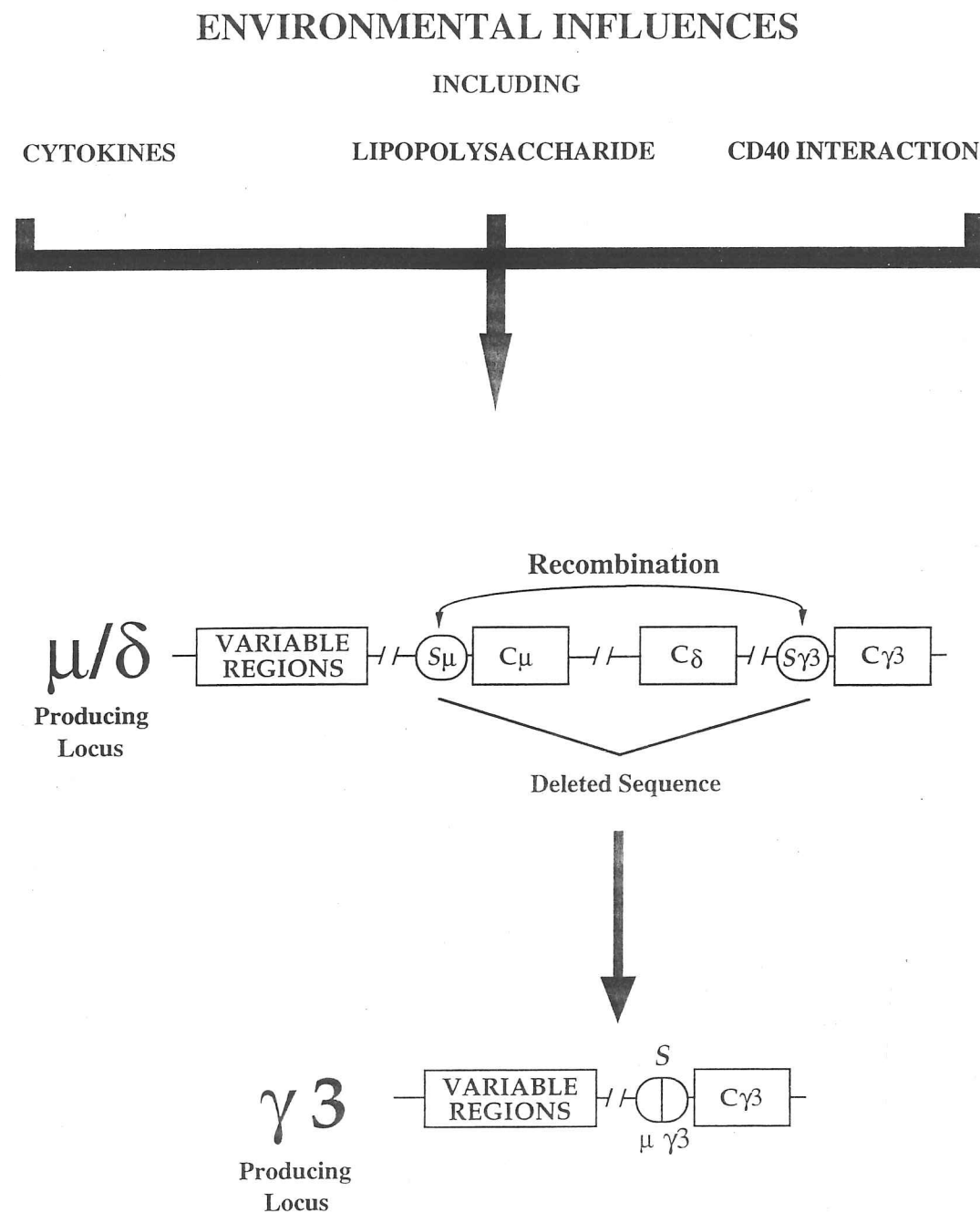


Figure 3

Schematic Diagram of B-Cell Class Switching

B cell class switching to IgA, IgE or IgG involves switch regions that are located 5' to constant regions. A switch to the $\gamma 3$ constant region by a locus capable of producing μ or δ chains is illustrated.

encoding mRNA is produced from nascent transcripts that contain μ and δ constant region exons (Liu et al., 1980). IgM is often produced early in immune responses, for example during primary immune responses against antigenically complex organisms. It is usually secreted as a pentamer containing five (HL)₂ units and a J (joining) chain. This arrangement, which contains 10 antigen binding sites, is probably important in several ways including facilitating the capture of antigen. IgD, in contrast, is only found in relatively low concentrations in the serum and it is unclear whether secreted IgD has a specific function. There is, however, evidence that a membrane bound form of IgD does have an important role; this will be reviewed later.

As an immune response proceeds, the predominant class of serum antibody often shifts from IgM to IgG. IgG is secreted as a monomer and is believed to possess more specialised effector functions that may be tailored to the requirements of more mature immune responses. Nossal *et al* proposed that the sequential expression of different classes of immunoglobulin by individual B cells accounts for the IgM to IgG change (Nossal et al., 1964). This idea was subsequently extended to account for the production of IgA and IgE (Kishimoto and Ishihara, 1972). IgA, in addition to being secreted as a monomer, is also produced in (IgA)₂ dimers containing a J chain and a molecule called secretory component which protects against proteolysis. Secretory component also facilitates the transport of IgA across epithelial cell layers, thus helping IgA to function as the major immunoglobulin in body secretions such as in the gut, and in saliva, milk and colostrum. IgE (secreted as a monomer) is specialised to protect against parasitic infections and is also important in allergic responses. It is present in relatively low quantities in the serum but can be seen concentrated on the surface of basophils and mast cells.

In fact, as had been predicted, individual B cells do sequentially express different classes of immunoglobulin. This is accomplished by a process called switch recombination or class switching where a heavy chain variable region (corresponding to

VDJ sequence) may be relocated upstream of an alternative heavy chain constant region (Figure 3). B-cell class switching can be modulated by environmental cues such as lipopolysaccharide (Kearney et al., 1975) and some cytokines (reviewed by Schulz and Coffman, 1991). Thus the identity of the heavy chain produced can be dictated by the stimulus, and therefore B cells can respond to their environment and switch to an isotype whose effector functions are potentially more appropriate to the immune response. One of the best characterised isotype switching mechanisms is the change to IgE production which is potentiated by the cytokine, interleukin-4 (IL-4). Incubation of B cells in growth medium containing IL-4 can induce the production of sterile ϵ germline mRNA transcripts (Jabara et al., 1990). The production of ϵ mRNA capable of encoding complete ϵ heavy chain protein can be induced by a second signal that is supplied by T cells (Shapira et al., 1991; Verceli et al., 1989). This T cell signal appears to be provided by the cell surface protein CD40L which, in addition to being expressed on activated T cells, is also present on several other cell types (Fanslow et al., 1992; Noelle et al., 1992) suggesting that it is not just T cells that can provide this stimulus to B cells. CD40L binds to the B cell surface protein, CD40. CD40 is a glycoprotein that has homology in its extracellular domains to other receptors such as the nerve growth factor receptor and is expressed on both immature and mature B cells (Braesch-Anderson., 1989; Stamenkovic et al., 1989). A critical role for the CD40L/CD40 interaction *in vivo* is suggested by the symptoms of humans who have the genetic defect; X-linked immunodeficiency with hyper-IgM which is caused by mutations in the CD40L gene (Allen et al., 1993; Armitage et al., 1992; Aruffo et al., 1993; DiSanto et al., 1993; Fuleihan et al., 1993; Korthauer et al., 1993; Ramesh et al., 1993). This rare disease is characterised by recurrent infections, normal to increased levels of serum IgM and low levels of serum IgA and IgG. (Rosen et al., 1961; Notarangelo et al., 1992; Geha et al., 1979; Levitt et al., 1983). CD40 itself appears to be important because mice which have been engineered to lack CD40 efficiently mount IgM but not IgG, IgA or IgE responses to T-cell dependent antigens (Kawabe et al., 1994). However, IgG responses to T-cell independent antigens appeared to be normal

in this study, thus suggesting that *in vivo* there are CD40-independent switching mechanisms.

Examination of cloned DNA representing the recombination products thought to have been generated by class switching suggests that this somatic rearrangement process exploits switch (S) regions which are located 5' to heavy chain constant regions (Kataoka et al., 1980; 1981; Nikaido et al., 1982; reviewed by Esser and Radbruch, 1990). The manipulation of the mouse genome using gene targeting techniques has confirmed the importance of S regions for switch recombination because deletion of a switch region located 5' to a heavy chain constant region in mice diminishes switch recombination to that isotype but not to others (Jung et al., 1993). Evidence that S regions are not the only DNA elements involved in switching comes from the finding that deletion of an enhancer element that is normally located 3' to the heavy chain coding region, abolishes switching to some isotypes (Cogne et al., 1994). It therefore appears that multiple species of DNA element in the heavy chain locus coordinate this rearrangement. It is not known how these elements are controlled by surface proteins such as CD40.

Membrane-Anchored Antibody

The predictions of Ehrlich and Burnet suggest that there is a receptor on B cells which regulates antibody production in response to antigen binding. The receptor is, in fact, a membrane-anchored form of antibody which is known as the B-cell antigen receptor. Upon binding of antigen, it can regulate B-cell proliferation and differentiation by transducing signals into the cytoplasm.

In 1965, an important aspect of Burnet's theory, namely the presence of a signalling receptor on B cells was strongly supported by the growth-promoting property of anti-immunoglobulin antisera on rabbit lymphocytes (Sell and Gell, 1965).

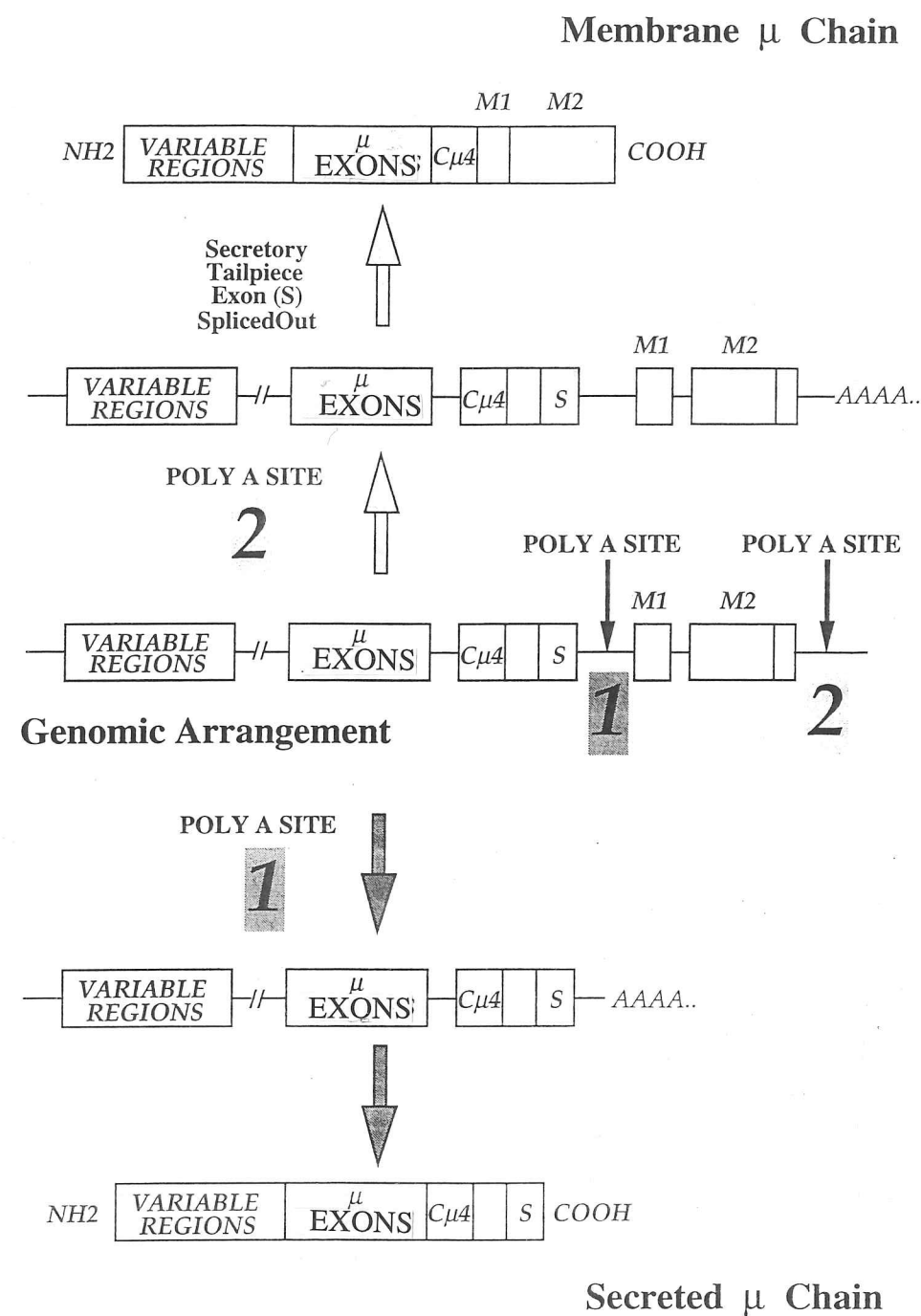


Figure 4

Schematic Diagram of the Production of Secreted or Membrane IgM by Differential RNA Processing

Route 1 => Secreted
Route 2 => Membrane

The specificity of the antisera suggested the existence of receptors containing antibody-like molecules. It was the development of molecular cloning technology in the 1970s, that allowed the nature of the B-cell antigen receptor to be properly assessed. Analysis of the cloned immunoglobulin μ heavy chain locus implied that, by alternative splicing, mRNA could give rise to either a secreted or membrane bound form of IgM (Alt et al., 1980; Early et al., 1980; Rogers et al., 1980). The alternative forms being generated by including, in the mRNA sequence specifying the constant region, an exon encoding a secretory tailpiece or exons predicted to encode a transmembrane anchor and cytoplasmic tail (Figure 4). Following the molecular cloning of other constant regions, it became apparent that all five classes of immunoglobulin could theoretically be processed into soluble or membrane forms by the use of optional exons (reviewed by Calabi and Neuberger, 1987). Therefore in 1980, the B-cell antigen receptor appeared to be composed of disulphide-linked antibody heavy and light chains anchored in the membrane in a $(HL)_2$ structure, with the ligand binding domains extending from the cell surface almost in anticipation of binding to antigen.

The Discovery of the Heterodimer

Early studies provided evidence that membrane immunoglobulin associates with other proteins (Koch and Haustein, 1983; Haustein and Von der Ahe, 1986). However, significant progress in this area was made following studies of immunoglobulin processing when it was shown that the membrane form of IgM (mIgM), when transfected into a myeloma cell line that is representative of a late B-cell differentiation stage, is not transported to the cell surface (Sitia et al., 1987). This suggested that myeloma cells lack factors that are important for the surface expression of mIgM. In an attempt to identify the cause of this abnormal processing of mIgM, Hombach *et al* isolated a mutant of this myeloma line that was capable of transporting mIgM to the cell surface (Hombach et al., 1988). Subsequent biochemical analyses showed that the membrane immunoglobulin, as well as having acquired the ability to be

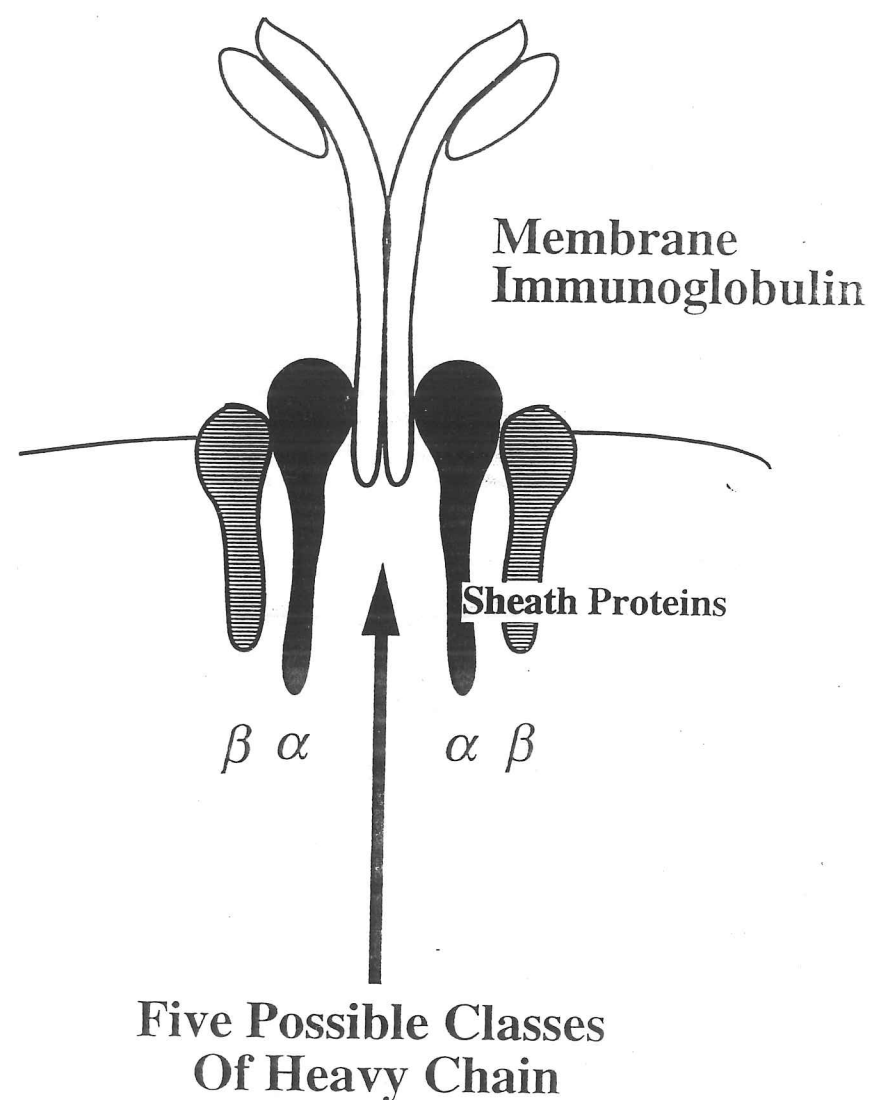


Figure 5

Basic Model of the Structure of the B-Cell Antigen Receptor

Membrane immunoglobulin is anchored in the cell membrane in a $(HL)_2$ structure where it can be complexed with the α/β sheath. There may be two α/β heterodimers per $(HL)_2$. IgD and IgG can exist stably on the cell surface in the absence of the sheath.

stably expressed on the cell surface, was now non-covalently associated with a disulphide-linked heterodimer. The cell variant had also become able to transcribe the B-cell specific gene, *mb-1* that had originally been cloned from a subtractive cDNA library and was predicted to encode a transmembrane protein product of an appropriate molecular weight to be, if glycosylated, a protein already seen associated with mIg. (Hombach et al., 1988; Sakaguchi et al., 1988). Hombach *et al* therefore proposed that mIgM may associate with the *mb-1* gene product (Hombach et al., 1988). This conclusion was consistent with the subsequent finding that co-expression of MB-1 with mIgM in a myeloma cell line allowed surface expression of heterodimer-associated mIgM molecules (Hombach et al., 1990a). Amino-terminal sequencing of the heterodimer proteins indicated that one was indeed MB-1, and the other, a B-cell specific protein also first cloned from a subtracted cDNA library, called B29 (Hombach et al., 1990b; Campbell et al., 1991; Hermanson et al., 1988); thus mIgM appears to be associated with two distinct disulphide-linked proteins. They may facilitate surface expression by acting as a sheath that masks the polar residues present in the transmembrane of mIgM and allow it to stably incorporated into the lipid bilayer (Williams et al., 1990; Neuberger et al., 1993).

Having refined the structure of the IgM class antigen receptor, the structures of the other classes of the antigen receptor remained to be examined in a similar manner. In 1991, it transpired that all classes of membrane immunoglobulin can associate with MB-1 and B29 (Venkitaraman et al., 1991). Notably, in a myeloma line, although the sheath proteins are essential for the transport of IgM, IgA and IgE to the cell surface, mIgD and mIgG can exist stably on the cell surface either complexed with or without the sheath (Figure 5) (Venkitaraman et al., 1991; Williams et al., 1993; Wienands and Reth, 1991). However as yet, there is no significant evidence for the existence of sheathless variants *in vivo*. MB-1 and B29 have been known by a number of designations but for the remainder of this work, they shall be called α and β respectively. They are B-cell specific transmembrane proteins and both are significantly

glycosylated (Sakaguichi et al., 1988; Hermanson et al., 1988). Indeed, the degree of glycosylation and thus the molecular weight of α varies according to the isotype of membrane immunoglobulin with which the sheath is associated (Venkitaraman et al., 1991). Furthermore, they each contain within their cytoplasmic tails, a conserved motif of leucine and tyrosine residues that was first noticed by Michael Reth and is of the general structure; D/E-X₇-D/E-X₂-Y-X₂-L/I-X₇-Y-X₂-L/I (Reth, 1989). This *Reth motif* also exists in the tails of a number of other proteins involved in signal transduction in lymphoid cells.

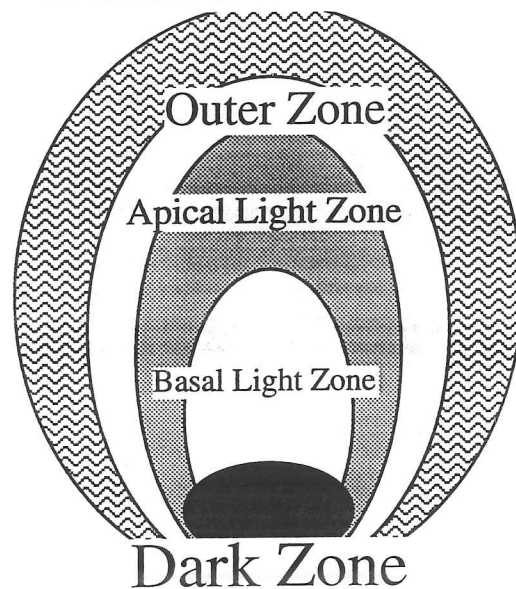
As will be subsequently discussed, the ability of the B-cell antigen receptor to transduce signals can be attributed to the α/β sheath. However, before considering the mechanisms involved in signalling, the contribution of mIg and the sheath to B-cell development will be reviewed.

B-Cell Development: An Overview

Cells of the B cell lineage are formed from pluripotent stem cells that appear to be present in a variety of sites in the embryo. The generation of B cells from these sites constitute the first phase of B cell generation which is responsible for the earliest B cells. The second phase is responsible for the maintenance of B cell numbers. In the mouse, this occurs predominantly in the bone marrow with contributions from the neonatal spleen and intestine associated Peyer's Patches (reviewed by Rolink and Melchers, 1993). Development in these organs consists of regulated proliferation, differentiation and cell migration. Following dedication to the B cell lineage, B cell development can be divided into three major stages based on the expression of immunoglobulin. These are pro-B (no significant expression of heavy or light chain genes), pre-B (expression of μ heavy chain) and B (both heavy and light chain expression). B cells may terminally differentiate into plasma cells. This cell type is specially adapted to produce soluble antibody and generally lacks expression of many B

Germinal Center Zones

Follicular Mantle



Major B Cell Morphology

Small, Recirculating

CD75 High, Mixed Phenotype

Small Centrocytes

Large Centrocytes

Centroblasts

Figure 6

Human Tonsil Germinal Center Zones

(Adapted from MacLennan et al., 1992)

cell surface molecules including the B-cell antigen receptor. Presumably from some stage in the differentiation process, memory B cells are derived. By convention, memory B cells express mIg, have undergone antigenic selection, are somatically mutated and can be recruited in secondary immune responses. They are also likely to have undergone class switching and thus express IgG, IgA or IgE. The actual nature of the phenomenon known as B cell memory is not understood. It has been speculated that there may be a special class of long lived memory B cells or even that memory stems from conventional B cells that have remained in the body as a consequence of interactions with persistent antigen (reviewed by Freitas et al., 1986; MacLennan and Gray, 1986).

Aggregations of lymphocytes known as germinal centers can be seen in peripheral lymphoid tissues and in the spleen. Recruitment of activated lymphoid cells and cellular proliferation probably contributes significantly to the formation of these sites. It is believed they facilitate the generation of immune responses in a number of ways. These include allowing antigen to be concentrated and presented by follicular dendritic cells and providing a conducive environment for B cells to interact with antigen and T cells. Fluorescence analyses have led to germinal centers from human tonsils being classified into several distinct compartments which contain populations of B cells that differ significantly in their morphology (Hardie et al., 1993; reviewed by MacLennan et al., 1992) (Figure 6). These include B cells that seem to express little or no surface immunoglobulin, B cells in various stages of the cell cycle, B cells that are not in cycle and some that appear to be dying. The significance of these zones and the involvement of these B cell types in immune responses remains to be determined. However, consistent with an important role, germinal centres are associated with B cell proliferation, somatic hypermutation, class switching and differentiation into plasma and memory cells.

The Involvement of Membrane Immunoglobulin

The μ heavy chain is the first isotype expressed during development. This isotype appears to be essential for normal B-cell differentiation because disruption of the membrane exon of the μ heavy chain abolishes B cell production (Kitamura et al., 1991). Mice exhibiting this defect have cells that are arrested at a pro-B cell stage suggesting that membrane anchored μ is essential for subsequent B-cell differentiation. In heterozygous ($\mu^{\text{wt}}/\mu^{\text{membrane exon knockout}}$) mice, there is a significant increase in the number of B cells expressing both heavy chain alleles (Kitamura and Rajewsky, 1992). Thus μ may also be involved in allelic exclusion by ensuring that only a single heavy chain locus is productively rearranged and expressed in each B cell.

Some strains of mice do not express significant endogenous immunoglobulin because they have non-functional antibody recombination machinery. The B cells in these mice are arrested at a pro-B cell stage. Introduction of a transgene that expresses μ protein allows B cells to pass through the pro-B stage but they then predominantly arrest at a pre-B cell stage. If both μ and light chain encoding transgenes are expressed, mature B cells are produced (Reichman-Fried et al., 1993; Spanopoulou et al., 1994; Young et al., 1994). One interpretation of these findings is that they imply μ is important at two stages during the production of B cells. It may not only drive pro-B to pre-B changes (as is also suggested by the phenotype of μ membrane exon knockout mice above) but could also be important for the differentiation of pre-B cells into mature B lymphocytes. The μ chain may operate in association with light chain in the latter process but with other proteins earlier in ontogeny when the antibody light chain loci are not productively rearranged and expressed.

How could μ function in the absence of κ or λ light chain? The answer appears to involve the discovery that μ chain can be seen associated with other novel proteins in early B cells and some cell lines. In pre-B cells, a proportion of μ is associated with

two proteins called Vpre-B and $\lambda 5$. These are called surrogate light chains and contain significant homology to the variable and constant regions of light chain but in contrast, they exhibit relatively little genetic variation (reviewed by Rolink and Melchers, 1993). Irrespective of its function, evidence suggestive of an important role for this pre-B cell immunoglobulin complex during B cell differentiation comes from findings which show that mice that are deleted for the $\lambda 5$ gene have few B cells in early life, however as the mice mature they acquire significant numbers of peripheral B cells (Kitamura et al., 1992). The complex may therefore drive a developmental process that ensures new born animals have a fully competent B cell compartment that can be easily maintained, although it remains a possibility that the incomplete block in development occurs because there are other proteins that partially compensate for the absence of $\lambda 5$ (Kitamura et al., 1991; Desiderio, 1994). Indirect evidence that $\mu/\lambda 5$ /VpreB assemblies function by transducing signals comes from the findings that they can be detected associating with an α/β sheath and that binding of antisera can induce intracellular biochemical changes associated with signal transduction (Nomura et al., 1991; Matsuo et al., 1993; Spanopoulou et al., 1994; Young et al., 1994; Brouns et al., 1993). The significance of these results is unclear because similar biochemical changes have not been reported in pure populations of normal pre-B cells (which seem to have a relatively low expression of this μ complex on their surface).

The δ heavy chain is not believed to be significantly expressed during early B-cell development and, consistent with this assumption, gross developmental abnormalities are not present in mice whose δ constant regions are disrupted so that IgD production is abolished (Roes and Rajewsky, 1993). However, these δ^- mice have a slower rate of antibody affinity maturation; the phenomenon whereby the affinity of serum antibody to antigen increases during an immune response, suggesting that IgD is involved in antibody production. In addition, in heterozygous (δ^-/δ^+) mice, the δ^- deficient cells are under-represented in the periphery suggesting they are at a selective disadvantage. Since IgD is only present in relatively small quantities in the serum but is

significantly expressed on the surface of B cells, the defects seen in δ -deficient mice may simply reflect abnormal responses to antigen by δ -deficient B cells. How mIgD functions and the rationale behind B cells expressing both mIgM and mIgD is not clear but these two isotypes do differ structurally in several ways. These include the ability of mIgD to be surface expressed in the absence of the α/β sheath (Venkitaraman et al., 1991; Wienands and Reth, 1992; Williams et al., 1993) and the presence within its extracellular domains of a flexible region that is often called a hinge. The differential sheath association properties of mIgD compared to mIgM and the flexibility of the hinge may contribute to unique signalling properties, for example the hinge may facilitate the capture of antigen and the ability of polyvalent antigen to crosslink mIgD with other mIg molecules or even with co-receptors such as the CD19/CD21 complex (see later).

B-Cell Programmed Death and Tolerance

Cytotoxic CD4⁺ T cells can induce B cell death by triggering a pathway that is dependent on the interaction of the B cell surface protein, Fas with a T-cell surface ligand. Stimulation *in vitro* of B cells via the antigen receptor (but not via CD40 or with LPS) protects them from this Fas-mediated apoptosis (programmed cell death) (Rothstein et al., 1995; references therein). Whilst it is unknown whether this process has a role *in vivo*, the protection conferred by the antigen receptor could promote the survival of antigen-specific B cells and retard non-specific B cell responses.

Stimulation of the B-cell antigen receptor does not always inhibit B cell death or induce events associated with activation such as proliferation because it may also cause some primary B cells and B cell lines to undergo apoptosis (reviewed by Green and Scott, 1994). The significance of this apoptotic pathway is unclear but may, at the simplest level, reflect that B cell death is triggered upon inappropriate activation through the receptor. Clearly such a process could serve to destroy B cells that are reactive with self-antigens thus presumably helping to prevent autoimmune disease. Theoretically

such self-reactive B cells could be generated after expression of newly assembled immunoglobulin genes, after class switching (a constant region may affect antibody binding), or somatic mutation. Recently it has become apparent that some stimuli can suppress antigen receptor initiated B-cell apoptosis. These include ligation of CD40 (Valentine and Licciardi, 1993; Tsubata et al., 1993), CD21 (Bonney et al., 1993) and CD2 (which is expressed on murine B cells) (Genaro et al., 1994; Yagita et al., 1989). Thus it seems that, certainly in some cases, stimulation of the antigen receptor causes apoptosis in the absence of appropriate co-stimulatory signals.

A number of murine models have been used to examine B-cell reactivity to self-antigens. These animals contain antibody transgenes that direct the expression of mIg with binding specificity for antigens that are expressed endogenously by the mouse (Nemazee et al., 1991; Hartley et al., 1991; Erikson et al., 1991; Okamoto et al., 1992; Brombacher et al., 1991; Goodnow et al., 1988; 1989; reviewed by Goodnow, 1992). The fate of the reactive B cells seems to depend on the ligand interaction, thus often B cells may be deleted (possibly by antigen receptor-mediated apoptosis) or driven into a state of inertia called tolerance. The *tolerant* B cells characterised by Goodnow *et al* which express anti-lysozyme antigen receptors and are reactive with endogenously produced lysozyme appear to be less efficient at entering lymphoid tissue from the periphery compared to normal B cells; thus suggesting that self-reactive B cells are actively excluded from sites important for B cell responses (Cyster et al., 1994). How this exclusion occurs is not known but there does appear to be some degree of competition between cells because in the absence of normal B cells, the anti-self B cells can efficiently enter lymphoid follicles (Cyster et al., 1994). Interestingly, lysozyme tolerant B cells are less efficient at producing signals when antigen binds to their B cell antigen receptors (Cooke et al., 1994; Ho et al., 1994). It is not clear whether these defects are a cause or a consequence of the B cell tolerance however they could result from the presence of abnormal antigen receptor structures that do not signal properly. In this regard, the tolerant B cells do express unusually low quantities of mIgM and

relatively large quantities of mIgD on their surfaces (Goodnow et al., 1988; 1989). Thus any signalling defect could simply be explained by the inability of mIgD to signal efficiently. This could reflect an uncoupling of IgD from the α/β sheath because mIgD can exist on the cell surface in an unsheathed state (Venkitaraman et al., 1991; Williams et al., 1993). Analyses of these cells show that mIgD does bind to α and β but the relative proportions of mIgD present in its sheathed or unsheathed state remains to be determined (Bell et al., 1994). As for the abnormally low production of IgM; this appears to arise from a processing defect in these cells resulting in mIgM being retained intracellularly. This is apparently not due to a deficiency in α or β production because they both seem to be present in normal quantities (Bell et al., 1994). It therefore seems likely that the retention is caused by aberrant post-translational modifications such as the inappropriate processing of carbohydrate side chains (Bell et al., 1994). In support of this hypothesis, there is evidence that mIgM requires α to be glycosylated for it to be efficiently transported to the surface (Neuberger et al., 1993). Membrane IgD may escape this retention because it does not require the sheath or sheath glycosylation for cell surface expression (Venkitaraman et al., 1991; Williams et al., 1993; Neuberger et al., 1993).

Antigen Presentation by the B-Cell Antigen Receptor

In addition to transducing signals, the B-cell antigen receptor can bind and internalise antigen, thus potentiating the degradation of antigen into peptide fragments that can be presented to T cells by MHC class II (reviewed by Lanzavecchia, 1990). This pathway can be 10^3 -fold more efficient than the presentation mediated by other types of antigen presenting cells and non-specific B cells (Kakiuchi et al., 1983; Rock et al., 1984; Tony and Parker, 1985; Lanzavecchia, 1985) and is therefore a potent mechanism for promoting the interaction of activated T cells with B cells. Thus antigen receptor-mediated antigen presentation, by promoting T/B collaboration of this nature may be important for efficient B cell antibody production; for example in class

switching and the suppression of apoptosis; processes which are regulated by the B cell-T cell CD40/CD40L interaction (Noelle et al., 1992; Fanslow et al., 1992; Valentine et al., 1992; Tsubata et al., 1993). The antigen receptor may be particularly suited to this role in antigen presentation because it can bind tightly to antigen and efficiently internalise bound ligands (Taylor et al., 1971; Metezeau et al., 1984) possibly by virtue of its ability to undergo constitutive endocytosis (Watts et al., 1988).

Analyses of mutant receptors suggests that the potent presenting properties and the ability to efficiently internalise antigen can be ascribed to the presence of the α/β sheath in the receptor (Patel and Neuberger, 1993). How the sheath mediates these properties remains a mystery. The B-cell antigen receptor may internalise via clathrin-coated pits (Davidson et al., 1988) but, interestingly, the α/β sheath does not obviously contain any previously characterised internalisation motifs which could mediate this interaction. One arrangement of amino acid residues which can potentiate the association of receptors with clathrin-coated pits consists of the sequence NPXY. This NPXY motif is often found in the cytoplasmic domains of transmembrane proteins and is essential for efficient clathrin-coated pit internalisation of the low density lipoprotein (LDL) receptor (Chen et al., 1990). Although mIgM and mIgD possess short cytoplasmic tails (usually considered to be a KVK sequence), the longer cytoplasmic tails of some of the other classes of mIg including mIgG contain an NPXY sequence (reviewed in Calabi and Neuberger, 1987). Whilst it has been proposed that this may mediate the interaction of antigen receptors with clathrin-coated pits thus promoting internalisation (Pearse, 1988), at present, no specific presentation or internalisation function has been directly attributed to any of the tails of membrane immunoglobulin. Provocatively, mIgM has been co-precipitated with a protein (BAP37) which also contains an NPXY motif (Terashima et al., 1994). This protein is one of five proteins that have recently been co-precipitated with mIgM or mIgD (Terashima et al., 1994; Kim et al., 1994) but it is presently unclear whether these associations occur under physiological conditions and their relevance to membrane immunoglobulin function has

not been determined. However, their predicted sequences and the fact that mutation of the transmembrane of mIg inhibits association (Terashima et al., 1994; Kim et al., 1994) suggests that they could be localised in the cytoplasm close to the membrane in a manner reminiscent of the viral protein, Nef which is anchored to the membrane by lipid and can mediate the downregulation of CD4 in T cells (Aiken et al., 1994). It is therefore tempting to speculate that they may be involved in receptor internalisation and intracellular trafficking.

To summarise, membrane immunoglobulin appears to be important for many aspects of B cell function. Its ability to transduce signals may be essential for it to perform these roles. Before reviewing how it signals, some general concepts of the signal transduction process will be introduced using a class of growth factor receptors and the T-cell receptor as models.

1 INTRODUCTION

1.2 SIGNAL TRANSDUCTION

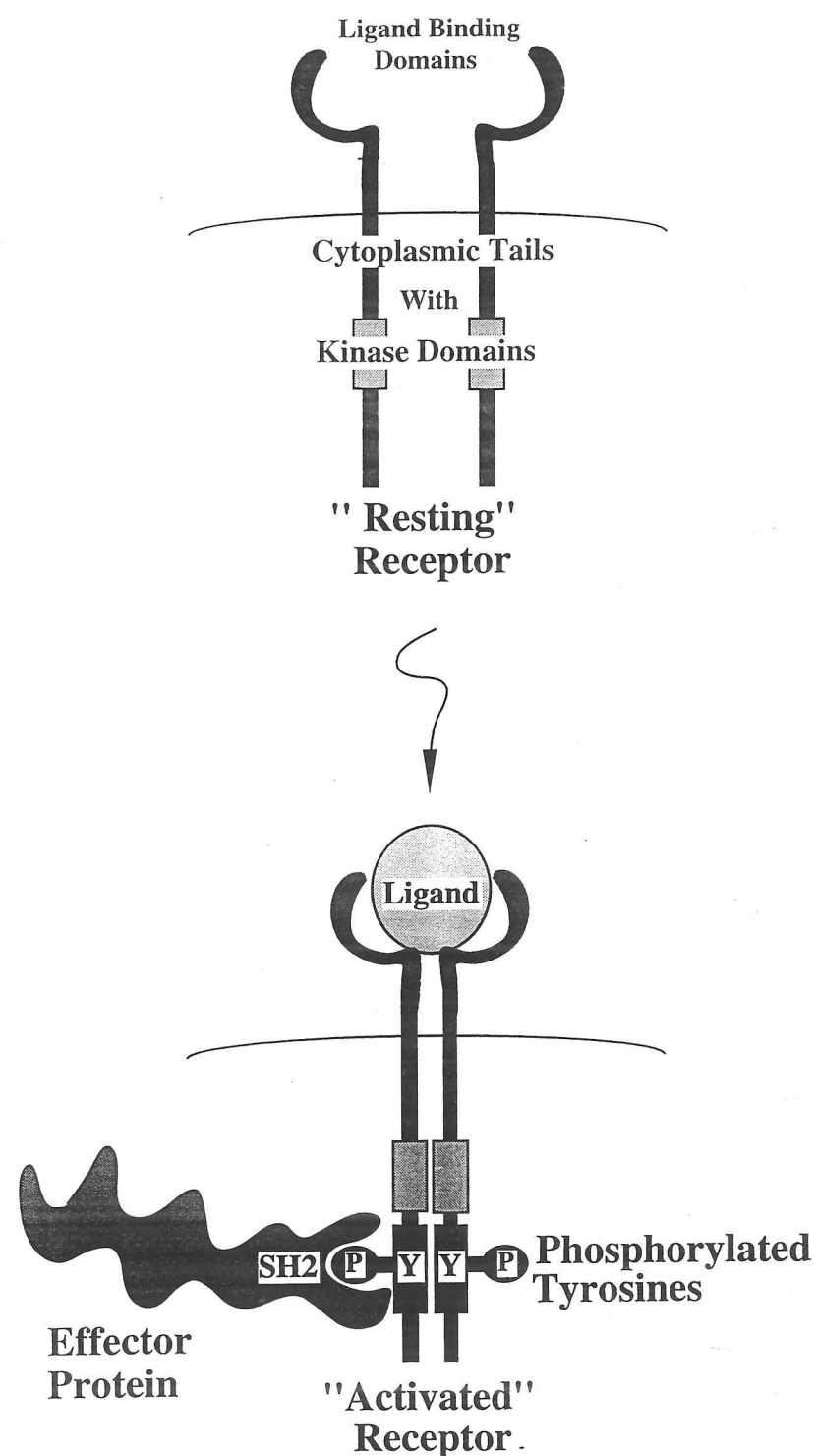


Figure 7

How a Hypothetical Growth Factor Receptor is Activated

Receptors That DO Contain Intrinsic Tyrosine Kinase Activity

Introduction

To develop and function normally, many cells must respond to environmental stimuli. As one means of achieving this, cells express cell surface receptors that may transduce signals when bound by appropriate ligands. One large group of these receptors exhibit a common basic structure which consists of an extracellular domain that mediates ligand binding, a transmembrane domain, and a cytoplasmic domain that contains a catalytic tyrosine kinase. Because of this arrangement they are called receptor tyrosine kinases. Members of this group include platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), insulin receptor (IR) and neuronal growth factor receptor (NGFR) (reviewed by Schlessinger and Ullrich, 1993). Ligand binding can induce a myriad of biochemical signals which can modulate cell proliferation, differentiation and growth arrest. The nature of the response seems to depend on the type of ligand, the receptor bound and the cell type stimulated.

Receptor Activation

Binding of ligand may induce these receptors to dimerise with themselves which, in turn, triggers a signalling cascade (Figure 7). This is, however, a generalisation and in fact, different receptors are activated in subtly different ways. For example PDGF is a dimeric molecule and activation is believed to be achieved by this growth factor forming a bridge between two receptor molecules (Hart et al., 1988; Heldin et al., 1988; 1989). In contrast, EGF does not bridge two receptor molecules in an obvious manner because it can only apparently bind a single EGFR molecule (Weber et al., 1984). Thus it has been proposed that the binding of EGF stabilises receptor molecules on the cell surface possibly as activated dimeric forms by inducing

conformational change (Greenfield et al., 1989). Although these receptors are activated by diverse means, ligand binding is generally followed by activation of the intrinsic protein tyrosine kinase and presumably by an intermolecular phosphorylation mechanism involving this kinase, the receptor is phosphorylated on tyrosines within its cytoplasmic domains (Honegger et al., 1989; 1990; Lammers et al., 1990). This process is important because both the tyrosine kinase and the presence of phosphorylated tyrosines appear to be essential for the generation of a full repertoire of signals. This is predominantly because the phosphorylated tyrosines mediate the docking of the receptor to important cytoplasmic effector molecules. Indeed, mutational analyses have defined distinct phosphotyrosines in growth factor receptor tails that bind different effector molecules. It therefore appears that the receptor tails function as a scaffold, on which signalling molecules can congregate.

Interactions with Cytoplasmic Signalling Machinery

Although signalling molecules may bind to the tails via a variety of mechanisms, the best characterised interactions are those involving the binding of phosphorylated tyrosines in the tails to SH2-domains in effector proteins. SH2-domains are considered to be motifs of around 100 amino acids that bind specifically to phosphotyrosine-containing sequences. Many proteins aggregate using this mechanism and, according to Schlessinger and Ullrich, they can be divided into two groups (Schlessinger and Ullrich, 1993). The designation type I refers to proteins that contain distinct enzymatic activities such as PLC- γ and GAP. Type II proteins such as GRB2 and Nck contain no defined enzymatic activity and may function as intermediaries that allow other molecules to interact indirectly with the receptor tail.

As a supplement to the intrinsic kinase activity, some growth factor receptors including the PDGFR recruit Src-related tyrosine kinases. It is worthwhile considering these Src-related kinases in more detail because, as will become evident later, the T- and

B-cell antigen receptors also associate with members of this kinase group. They constitute a family of at least nine members that were either originally characterised as retroviral oncogenes or isolated by screening cDNA libraries with appropriate probes. They all share an homologous structure that contains a motif believed to bind to proline-rich sequences (an SH3 domain), an SH2-domain, a kinase domain and, at the amino terminus, a sequence onto which lipid can be introduced so that these kinases can be anchored in the cytoplasmic side of plasma membrane. Binding of PDGF onto its receptor normally stimulates the phosphorylation of several tyrosine residues in the carboxyl tail of three of these kinases (Src, Fyn and Yes) that may serve to increase their activity (Gould and Hunter., 1988; Kypta et al., 1990; Ralston and Bishop, 1985). There may be a direct interaction with the receptor because *in vitro* these Src-related kinases associate at a low stoichiometry via their SH2-domains to phosphotyrosines in peptides corresponding to the juxtamembrane region of the cytoplasmic tail (Mori et al., 1993).

Although some receptors contain intrinsic tyrosine kinase activity, many receptors competently transduce signals, yet contain no intrinsic kinase. As a model system of this receptor type, the T-cell antigen receptor will be examined.

Receptors That DO NOT Contain Intrinsic Tyrosine Kinase Activity

Introduction to the T-Cell Antigen Receptor

T cells play important roles in the protection against pathogens. Following early T cell development, T cells may express on their surface either CD4 or alternatively, CD8. CD4⁺ T cells generally exhibit helper cell function i.e. they modulate the activity of other cells. CD8⁺ T cells are primarily cytotoxic and can mediate cell killing. In order to effectively perform these functions, T cells recognise antigen that has been processed

and presented by antigen presenting cells as peptide associated with MHC molecules. The T cell antigen receptor (TCR) can recognise and bind these antigens and mediate T cell activation by modulating and transducing signals into T cells (reviewed by Weiss and Littman, 1994). The best characterised T cell antigen receptor has an analogous structure to the B-cell antigen receptor because it similarly contains ligand binding molecules (α and β) which are non-covalently associated with disulphide-linked chains (the CD3 chains: δ , ϵ and γ and a ζ -containing dimer where ζ may be paired with another ζ , with an η or Fc ϵ RI γ chain). Although the stoichiometry of these interactions with the ligand binding domains is not known, I have depicted and will consider a generally accepted model of the TCR (Figure 8). Binding of the TCR to peptide antigen in the context of MHC is often not sufficient to induce the receptor to signal efficiently. To facilitate signalling, the TCR utilises the surface proteins CD4 or CD8 (which can bind MHC molecules) as co-receptors .

A simplistic model suggests that an activated T-cell antigen receptor consists of a T-cell receptor, antigen, MHC and CD4 or CD8. How does this assembly transduce signals? The CD3 and ζ chains contain motifs in their cytoplasmic tails that have been shown to be sufficient to transduce signals in T cells (Irving and Weiss, 1991; Irving et al., 1993; Letourneur and Klausner, 1992; Romeo et al., 1991). This motif consists of a conserved arrangement of tyrosine and leucines and is also present in the B-cell antigen receptor sheath proteins (Reth, 1989). Following TCR stimulation, these tyrosines can be phosphorylated (Irving et al., 1993; Letourneur and Klausner, 1992) thus providing potential docking sites for SH2-domain containing proteins analogous to the docking interactions that occur during growth factor receptor signalling. Unlike growth factor receptors, the T-cell receptor does not contain an intrinsic tyrosine kinase domain but it has been shown to co-precipitate with four cytoplasmic tyrosine kinases; namely Fyn, Lck, ZAP-70 and Syk (Chan et al., 1992; Couture et al., 1994; Samelson et al., 1990; Sarosi et al., 1992; Strauss and Weiss, 1993). These kinases belong to two classes; Fyn and Lck are Src-related tyrosine kinases, whereas ZAP-70 and Syk

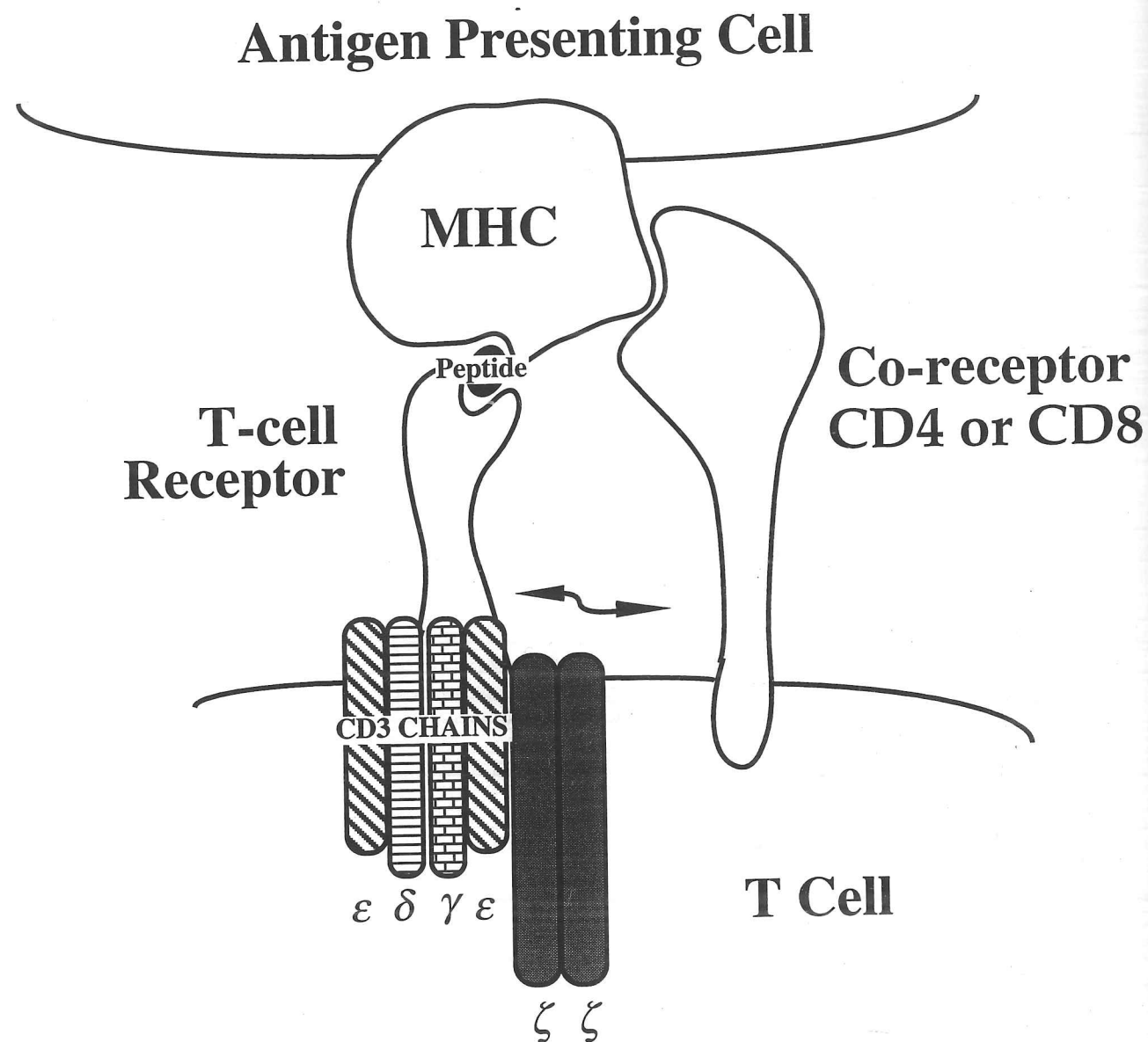


Figure 8

The T-Cell Receptor and its Interaction with Antigen, MHC and CD4 or CD8

are tyrosine kinases that lack obvious membrane attachment regions and characteristically contain two SH2-domains (Chan et al., 1992; Taniguchi et al., 1991).

T-Cell Antigen Receptor Associated Tyrosine Kinases

Fyn

Genetic evidence for the importance of Fyn during TCR signalling comes from findings that Fyn knockout mice show reduced sensitivity (Appleby et al., 1992; Stein et al., 1992), and Fyn overexpressing cells (Davidson et al., 1992) increased sensitivity, to stimulation via the TCR. There appears to be a physical and functional interaction because Fyn co-caps and co-precipitates (at a low stoichiometry) with the receptor (Samelson et al., 1990; Gassman et al., 1992; Sarosi et al., 1992). Furthermore, TCR-mediated activation can increase the activity of Fyn although only by around two fold (Da Silva et al., 1992; Tsygankov et al., 1992).

Lck

Consistent with an important role for this Src-related tyrosine kinase, Lck knockout mice, and transgenic mice expressing dominant negative mutants of Lck, despite exhibiting profound blockages in thymocyte development, do produce a few peripheral T cells that are defective in TCR signalling (Levin et al., 1993; Molina et al., 1993). In addition, TCR signalling can increase the activity of Lck by two fold (Danielian et al., 1992) and in an Lck-deficient T cell line, signals initiated by the TCR are significantly retarded (Karnitz et al., 1992; Strauss and Weiss, 1992).

Zap-70

Evidence that this kinase is essential to the immune system derives from studies which show that humans who have mutations in their Zap-70 genes are severely immunodeficient and possess defective T-cells (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994). Part of this importance may arise because ordinarily ZAP-70 is recruited by the CD3 and ζ chains of the TCR after receptor stimulation; an interaction probably mediated by its two SH2-domains binding to paired phosphotyrosines located within these chains (Chan et al., 1992; Irving and Weiss, 1993; Wang et al., 1993). It can be envisaged that the interaction of this kinase with the cytoplasmic tails of transmembrane proteins activates the enzyme and localises ZAP-70 to just beneath the cell membrane where favoured substrates and important cofactors could be located. One of these may be the 120 kD protein, fakB which has been shown to stably interact with ZAP-70 and is related to focal adhesion kinase (Kanner et al., 1994).

Syk

Syk has a similar structure to ZAP-70. It can also be co-precipitated with the TCR and an 120 kD protein and be activated following TCR receptor stimulation (Couture et al., 1994; Sidorenko et al., 1995).

How do these kinases act to initiate signals? The relative inability of an Lck mutant T cell line to phosphorylate TCR chains after TCR stimulation suggests that tyrosine phosphorylation of the CD3 and ζ chains can be performed by the Lck tyrosine kinase (Staass and Weiss, 1992). In support of this hypothesis, transfection experiments using non-lymphoid monkey cos cells have shown that ζ tyrosine phosphorylation can be mediated by the Src-related tyrosine kinases Lck and Fyn but not apparently by ZAP-70. Furthermore, only in the presence of significant Src-kinase activity does ZAP-70 detectably associate with the ζ chain (Chan et al., 1992). These

data suggest a model for TCR signal transduction where Src kinase phosphorylation of the CD3 and ζ tails allows the recruitment of ZAP-70 (and possibly Syk) (Chan et al., 1992). How phosphorylation of the tails is induced and how the signalling cascade is propagated is not clear. Clues may be gleaned from studying the roles of TCR co-receptors such as CD4 and CD8.

T-Cell Receptor Accessory Transmembrane Proteins

CD4 and CD8

The presence of CD4 or CD8 can facilitate TCR signalling by increasing the overall affinity that a TCR has for its peptide ligands. In addition, these coreceptors also function by recruiting the Src-related tyrosine kinase Lck into the complex because Lck interacts with CD4 and CD8 via distinct motifs within their tails (Rudd et al., 1988; Turner et al., 1990; reviewed by Weiss and Littman, 1994). It may function as a linker that facilitates the aggregation of CD4 with the TCR because CD4 attached to an Lck molecule containing a non-functional kinase domain can increase the efficiency of TCR signalling in response to MHC-associated antigen (Xu and Littman, 1993). However, the catalytic activity of the Lck associated with CD4/CD8 is important in some experimental systems (Glaichenhaus et al., 1991; Zamoyska et al., 1989). This may be because the enzyme, when brought in close proximity to the TCR by a co-receptor, increases the efficiency of CD3, ζ and ZAP-70 tyrosine phosphorylation; thus potentiating efficient signal transduction.

CD45

CD45 is a transmembrane protein that is expressed on many hematopoietic cells and contains intrinsic tyrosine phosphatase activity in its cytoplasmic tail (Charbonneau et al., 1988; Tonks et al., 1988). It is expressed in a variety of isoforms that arise

through alternative splicing of multiple exons that encode extracellular domains of the protein (reviewed by Penninger et al., 1993). Recently Novak and co-workers have found that different isoforms of CD45 modulate TCR signalling to different extents (Novak et al., 1994); thus implying that the extracellular domain of CD45 may influence the ability of CD45 to interact with particular proteins. Certainly the observation that CD45-deficient T cell lines exhibit defective TCR signalling suggests that this molecule has an important role in TCR-mediated activation (Pingel et al., 1989; Koretzky et al., 1990; 1991; Weaver et al., 1991; Kishihara et al., 1993). These signalling deficits can be attributed, at least in part, to the reduced activity of Src-related tyrosine kinases that is evident in CD45-deficient cell lines (Ostergaard et al., 1989; Mustelin et al., 1990; Shiroo et al., 1992; McFarland et al., 1993; Hurley et al., 1993; Sieh et al., 1993). It is believed that CD45 acts directly on these kinases to dephosphorylate regulatory tyrosines that are present in the carboxy-terminal region of Fyn and Lck thus priming them to participate in the signalling cascade. Consistent with this hypothesis, CD45 has been reported to co-cap and co-precipitate with Src-related tyrosine kinases (Guttinger et al., 1992; Mustelin et al., 1992; Schraven et al., 1991). However, CD45 has also been shown to co-precipitate with other proteins which may also be important for its function (Schraven et al., 1991; 1994; Takeda et al., 1992; 1994; Arendt and Ostergaard, 1995).

CD2

CD2 is a T cell surface protein that has been shown to associate with the T-cell receptor (Brown et al., 1989). Expression of CD2 can enhance, and anti-CD2 antibodies can inhibit antigen-dependent activation, proliferation, cytokine production and cytotoxicity *in vitro* (reviewed by Collins et al., 1994). The CD2 tail is important for its ability to transduce signals (He et al., 1988) but contains no intrinsic kinase activity or tyrosines that could dock with SH2-domains. Instead CD2 may exploit the TCR to produce signals (Spruyt et al., 1990) and consistent with this idea, tyrosines

within ζ are phosphorylated following CD2 crosslinking (Monostori et al., 1990). Thus CD2 ligation could modulate TCR signalling by influencing the activity of ζ and ζ -associated molecules when the TCR is interacting with MHC molecules. Under these circumstances CD2 may operate when bound by ligands such as LFA-3 which is often expressed on antigen presenting cells (reviewed by Dustin and Springer, 1991).

CD5

CD5 is another T cell surface protein that has been shown to co-precipitate with the TCR complex (Beyers et al., 1992a; Burgess et al., 1992). It is tyrosine phosphorylated after TCR ligation and contains a Reth motif in its cytoplasmic tail. (Burgess et al., 1992; Beyers et al., 1992b; Reth, 1989). Thus CD5 may modulate TCR signal transduction by recruiting SH2-domain containing proteins. Its only characterised ligand is CD72 (van de Velde et al., 1991) which is a B cell surface protein however it is unclear whether CD5/CD72 interactions are actually important *in vivo*. Preliminary characterisation of CD5 knockout mice has not revealed gross immune system abnormalities (Tarakhovsky et al., 1994).

Having considered how growth factor receptors and the T-cell receptor form activated complexes and transduce signals, B-cell antigen receptor signalling will now be discussed.

B-Cell Antigen Receptor Signal Transduction

Introduction

Whilst the B-cell antigen receptor is clearly capable of binding antigen, it is implicit from the proposals of Ehrlich and Burnet that it should also be able to transduce signals. The first evidence that this is the case came from experiments demonstrating

that anti-immunoglobulin antisera could stimulate the growth of rabbit lymphocytes (Sell and Gell, 1965). Subsequent work has shown that receptor stimulation can mediate a variety of biochemical signals including changes in intracellular calcium, inositol trisphosphate turnover and cellular tyrosine phosphorylation. These events are considered in more detail below.

An important role for tyrosine phosphorylation during B-cell receptor signalling is suggested by the observations that tyrosine kinase inhibitors retard signal generation (Carter et al., 1991) and the rapid phosphorylation of tyrosines in a variety of cellular proteins that occurs after receptor stimulation (Gold et al., 1990; Campbell and Sefton, 1990). Consistent with these findings, several tyrosine kinases appear to be activated after receptor stimulation. These include the Src-related tyrosine kinases, Lyn, Fyn, Lck and Blk (Burkhardt et al., 1991; Gold et al., 1994; Yamanashi et al., 1991), Syk (Hutchcroft et al., 1991; Yamada et al., 1993) and a third type of tyrosine kinase called Btk (Aoki et al., 1994; Weers et al., 1994). Several of the tyrosine phosphorylated proteins have been identified and include proteins such as the tyrosine kinases mentioned above (Aoki et al., 1994; Weers et al., 1994; Burkhardt et al., 1991; Gold et al., 1994; Yamanashi et al., 1991; Hutchcroft et al., 1991; Yamada et al., 1993), PLC- γ_1 and PLC- γ_2 (Coggeshall et al., 1992; Carter et al., 1991; Hempel et al., 1992), GAP (Gold et al., 1993), MAP-2 kinase (Casillas et al., 1991) and Vav (Bustelo and Barbacid, 1992; Gulbins et al., 1994). A variety of proteins (no characterised enzyme activity) which may be cytoplasmic or membrane proteins are also tyrosine phosphorylated. These include fakB (Kanner et al., 1994), Nck (Park and Rhee, 1992), SHC (Saxton et al., 1994; Smit et al., 1994), a 62 kD protein that interacts with GAP and contains homology to RNA-binding proteins (Gold et al., 1993), the regulatory subunit of PI3-Kinase (Gold et al., 1992), the BCR sheath proteins α and β (Gold et al., 1991), CD19 (Tuveson et al., 1993; Chalupney et al., 1993; Roifman and Li, 1993), CD22 (Schute et al., 1992; Peaker and Neuberger,

1993; Leprince et al., 1993), CD32/Fc γ RIIb1 (Muta et al., 1993) and the putative transcription factor HS1 (Yamanashi et al., 1993).

Signalling also induces the hydrolysis of phosphoinositides. Phosphatidylinositol 4,5-bisphosphate (PtdInsP2) is hydrolysed by phospholipase C (PLC) to produce inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) which mediate different effects (Coggeshall and Cambier 1984; Bijsterbosch et al., 1985). DAG induces the activation and translocation of protein kinase C to the membrane whereas the IP3 generation is followed rapidly by the release of calcium ions from stores in the endoplasmic reticulum after which there is an influx of extracellular calcium (Ransom et al., 1986; 1988; reviewed by Nishizuka, 1992).

In the hours following antigen receptor stimulation, increased surface expression of proteins which mediate the interaction of B cells with T cells can be detected (including MHC class II, Fas, B7 and B7.2) (Boussiotis et al., 1993; Freeman et al., 1993; Ho et al., 1994; Rothstein et al., 1985; Tsubata et al., 1993; reviewed by June et al., 1994). Additionally, the expression of Cdk2 - a protein involved in cell cycle control is also significantly increased (Tanguay and Chiles, 1994) and cytokine molecules are secreted (Justement et al., 1989). These changes may arise, at least in part, because signalling can modulate transcription. For example, transcription of the *egr-1*, *c-fos*, *c-myc* and human tumour necrosis factor α (TNF- α) genes is increased (Monroe, 1988; Klemsz et al., 1989; Seyfert et al., 1989; Goldfield et al., 1992). Furthermore, the transcription factors BSAP, NF-AT and NF κ B are activated (Choi et al., 1994; Venkataraman et al., 1994; Wakatsuki et al., 1994; Rooney et al., 1991). Other molecules that are activated include PI3-kinase (Tuveson et al., 1993; Gold et al., 1994), PI4-kinase (Gold et al., 1994), ras (Harwood and Cambier, 1993; Lazurus et al., 1993), p42 MAP-kinase (Casillas et al., 1991), and Vav (Gulbins et al., 1994). The stimulation of a novel serine/threonine kinase that phosphorylates c-Fos (Nel et al., 1994), calmodulin kinase activity (Fisher et al., 1991) and of protein kinase C (see

above) may contribute to increases in serine phosphorylation that can be detected on Lck, Ets-1 and CREB (both DNA-binding proteins) and Fc γ RII (Gold et al., 1994; Fisher et al., 1991; Hunziker et al., 1990; Xie and Rothstein, 1995). In addition, trimeric G proteins have been implicated in the signal transduction process. This is because of the effect of synthetic analogues of GTP which can enhance or inhibit the hydrolysis of phosphoinositide after receptor signalling (Gold et al., 1987; Harnett and Klaus, 1988; Harnett and Rigby, 1992) and the inhibitory nature of pertussis toxin on antigen receptor signal transduction (Monroe and Halder, 1989; Blank et al., 1990; Melamed et al., 1992).

The α/β Sheath Can Mediate Intracellular Signalling

How can the B-cell antigen receptor transduce such signals? Several investigations designed to determine which components of the B cell antigen receptor are involved in signalling have involved the expression of mutant antigen receptors and, after stimulation, assaying for the induction of a variety of intracellular signals. These studies demonstrate that the sheath proteins are primarily responsible for the signal generation observed (Alber et al., 1993; Blum et al., 1993; Flaswinkel and Reth., 1993; Kim et al., 1993; Sanchez et al., 1993; Law et al., 1993; Williams et al., 1993). This is concluded because mutant antigen receptors that do not associate with the sheath are ineffective at signalling, and fusions of the α or β cytoplasmic domains to other transmembrane proteins can signal independently of the normal antigen receptor complex. In some studies (Alber et al., 1993; Blum et al., 1993; Flaswinkel and Reth., 1993; Kim et al., 1993; Sanchez et al., 1993), but not all, (Law et al., 1993), the α cytoplasmic tail is more effective at initiating signals than the tail of β . Consistent with the sheath proteins possessing dissimilar signalling potencies, *in vitro*, the α tail, compared with the β tail associates with different affinities to several phosphoproteins (Clark et al., 1992). However, such differential associations have not been reproduced

in cellular systems thus overall it is unclear whether α and β have distinct roles in signal generation.

Tyrosines in the α and β tails appear to be important because signal generation is inhibited when they are mutated (Flaswinkel and Reth, 1993; Williams et al., 1993; Sanchez et al., 1993). This importance possibly reflects the fact that, after antigen receptor stimulation, they are phosphorylated and thus may provide binding sites for SH2-domain containing proteins (Gold et al., 1991). In this regard, the B-cell antigen receptor has been co-precipitated with several tyrosine kinases that contain SH2-domains (albeit detectably only at low stoichiometries). These are Syk and the Src-related tyrosine kinases, Lyn, Fyn, Lck and Blk (Hutchcroft et al., 1992; Yamanashi et al., 1991; Yamada et al., 1993; Campbell and Sefton, 1992; Burkhardt et al., 1991). These kinases, together with another tyrosine kinase called Btk are phosphorylated and activated after receptor stimulation (reaffirming what has been noted previously, references also shown earlier). Further analyses of the function of these molecules are summarised below.

B-Cell Antigen Receptor Associated Tyrosine Kinases

Src-Related Tyrosine Kinases; Blk, Fyn, Lck & Lyn

Evidence that Lyn is particularly important for antigen receptor signal transduction comes from results that show that a mutant chicken B cell line which does not express Lyn, displays a delayed and slow calcium flux and reduced tyrosine phosphorylation after receptor stimulation (Takata et al., 1994). Blk is the only kinase of those discussed here that is believed to be expressed exclusively in the B cell lineage (Dymecki et al., 1990). Expression appears to be limited predominantly to immature B cells suggesting that it has an important role in early B cell ontogeny. In a B cell line that dies after B-cell antigen receptor stimulation, incubation with antisense *blk* RNA

decreases receptor initiated cell death (Yao and Scott, 1993) thus suggesting that Blk has a role in mediating apoptosis.

Syk

Although Syk is similar in structure to the kinase ZAP-70, unlike ZAP-70, it is significantly expressed in B cells (Taniguchi et al., 1991; Chan et al., 1992). Evidence of the importance of Syk for receptor signalling has been obtained from studying mutant chicken B cells that lack Syk. Following antigen receptor stimulation, these cells do not efficiently mobilise calcium nor mediate increases in IP₃, and do not efficiently mediate the tyrosine phosphorylation of several proteins including not detectably tyrosine phosphorylating PLC- γ_2 (Takata et al., 1994). This phenotype is distinct from the phenotype of similar cells that lack Lyn (which do mediate PLC- γ_2 tyrosine phosphorylation, produce IP₃ and show a different calcium mobilisation pattern) suggesting that Syk and Lyn have distinct roles in the B-cell antigen receptor signalling cascade (Takata et al., 1994). Consistent with an involvement in phospholipid metabolism, Syk has been reported to co-precipitate with PLC- γ_1 (Sidorenko et al., 1995).

Btk

Btk is an acronym of Bruton's tyrosine kinase. It was named after Bruton who, in 1952, described a young male with hypogammaglobulinaemia and multiple bacterial infections; symptoms that also occur in humans that have defective Btk function (Bruton, 1952; Tsukada et al., 1993; Vetrie et al., 1993). Btk deficiency manifests as X-linked agammaglobulinemia (XLA) in humans (Tsukada et al., 1993; Vetri et al., 1993) and results in X-linked immunodeficiency (xid) in mice (Thomas et al., 1993; Rawlings et al., 1993; reviewed by Conley et al., 1994 and Rawlings et al., 1994). XLA patients have significantly decreased levels of serum immunoglobulin and have a

severe deficit of B cells which appears to result from B cell developmental defects (Siegal et al., 1971; Geha et al., 1973 and references above). Xid mice, in contrast, do not show such significant decreases in peripheral B cell numbers but those that are present respond abnormally to stimulation via a variety of surface antigens including membrane immunoglobulin. The differences in B cell defects between mouse and humans suggest that the specific contributions of Btk to B cell development differ in these two mammals. Although Btk is expressed in both the myeloid and B cell lineages, no myeloid defects have yet been characterised in Btk-deficient animals.

Btk, like Src-related tyrosine kinases, contains a tyrosine kinase domain, SH2 and SH3 domains, but lacks consensus lipid anchoring sites and shares much similarity in its other regions to a *Drosophila* tyrosine kinase, Tec and the T-cell kinase Itk/Tsk/Emt (Tsukada et al., 1993; Vetri et al., 1993; Mano et al., 1993; Siliciano et al., 1992; Heyech and Berg, 1993). This similarity includes a pleckstrin homology unit which has been found in other signalling molecules (Mayer et al., 1993; Haslam et al., 1993; Musacchio et al., 1993) and is so named because it was first described in the protein kinase C substrate, pleckstrin (Tyers et al., 1988). The mutations that have been characterised in the Btk gene suggest that the SH2, SH3 and pleckstrin domains are all important for function.

The view of the B-cell antigen receptor which has been emphasised so far, is that of membrane immunoglobulin that is capable of associating with tyrosine kinases of two distinct classes (Figure 9) and activating tyrosine kinases of three classes. How do these kinases co-operate with the sheath proteins during signal transduction? The similarities between the B-cell antigen receptor and the TCR do suggest they signal in a similar way and in favour of this assumption, Burkhardt *et al* have shown that α and β tails can efficiently signal in T cells (Burkhardt et al., 1993). Thus an attractive hypothesis is that Src-related tyrosine kinases phosphorylate the sheath tyrosines which then allows the recruitment of effector proteins containing SH2-domains such as Syk; a

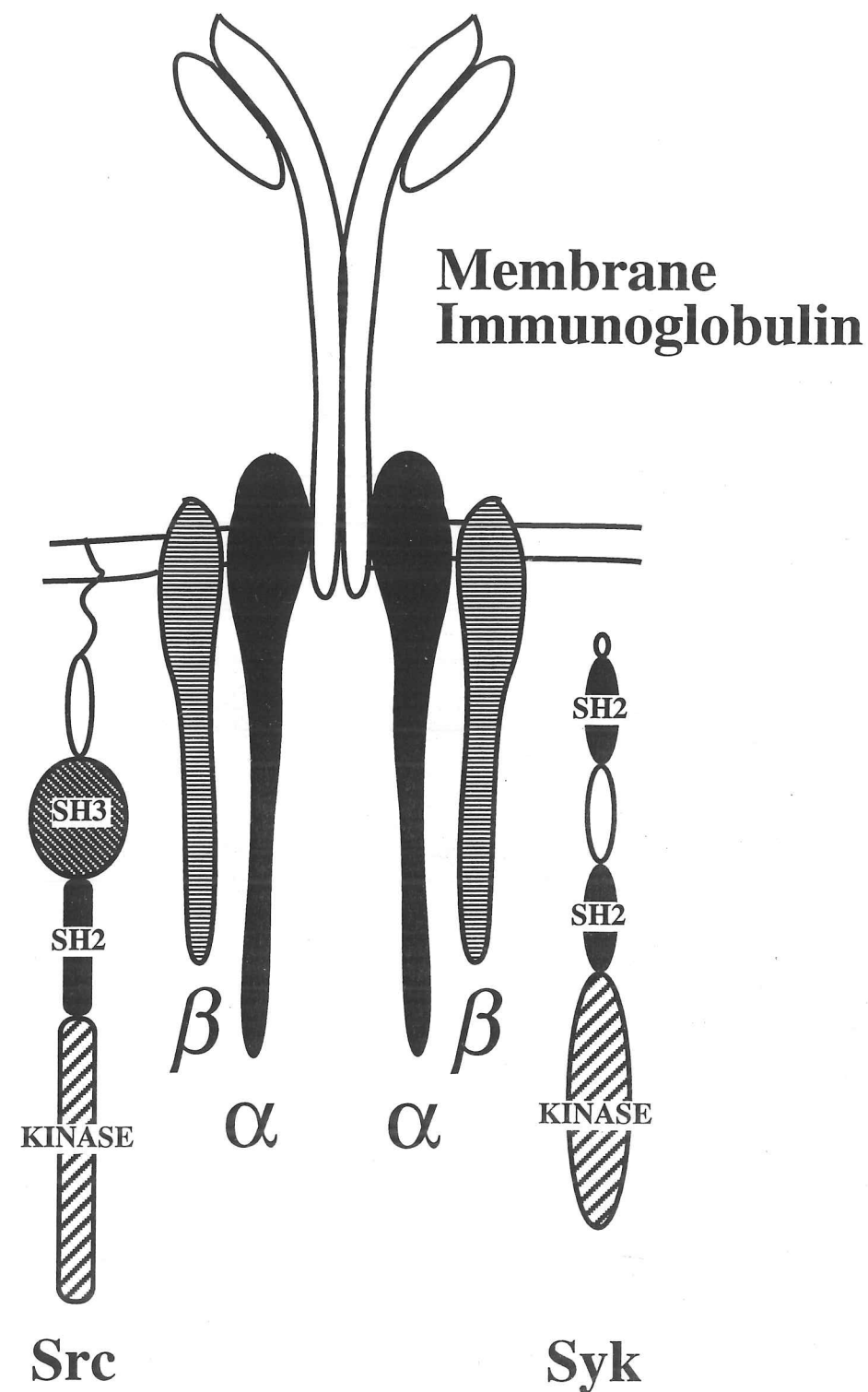


Figure 9

The B-Cell Antigen Receptor Complex and its Associated Tyrosine Kinases

model analogous to that proposed for T-cell receptor signal generation (Chan et al., 1992). It is unclear how the cascade continues, however extrapolation from the phenotype of Syk-deficient, mutant chicken B cells, suggests that Syk catalyses phospholipid hydrolysis by phosphorylating and activating PLC- γ_2 (Takata et al., 1994).

Interestingly, mutagenesis and expression of CD4 in non-lymphoid cells has suggested that its association with the Src-related tyrosine kinase, Lck inhibits CD4 endocytosis (Pelchen-Matthews et al., 1992; 1993; Sleckman et al., 1992). Evidence that tyrosine kinases may similarly regulate antigen receptor internalisation derives from the findings that treatment of primary B cells with tyrosine kinase inhibitors significantly retards receptor endocytosis (Pure and Tardelli, 1992). However, although several tyrosine kinases have been co-precipitated with the B-cell antigen receptor and are activated after signalling, it is not known if they are involved in regulating antigen receptor endocytosis (Campbell and Sefton, 1992; Burkhardt et al., 1991; Yamanashi et al., 1991; Hutchcroft et al., 1991). Studies using a variety of mutant antigen receptors have implied that the ability of the β tail to potentiate internalisation and presentation are not dependent on its ability to efficiently induce tyrosine phosphorylation; suggesting that activation of tyrosine kinases by the receptor is not essential for receptor endocytosis or presentation (Patel and Neuberger, 1994; Williams et al., 1994). Notably these experiments were performed using transfectants of a transformed B cell line so that it is not clear whether these conclusions apply to the normal antigen receptor in primary B cells. This serves to emphasise that it remains perfectly feasible that receptor signalling may drive internalisation at some stages of B-cell development and also that the receptor may utilise multiple internalisation mechanisms.

Whilst the ability of the B-cell antigen receptor to transduce signals can be ascribed to the α/β sheath, there is much evidence that the B-cell antigen receptor, like

the TCR, interacts with variety of accessory transmembrane proteins that may modulate its activity *in vivo*; this is reviewed below.

B-Cell Antigen Receptor Accessory Transmembrane Proteins

CD22

The B-cell specific, transmembrane protein CD22 is rapidly tyrosine phosphorylated on its cytoplasmic tail after antigen receptor crosslinking (Schulte et al., 1992; Peaker and Neuberger, 1993; LePrince et al., 1993). Like the sheath, it can also be co-precipitated with membrane immunoglobulin, although in detergents lysates, the interaction is of a much lower stoichiometry than that of membrane immunoglobulin with the α/β sheath (Peaker and Neuberger, 1993). CD22 may therefore recruit additional cytoplasmic effector proteins into the complex. In support of this suggestion, it has been found that the cytoplasmic tail contains a sequence with significant homology to the activation motifs present in the α/β sheath (Peaker and Neuberger, 1993; LePrince et al., 1993). The physiological functions of CD22 are not yet known, however, its extracellular domain contains homologies to a variety of adhesion molecules and, indeed, it can mediate homotypic B-cell to B-cell interactions and heterotypic B cell adhesion to a variety of cell types (Stamenkovic and Seed, 1990; Wilson et al., 1991; Torres et al., 1993). At present, it appears CD22 binds to a variety of proteins that contain sialylated carbohydrate groups, including CD45RO (a T-cell isoform of the transmembrane phosphatase) and CD75 (Stamenkovic et al., 1991; Sgoi et al., 1993; Powel et al., 1993). CD22 may function when co-crosslinked with the antigen receptor on the cell surface. Indeed, consistent with this hypothesis, anti-CD22 antibodies have been reported to amplify antigen receptor-mediated calcium mobilisation and proliferation (Pezzuto et al., 1987).

The CD19/CD21 Complex

The transmembrane proteins CD19 and CD21 are components of a multi-molecular complex on the surface of human B lymphocytes (reviewed by Fearon, 1993). The complex also contains proteins called TAPA-1 and Leu-13 (Bradbury et al., 1992), possibly CD38 (Funaro et al., 1993) and co-precipitates with Src-related tyrosine kinases (Uckun et al., 1993; van Noesel et al., 1992). CD19 has been proposed to interact with the cell surface antigen, CD77; a glycosphingolipid globotriaosyl ceramide, suggesting that such interactions may be important at certain stages in B-cell development (Maloney et al., 1994). CD21 is apparently capable of interacting with several ligands including C3 fragments of complement. Thus binding of antigen-antibody complexes containing complement to B cells may provide a convenient means of inducing co-crosslinking of the CD19/CD21 complex with the antigen receptor. An important role for this process is suggested by findings that CD19 can amplify antigen receptor signals when co-crosslinked with the B-cell antigen receptor (Carter and Fearon, 1992). This amplification can substantially reduce the amount of antigen receptor crosslinking needed to initiate signals suggesting that the CD19/CD21 complex is important for B-cell activation at low antigen concentrations (Carter and Fearon, 1992). Other evidence that this complex operates in partnership with the antigen receptor comes from findings that demonstrate that CD19 aggregates and co-modulates with membrane immunoglobulin (Pesando et al., 1989) and, like CD22, is rapidly tyrosine phosphorylated after antigen receptor stimulation (Tuveson et al., 1993; Chalupny et al., 1993; Uckun et al., 1993). This phosphorylation of CD19 facilitates its association with the cytoplasmic effector molecule, PI3-kinase because PI3-kinase binds, via two SH2-domains in its regulatory subunit, to a pair of phosphotyrosines in the CD19 tail (Tuveson et al., 1993). The significance of this interaction for B cell function is unclear but it could be involved in the ability of CD19 to modulate signals. These have been reported to include its ability to stimulate DNA synthesis, calcium mobilisation, tyrosine kinase activation and the tyrosine

phosphorylation and translocation to the membrane of SHC (Carter and Fearon, 1992; Matsumoto et al., 1993; Bradbury et al., 1993; Lankester et al., 1994; Uckun et al., 1993).

CD32

CD32 (also known as the Fc γ R11b1 receptor) is a surface protein that binds to the constant regions of IgG. Stimulation of the antigen receptor with antisera can result in antigen receptors co-aggregating with CD32 on the cell surface. This probably occurs because the soluble antibody can bind to mIg with its variable regions and simultaneously interact with CD32 via the Fc portions of its constant regions. Evidence of an important role for this phenomenon comes from studies which show that co-crosslinking of the antigen receptor with CD32 can inhibit antigen receptor signalling. It reduces calcium mobilisation, DNA synthesis, inositol phosphate metabolism, interleukin-2 (IL-2) production and inhibits B-cell maturation and transcription of the *egr-1* and *egr-2* genes (Bijsterbosch and Klaus, 1985; Phillips et al., 1984; Wilson et al., 1987; Gottschalk et al., 1994; Muta et al., 1994; reviewed by Friedman, 1993). Furthermore, it causes the hyperphosphorylation of a 62 kD protein of unknown function that interacts with GAP (Gottschalk et al., 1994). Thus CD32 may dampen B cell responses *in vivo*.

CD32 may mediate its effects on signalling through its cytoplasmic tail which is phosphorylated on serine and tyrosine residues upon co-crosslinking (Hunziger et al., 1990; Muta et al., 1994). The relevance of the serine phosphorylation is not known however evidence that the tyrosine phosphorylation is important comes from findings which demonstrate that a single phosphorylatable tyrosine in the cytoplasmic tail of CD32 is essential for the inhibition of calcium mobilisation and IL-2 production (Muta et al., 1994) (The importance of this tyrosine for the other effects have not yet been tested). Indeed, it is present within a 13 amino acid motif in the tail which modulates

antigen receptor signalling (Muta et al., 1994). This implies that the regulatory activity of this Fc receptor is not merely based on sequestration of the antigen receptor away from important signalling apparatus but may also reflect the recruitment of SH2-domain containing proteins by the CD32 cytoplasmic tail.

CD38

CD38 co-caps with the T-cell antigen receptor on T cells, CD16 on natural killer cells, and with the CD19/CD21 complex and possibly membrane immunoglobulin on B cells (Funaro et al., 1990). It is a transmembrane protein containing within its extracellular domain, homology to the enzyme, ADP-ribosyl cyclase (States et al., 1992) and has been shown to be capable of catalysing the formation of cyclic ADP-ribose, a molecule that is believed to function as a second messenger during signal generation (Meszaros et al., 1993; Gallone et al., 1993). Evidence for a direct role in mediating signals derives from findings which show that CD38 can elicit a mitogenic response when it is bound by anti-CD38 antibodies (Funaro et al., 1993). However, further characterisation is clearly necessary to ascertain the significance of these findings.

CD45

In CD45-exon6 knockout mice, B lymphocytes do not express CD45 (Kishihara et al., 1993). The behaviour of these CD45-deficient B cells, which unlike normal B cells, do not proliferate in response to anti-immunoglobulin antisera implies that CD45 is essential for normal antigen receptor function. These mice do, however, have a normal sized B cell compartment and produce antibody (although in unspecified quantities), thus the contribution of CD45 to B cell function *in vivo* is unclear (Kishihara et al., 1993). Both a functional and physical interaction of CD45 with the BCR is suggested by a study which reports that CD45 and the antigen receptor co-cap

and co-modulate and that CD45 expression can increase the antigen receptor-mediated calcium mobilisation in a CD45-deficient cell line (Justement et al., 1991). In B cells, analogously to its role in T cells, CD45 may regulate the activity of Src-related tyrosine kinases. Indeed, consistent with an ability to regulate tyrosine phosphorylation, CD45-deficient variants of the WEHI-231 B cell line (often considered to represent an immature B cell) have high levels of constitutive cellular tyrosine phosphorylation (Ogimoto et al., 1994).

Conclusion

The B-cell antigen receptor seems to play important roles in driving the production of B cells, inducing B cell activation, both preventing and causing B cell apoptosis and mediating antigen presentation. It appears likely that many of these properties are mediated by the α/β sheath acting in concert with a variety of accessory transmembrane proteins and cytoplasmic effector molecules.

Aims of this Work

This thesis investigates the structure and function of the B-cell antigen receptor complex. It covers work performed since 1991, a few months after it was discovered that all five classes of membrane immunoglobulin associate with the α/β sheath.

MATERIALS AND METHODS

Chemicals/Materials

All basic chemicals from BDH, Poole, UK or Sigma Chemical Company, Poole, UK, unless otherwise stated. Restriction enzymes were from Boehringer Mannheim or New England Biolabs.

40% (w/v) acrylamide/bisacrylamide 19:1 - **Molecular Biology Products**

agarose - **Gibco/BRL**

cyanogen bromide-activated Sepharose - **Pharmacia**

DMEM lacking essential amino acids, glucose and phosphate - **Gibco/BRL**

DMEM supplements - **Gibco/BRL**

endoglycosidase F/poly-N-glycosidase F - **Boehringer Mannheim**

Enhanced Chemluminescence System - **Amersham International**

fetal calf serum - **PAA Labor-und Forschungsgesellschaft mbH**

FITC-conjugated streptavidin - **Pierce**

[γ -³²P]-ATP (3000 Ci/mmol) - **Amersham International**

Geneticin (G418 sulphate) - **Gibco/BRL**

glacial acetic acid - **Fisons**

hepes - **Gibco/BRL**

HRP-conjugated streptavidin - **Pierce**

hygromycin - **Calbiochem**

¹²⁵I-protein A - **Amersham International**

Immobilon P - **Millipore**

methanol - **Rhone-Poulenc**

mycophenolic Acid - **Gibco/BRL**

NHS-biotin (10 mg/ml in DMF) - **Amersham International**

NHS-SS-biotin - **Pierce**

nitrocellulose - **Schleicher and Schuell**

NIP-conjugated BSA- gift of G.T. Williams

orthophosphate (10 mCi/ml) - **Amersham International**

Rainbow markers ^{14}C 14300 kD-200000 kD - **Amersham International**

Rainbow markers 14300 kD-200000 kD- **Amersham International**

Saranwrap - **Dow**

Spectra/Por membrane mwco 6-8000 - **Spectrum Medical Industries inc**

sulfo-NHS-biotin - **Pierce**

sulfo-SHPP - **Pierce**

Tween 20 - **NBS Biologicals**

ethanol (95% or absolute) - **Hayman**

methanol - **Rathburn**

Cells

All cells were grown in DMEM, 10% FCS, 50 mM β -mercaptoethanol at 37°C in an atmosphere of 10% CO_2 . The following cell lines from the laboratory collection were used: A20 (murine B-cell lymphoma) (Kim et al., 1979), BCL₁ (murine B-cell lymphoma), J558L (murine myeloma) (Oi et al., 1983), Daudi (Burkitt lymphoma), Raji (Burkitt lymphoma), NIH3T3 (murine fibroblast) (Jainchill et al., 1969), WEHI-231 (murine B-cell lymphoma). A second clone of WEHI-231 was obtained from G. Klaus. The TIB163 hydridoma (Symington et al., 1982) was obtained from the ATCC (American Type Culture Collection). For additional details on any of these cell lines see the ATCC catalogue (Hay et al., 1988). A list of the transfectants used in this study can be found in the Appendix. Tonsils were obtained from patients following routine tonsilectomy and the cells isolated by gently teasing them from the tissue. The cellular contents from a single tonsil were then resuspended in 5 ml PBS, gently layered on 5 ml Ficoll and centrifuged at 2800 rpm at RT for 20 minutes. The cells at the interface were washed once in 10 ml PBS before being used for further analysis. This was done by centrifugation at 1000 rpm for 5 minutes at RT and the pellet resuspended. Mouse splenic cells were isolated by gently kneading the murine spleens. The cellular contents

from a spleen were resuspended in 5 ml PBS, gently layered on 5 ml Percoll and centrifuged at 2500 rpm at RT for 20 minutes. The cells at the interface were washed in PBS (as above) before being used for analysis.

Antisera

Rabbit anti-human μ antiserum (Fc specific), F(ab')₂ goat anti-human μ (Fc specific), rabbit anti-goat IgG (minimal cross-reaction to human serum proteins), goat anti-human γ antiserum (Fc specific), F(ab')₂ goat anti-mouse IgM, F(ab')₂ goat anti-mouse IgG, peroxidase-conjugated donkey anti-rabbit IgG, Rabbit anti-mouse IgM were obtained from Jackson ImmunoResearch. Goat anti-human δ antiserum, FITC-conjugated goat anti-mouse IgM, FITC-conjugated goat anti-mouse IgG1 were from Southern Biotechnology. Rabbit anti-phosphotyrosine antisera was purchased from ICN. Phycoerythrin-conjugated rat anti-CD45 (16A) mAb was from Gibco. Mouse monoclonal antibodies HD6 (IgG1 anti-human CD22), HD39 (IgG1 anti-human CD22). T29/33 (IgG2b anti-CD45) were from Boehringer Mannheim. HD37 (IgG1 anti-human CD19), To15 (IgG2b anti-human CD22) and 4KB128 (IgG2b anti-human CD22) were from Dako. IgG2b anti-CD21 was a gift from N. Ling. The mAb, HM47 (Anti- β polypeptide) was a gift from D. Mason. The rat anti-mouse transferrin receptor monoclonal antibody R17 was a gift from C. Hopkins. The IgG1 mAb used as a control was MOPC-21 from Sigma, the IgG2b mAb used as a control was AG10 provided by G.T. Williams. The IgG1 anti-murine CD22 mAb was prepared from the TIB163 hybridoma (Symington et al., 1982; Torres et al., 1993).

Standard Buffers

PBS

8 g NaCl, 0.2 g KCl, 1.44 g Na₂PO₄, 0.24 g
KH₂PO₄ in 1 litre of distilled water (pH 7.4)

from a spleen were resuspended in 5 ml PBS, gently layered on 5 ml Percoll and centrifuged at 2500 rpm at RT for 20 minutes. The cells at the interface were washed in PBS (as above) before being used for analysis.

Antisera

Rabbit anti-human μ antiserum (Fc specific), F(ab')₂ goat anti-human μ (Fc specific), rabbit anti-goat IgG (minimal cross-reaction to human serum proteins), goat anti-human γ antiserum (Fc specific), F(ab')₂ goat anti-mouse IgM, F(ab')₂ goat anti-mouse IgG, peroxidase-conjugated donkey anti-rabbit IgG, Rabbit anti-mouse IgM were obtained from Jackson ImmunoResearch. Goat anti-human δ antiserum, FITC-conjugated goat anti-mouse IgM, FITC-conjugated goat anti-mouse IgG1 were from Southern Biotechnology. Rabbit anti-phosphotyrosine antisera was purchased from ICN. Phycoerythrin-conjugated rat anti-CD45 (16A) mAb was from Gibco. Mouse monoclonal antibodies HD6 (IgG1 anti-human CD22), HD39 (IgG1 anti-human CD22), T29/33 (IgG2b anti-CD45) were from Boehringer Mannheim. HD37 (IgG1 anti-human CD19), To15 (IgG2b anti-human CD22) and 4KB128 (IgG2b anti-human CD22) were from Dako. IgG2b anti-CD21 was a gift from N. Ling. The mAb, HM47 (Anti- β polypeptide) was a gift from D. Mason. The rat anti-mouse transferrin receptor monoclonal antibody R17 was a gift from C. Hopkins. The IgG1 mAb used as a control was MOPC-21 from Sigma, the IgG2b mAb used as a control was AG10 provided by G.T. Williams. The IgG1 anti-murine CD22 mAb was prepared from the TIB163 hybridoma (Symington et al., 1982; Torres et al., 1993).

Standard Buffers

PBS

8 g NaCl, 0.2 g KCl, 1.44 g Na₂PO₄, 0.24 g
KH₂PO₄ in 1 litre of distilled water (pH 7.4)

2X PBS	8 g NaCl, 0.2 g KCl, 1.44 g Na ₂ PO ₄ , 0.24 g KH ₂ PO ₄ in 500 ml of distilled water
SDS-sample buffer (2X)	100 mM Tris-Cl, 4% SDS, 0.2% Bromophenol blue, 20% glycerol, (plus 5% β-mercaptoethanol for reducing conditions)
TE	10 mM Tris-HCl pH 7.4, 1 mM EDTA
TBE/Ficoll/dyes	0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol FF, 15% w/v Ficoll 400, in 5 X TBE
10X TBE	0.89 M Tris-HCl, 0.89 M boric acid, 25 mM EDTA (pH 8.3)
SDS/PAGE running buffer	25 mM Tris, 250 mM glycine, 0.1% SDS

In Vitro Kinase Assays

Cells (2×10^8 mononuclear cells from tonsil or 5×10^7 cells of the Burkitt lymphoma lines or mouse cells) were lysed in 1% digitonin, 150 mM NaCl, 10 mM triethanolamine, 1 mM PMSF, 1 mM EDTA, 0.5 mM iodoacetamide, 10 mM Tris-HCl, 1 mg/ml BSA (pH 7.4) in a volume of 1 ml (or 2 ml for tonsil cells). The lysates were then centrifuged at 13000 rpm for 10 minutes. The supernatant was then precleared with 20 µl of protein A-Sepharose or protein G-Sepharose for 1 hour at 4°C. Immunoprecipitates were prepared by incubating with the appropriate antisera (5-20 µg of antibody) for 1-3 hours at 4°C followed by the addition of 20 µl protein A-sepharose or protein G-sepharose. After three washes with lysis buffer (see above) and two

washes with assay buffer (25 mM hepes (pH 7.5) containing 0.1% digitonin), immunoprecipitates (on the Sepharose beads) were resuspended in 30 μ l kinase buffer (assay buffer containing 10 mM MnCl_2 and 5 μCi [γ - ^{32}P]-ATP), incubated at room temperature for 10 minutes and then washed three times in lysis buffer. All samples were boiled in SDS-sample buffer (with or without β -mercaptoethanol) (30 μ l) prior to SDS/PAGE. For reprecipitations of human antigens, samples were boiled in 1% SDS, diluted 1:9 in lysis buffer and precleared prior to precipitation with the relevant monoclonal antibody and protein G-sepharose followed by SDS/PAGE. This protocol was used for all *in vitro* kinase assays with some variations as follows: For primary cells, preclearing was performed with 20 μ l of protein A-Sepharose or protein G-Sepharose and 20 μ l protein A-Sepharose or protein G-Sepharose that had been preincubated for 0.5-1 hour with mouse serum (50-100 μ l) (for mouse monoclonal antibodies) or rabbit serum (50-100 μ l) (for rabbit antisera). For reprecipitations of murine CD22, samples were incubated with 1% Triton X100 lysis buffer (as above except with Triton X100 instead of digitonin) for 1 hour. The eluate was incubated with anti-murine CD22 mAb (10 μg) for 1 hour followed by the addition of 20 μ l of protein G-Sepharose. For the biotinylation *in vitro* kinase assay protocol, for reprecipitations of biotinylated proteins, samples eluted from the Sepharose by boiling were made up to a volume of 1 ml with lysis buffer and precleared with 20 μ l of rabbit Ig-conjugated agarose (1 mg/ml) for 30 minutes prior to precipitation with 20 μ l streptavidin-agarose (streptavidin conjugated to 6% beaded agarose with 12 atom spacer) for 45 minutes. Protein was eluted by boiling in non-reducing SDS-sample buffer.

Polyacrylamide Gel Electrophoresis

The resolving phase, reducing SDS/polyacrylamide gels contained either 7% or 10.6% acrylamide (19:1 acrylamide: bisacrylamide), 1% SDS and 0.375 M Tris-HCl (pH 8.8). The polymerisation was performed by the addition of 15 μ l of TEMED and 30 μ l of a 10% w/v solution of ammonium persulphate per 12 mls of 10.6% gel mix.

For 7% gels, polymerisation was catalysed by the addition of 15 μ l of TEMED and 0.4 ml of a 10% w/v solution of ammonium persulphate per 40 mls of gel mix. The stacking gel contained 5% acrylamide (19:1 acrylamide: bisacrylamide), 1% SDS and 0.125 M Tris-HCl (pH 6.8) and was polymerised using 15 μ l of TEMED and 30 μ l of a 10% w/v solution of ammonium persulphate per 5 mls. The resolving phase, *non-reducing* SDS/polyacrylamide gels contained 6% acrylamide (29:1 acrylamide: bisacrylamide), 1% SDS and 0.375 M Tris-HCl (pH 8.8). Polymerisation was catalysed by the addition of 40 μ l of TEMED and 0.5 ml of a 10% w/v solution of ammonium persulphate per 50 mls of gel mix. The stacking gel contained 5% acrylamide (29:1 acrylamide: bisacrylamide), 1% SDS and 0.125 M Tris-HCl (pH 6.8) and was polymerised using 10 μ l of TEMED and 100 μ l of a 10% w/v solution of ammonium persulphate per 10 mls. All SDS/PAGE was performed using SDS/PAGE running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) on Hoeffer Scientific Instruments apparatus (Mighty Small II or Mighty Tall models) using either the 1D or 2D accessories. Reducing 1D gels were run at 15 mA constant current using an LKB Bromna 2197 power supply for times that resolved the appropriate molecular weights as judged by examining the migration of coloured molecular weight markers. Non-reducing gels were run at 4°C, at 8 mA constant current until the proteins had stacked whereupon the current was increased to 12 mA. 2D gel electrophoresis was slightly more complex. The first dimension was run in a glass tube using the standard resolving gel mix at 1.5 mA/tube constant current until the bromophenol blue from the loading buffer reached the bottom of the tube. The gel was then extruded from the tube and immersed in 20 ml of 0.125 M Tris-HCl (pH 6.8) containing 150 μ l of β -mercaptoethanol for 15 minutes prior to overlaying on the top of the 2nd dimension. The tube gel was sealed on with molten 1.2% agarose in SDS/PAGE running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). The gel was then run at 15 mA constant current as before. Where appropriate, gels were fixed by immersion in 7% acetic acid, 35% methanol, 5% glycerol for 0.5 hr. They were then removed and placed between 3M paper and Saranwrap and vacuum dried at 80°C for 0.5-3 hours. Autoradiography

was performed at -70°C with preflashed Fuji RX medical X-ray film and an intensifying screen.

Western Blotting

Following SDS/PAGE, proteins were electroblotted onto membrane using a Hoeffer Scientific semi-dry blotting apparatus. For blots that were to be developed with ^{125}I -protein A, proteins were transferred onto Immobilon P membrane. The 'sandwiching' used was 3M paper/membrane/gel/3M paper/3M paper, with transfer performed at 250 mA for 30 minutes. For blots that were to be developed with ECL (Enhanced Chemiluminescence system), proteins were transferred onto nitrocellulose membrane. The 'sandwiching' used was 3M paper/membrane/gel/3M paper. The paper and membrane being soaked in 10% methanol/90% protein gel running buffer. The transfer was performed at 80 mA for 1 hour for all 'ECL' blots apart from those due to be probed with HRP-conjugated streptavidin. For these, transfer was performed by application of 120 mA for 0.6-1 hour. The probing procedure depended on the antisera and detection system used. Generally for all occasions when ^{125}I -protein A was used, the filter was washed three times in PBS (5 minutes per wash). It was then incubated overnight with a solution of 3% BSA in PBS with rabbit anti- μ antisera at 2.5 $\mu\text{g}/\text{ml}$ or 1/2000 dilution (0.5 $\mu\text{g}/\text{ml}$) of rabbit anti-phosphotyrosine antisera. Following three 5 minute washes in PBS, the filter was incubated for 3 hours with ^{125}I -labelled protein A (1/1000 dilution). The filter then was washed five times in PBS, allowed to dry, and the signal visualised by autoradiography. When the ECL system was used and probing performed with antisera, filters were prepared by washing three times in PBS (5 minutes per wash). Blocking was performed by incubating the filters for 3 hours with 3% BSA, 0.1% Tween 20 in PBS followed by washing as before with 0.1% Tween 20 in PBS. The filter was then incubated in 8 ml of 3% BSA, 0.1% Tween 20 in PBS with the primary antibody (rabbit anti-phosphotyrosine antisera (0.5 $\mu\text{g}/\text{ml}$) for 3 hours). Following washing as before in 0.1% Tween 20 in PBS, the filter was incubated in 3%

BSA, 0.1% Tween 20 in PBS with the secondary antibody (HRP-conjugated donkey anti-rabbit IgG (0.1 $\mu\text{g/ml}$) for 1 hour) followed by washing as before. The signal was visualised as recommended by the manufacturer of the ECL package (Amersham). This involved mixing, at a 1:1 ratio, solution 1 with solution 2 and immediately adding 0.125 ml/1 cm^2 of this solution to a membrane from which as much as possible of the PBS/0.1% Tween 20 had been poured off. The membrane was evenly covered with the developing solution and left for 1 minute. Excess solution was then poured off and the membrane wrapped in Saranwrap and exposed to film (Fuji RX medical X-ray film). When the ECL system was used and probing performed with HRP-conjugated streptavidin, filters were prepared as above except that blocking was performed for 1 hour. The filter was washed as above and then incubated in 10 ml of 3% BSA/0.1% Tween 20 containing 0.2 $\mu\text{g/ml}$ HRP-conjugated streptavidin for 1 hour followed by washing and developing as above.

Surface Biotinylation

For labelling with sulfo-NHS-biotin, Daudi cells (5×10^7) that had been on ice for 30 minutes were incubated in 5 ml PBS with or (as a control) without 0.3 mg/ml sulfo-NHS-biotin (a membrane impermeable agent) for 30 minutes at 4°C prior to gently washing three times in a total volume of 15 ml with PBS/0.5 mg/ml L-lysine. Cell viability was apparently unchanged by the procedure (as assayed by trypan blue exclusion). For labelling with NHS-SS-biotin, cells (5×10^6 - 2×10^7) after one wash in ice cold PBS were incubated in 2ml of ice cold PBS, containing 0.5 mg/ml NHS-SS-biotin, on ice for 30 minutes. The cells were then washed twice in 15 ml ice cold PBS containing 10% FCS. Washing, in this case, means gently resuspending cells in ice cold solution followed by centrifugation at 1000 rpm, at 4°C for 5 minutes and then discarding the supernatant. The NHS-SS-biotin proved difficult to dissolve and therefore was routinely prepared by dissolving the powder in distilled water at room temperature and then making up to the desired concentration of 0.5 mg/ml with 2X

PBS. The 2X PBS was mixed with sodium hydroxide so that after it was diluted to 1X PBS, it would have a pH of 7.4. This biotin solution was prepared a maximum of 45 minutes before its use and was cooled to 0°C before being added to cells.

Incubation of Cells with Antisera and Subsequent Treatments

To crosslink membrane immunoglobulin, Daudi ($2.5-5 \times 10^7$), A20/J558 (5×10^7) or NIH3T3 (2×10^8) cells were incubated at 5×10^7 cells/ml in DMEM at 37°C, with 20 µg/ml rabbit anti-µ antiserum or control antiserum. After various times, cells were washed once with 1 mM sodium vanadate in ice cold PBS and then lysed in lysis buffer (1% Triton X100 (or similar detergent), 150 mM NaCl, 10 mM triethanolamine, 5 mM EDTA, 1 mM PMSF, 5 mM iodoacetamide, 1 mM Na_3VO_4 , 10 mM NaF, 10 mM Tris-HCl, 1 mg/ml BSA, pH 7.4) before immunoprecipitation. This general protocol was used in all instances, with different antisera used where relevant for immunoprecipitation. Ligation of CD22 or CD19 was performed by incubation with 10 µg/ml of each mAb used for 5 minutes. Where appropriate, cells were then pulsed for 5 s in a microfuge and the pellet resuspended in medium containing goat anti-mouse IgG at 20 µg/ml for 5 minutes before washing as above. In some cases, the determination of the phosphorylation state of an immunoprecipitated protein was not the sole aim, and thus total non-nuclear tyrosine phosphorylation was assessed. In these circumstances, 2×10^6 Daudi cells were incubated with anti-µ antisera or control sera, and then lysed, under conditions scaled down, in proportion, from those above. Following lysis, cell membranes were pelleted by centrifugation at 14500 rpm for 10 minutes, and the supernatant boiled following the addition of an equal volume of 2 x SDS-sample buffer. One third of each preparation was then analysed by SDS/PAGE and blotted with anti-phosphotyrosine antisera and ^{125}I -protein A as detailed above.

Plasmid Preparation

For the preparation of plasmids, *Escherichia coli* (strain TG-1) (Gibson, 1984) was transformed using the method of Hanahan, 1983 using freshly prepared competent cells. Following transformation, cells were plated out on agar plates containing 100 mg/ml ampicillin, left to dry, inverted and incubated overnight at 37°C. Colonies were then picked into 15 ml of 2xTY medium, and propagated by incubation overnight at 37°C. Plasmid DNA was prepared by the alkaline/SDS lysis method (Maniatis et al., 1982). This DNA was used generally only for analytical digests. For greater yields of DNA that was to be used to transform eukaryotic cells, 100-500 ml cell cultures were prepared by picking cells from a single colony, inoculating 2xTY and shaking overnight at 37°C. Plasmid DNA was prepared using the method above (Maniatis et al., 1982) with some adaptations as follows; The ribosomal RNA was precipitated in an equal volume of 5 M LiCl. Following centrifugation, at 2500 rpm for 5 minutes, the supernatant was mixed with an equal volume of 95% ethanol to precipitate the DNA. The DNA was pelleted by centrifugation at 2500 rpm for 10 minutes and the supernatant discarded. To remove residual RNA, this pellet was suspended in TE containing 5 mg/ml RNase A and the reaction incubated at 37°C for 15 minutes. The DNA was then precipitated by the addition of 0.5 volumes of 20% polyethylene glycol 6000/2.5 M NaCl and incubated at -20°C overnight. The DNA was then pelleted by centrifugation as before, resuspended in 0.5 ml TE and extracted with phenol three times. The remaining DNA was then precipitated with ethanol, pelleted by centrifugation at 13000 rpm for 5 minutes and resuspended in TE. Plasmid DNA was analysed by restriction enzyme digestion in a volume of 15 µl using 0.5 - 1 mg DNA and 10% w/v of the 10X concentrated digestion buffer provided by the appropriate manufacturer. Digestion at 37°C was allowed to proceed for 1-3 hours after which 10% w/v of Ficoll/TBE/dyes was added to the samples. Analysis was performed by gel electrophoresis using 0.7-1.3% agarose (dissolved in 1xTBE) in a horizontal submarine gel apparatus connected to an LKB Bromna 2197 power supply. These analytical gels

were run in 1 x TBE containing 1 mg/ml ethidium bromide at 65 mA constant current. DNA was visualised under UV illumination (Chromato-vue, Transilluminator Model TM-20, UVP inc). Photographs were taken with a Polaroid CU-5 88-46 camera using Polaroid 667 iso 3000, 3.25 x 4.25 film. For details of plasmids used, see the appendix.

FACS Analysis and Cell Sorting

Generally FACS analyses and the labelling of cells for cell sorting was performed on $1-3 \times 10^6$ cells. Cells were prepared for labelling by washing once or twice in ice cold PBS. This washing was performed by suspension in PBS and then pelleting the cells by centrifugation at 1000 rpm at 4°C. Cells were labelled by incubation with antisera on ice for 20 minutes with 1% BSA/1% FCS (with 1% goat serum for goat antisera) in PBS in a total volume of 50 μ l. Generally 0.5 μ g of antibody or FITC-conjugated streptavidin was used. For multiple layer labelling, cells were washed once in 3 ml PBS and incubated with the appropriate antisera as before. Cells were then pelleted and washed once in PBS and then analysed on a Becton-Dickinson FACS SCAN by David Gilmore. Cells were sorted on a Becton-Dickenson FACS STAR by David Gilmore. Sorted cells were propagated in the appropriate medium containing 50 μ g/ml gentamycin for at least one week following sorting.

Endoglycosidase F/poly-N-Glycosidase F Digestion

Firstly, protein was precipitated by adding 20 volumes of 95% ethanol. The suspension was then incubated overnight at -20°C. Precipitated proteins were pelleted by centrifugation at 13000 rpm for 15 minutes. The pellet was then dissolved in digestion buffer (150 mM sodium phosphate buffer (pH 7.6), 25 mM EDTA, 0.1% SDS, 1% β -mercaptoethanol, 0.2% NP40) and the solution heated to 90°C and left at this temperature for 15 minutes before being allowed to cool down to 37°C. 1 unit of

EndoF/PNGF was then added and the mix incubated overnight at 37°C. Protein sample buffer was added prior to analysis by SDS/PAGE.

Transfection of Mammalian Cells using Calcium Phosphate

DNA/salt precipitates were prepared by gently mixing 0.56 ml TE and 0.62 ml 2.5 M CaCl_2 with 40 μg of plasmid DNA to which was slowly added, dropwise, over 30 seconds, with gently swirling, 0.62 ml of 2XHBS (280 mM NaCl, 50 mM hepes, 1.5 mM Na_2HPO_4 (pH 7.1)). This mix was then allowed to sit for 20 min to allow the precipitate to form. This was then added to a 150 cm^3 flask of semi-confluent NIH3T3 cells containing 15 ml of medium. Following incubation overnight in an atmosphere of 5% CO_2 at 37°C, the medium and excess precipitates were replaced with 15 ml fresh medium. The cells were then incubated overnight in an atmosphere of 10% CO_2 at 37°C. The following day, the adherent cells were washed twice with EDTA, removed by trypsinization and plated out in selective media (DMEM/10% FCS containing either 1 mg/ml G418 sulfate, 0.4 mg/ml hygromycin or 2 mg/ml mycophenolic acid selection). For details of transfectants used, see the appendix.

Transfection of Mammalian Cells using Electroporation

2×10^7 cells were washed in PBS, pelleted by centrifugation at 1000 rpm at 4°C and then resuspended in 200 μl of PBS and put into a plastic electroporation cuvette. 30-80 μg of plasmid DNA in 20 μl of distilled water was then added and the cell/DNA suspension left on ice for 10 minutes. The conditions used for electroporation were five 2 KV pulses at 1 second intervals for J558L or five 1KV pulses at 1 second intervals for Raji on an Appex Electropulsing unit. Following pulsing, the cell suspension was left for 20 minutes on ice, then resuspended in 50 ml medium and plated in either tissue culture 24 or 96 well plates. The following day, the normal media

was removed and selective media added. This method is an adaptation of the protocol of Potter et al., 1984. For details of transfectants used, see the appendix.

Protein Iodination

5×10^7 cells were washed twice in PBS with pelleting performed by centrifugation at 1000 rpm for 5 minutes. The cells were then resuspended in 1.5 ml PBS in a 50 ml tube. Iodination was performed using water-soluble, membrane impermeable Bolton-Hunter reagent. Basically, the following reagents were sequentially mixed in a 1.5 ml Tube; 20 μ l of 0.2 mg/ml sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate (sulfo-SHPP) in DMSO, 1 μ Ci 125 Iodine (aqueous sodium iodide (pH 8-10) 4736 Mbq/ml), 10 μ l of 5 mg/ml chloramine T in 0.5 M sodium phosphate buffer (pH 7.5), 100 μ l of 1 mg/ml hydroxyphenylacetic acid in distilled water and 10 μ l of 12 mg/ml sodium metabisulphite in 0.05 M sodium phosphate buffer (pH 7.5). This mix was then added to the cells, and the suspension left to incubate on ice for 30 min. The cells were then washed twice in cold PBS containing 1 mg/ml L-lysine. The cells were pelleted as before and lysed in buffer containing the appropriate detergent prior to immunoprecipitation.

Immunoprecipitation

The conditions used for immunoprecipitation depended principally on the nature of the target antigen and the cells used. Normally $2.5 - 5 \times 10^7$ cells (matched numbers for each sample per experiment) were lysed in lysis buffer, see *in vitro* kinase method, with the detergent being either 1% NP40, 1% Lubrol, 1% digitonin, 1% Brij 96, 1% Triton, 1% octyl glucoside. Cell membranes were pelleted by centrifugation at 13000 rpm for 10 minutes. Preclearing of the supernatant was performed by adding 20 μ l protein A-Sepharose or protein G-Sepharose, and the mix allowed to roll at 4°C for 1 hour. 5-20 mg of antibody was then added to the supernatant and allowed to roll at 4°C

for 1-3 hours. 20 μ l Protein A-Sepharose or protein G-Sepharose was then added and this was left rolling at 4°C for 1 hour. Generally the sepharose pellet was washed at least three times with lysis buffer and the immunoprecipitated proteins eluted by boiling in SDS-sample buffer. In some circumstances, modifications were made as follows; for the experiment involving immunoprecipitation of the IgM complex from Daudi using a variety of detergents, immunoprecipitation was performed by passaging cell lysates through a small column of rabbit anti- μ -covalently conjugated to protein A-Sepharose (50 μ l at 10 mg/ml) that had been immobilised in a blue plastic pipette tip using a plug of glass wool and then washing performed by passing through 10 ml of the appropriate lysis buffer. The sepharose conjugate was carefully removed and boiled in SDS-sample buffer to elute protein.

Sepharose Conjugation

For some experiments, antibody that was directly coupled to Sepharose was used. Anti-immunoglobulin antisera or control antisera was coupled to cyanogen bromide-activated Sepharose using the method of Harlow and Lane, 1988. Basically, 1 mg of antibody diluted 1:1 in 1 M sodium phosphate buffer was mixed with 100 ml of activated Sepharose beads that had been washed three times in 1 mM HCl and then twice in 0.5 M sodium phosphate buffer (pH 7.6). The beads and antibody were allowed to roll for 3 hours at RT. The beads were then washed twice in 1M NaCl, 0.05 M sodium phosphate buffer (pH 7.6) before resuspension in 10 volumes of 100 mM ethanolamine (pH 7.5) and incubated overnight at 4°C. The beads and coupled antibody were then washed 4 times in PBS and stored in PBS containing 0.02% sodium azide. Rabbit anti- μ antisera, anti-CD22 and anti-CD19 monoclonal antibodies were, for some experiments, covalently coupled to protein A- or protein G-Sepharose. Antibody was incubated with protein A- or protein G-Sepharose at the desired concentration by rolling for 1 hour at 4°C in a 1.5 ml centrifuge tube. The beads were then washed three times with 0.2 M sodium borate (pH 9.0) and finally resuspended in 2 ml 0.2 M sodium

borate. Dimethylpimelimidate was added to a concentration of 20 mM and then the mix allowed to roll for 30 minutes at RT. The beads were then washed in 0.2 M ethanolamine (pH 8.0) and then incubated overnight in 0.2 M ethanolamine at 4°C with gentle mixing. The beads were then washed three times in PBS and stored in PBS containing 0.02% sodium azide at 4°C prior to use.

Preparation of TIB163 Antibody

The TIB163 hybridoma that produces IgG1 anti-murine CD22 antibody (Symington et al., 1982; Torres et al., 1993) was grown to semi-confluence in 2 litres of DMEM/10% FCS/5 mM β -mercaptoethanol. The cells were then pelleted by centrifugation at 1000 rpm for 5 min and resuspended in 300 ml of DMEM and allowed to grow for 48 hours. The antibody was prepared essentially according to Harlow and Lane, 1988. In outline, the cells were pelleted by centrifugation at 3000 rpm for 30 minutes. The supernatant was removed and mixed 1:4 with a saturated solution of NH_4Cl (pH 7.0) and left at 4°C overnight. The following day the sample was centrifuged at 3000 rpm and the pellet discarded. The solution was then mixed 1:4 with a saturated solution of NH_4Cl (pH 7.0) as before and left at 4°C overnight. The solution was then centrifuged at 30000 rpm and the supernatant discarded. The pellet containing precipitated antibody was then dissolved in 15 ml PBS. The antibody was purified on a protein G-Sepharose column containing 0.5 ml of Sepharose conjugate. The solution was passed through three times, and the column then washed with 10 ml of 100 mM Tris-HCl (pH 8.0) and then 10 ml of 10 mM Tris-HCl (pH 8.0). Antibody was eluted in 100 mM glycine which, after passage through the column, was immediately mixed 10:1 with 1M Tris-HCl (pH 8.0). The protein concentration was measured by determining the optical density at a wavelength of 280 nm and was stored prior to use with 0.02% sodium azide. The antibody was biotinylated as follows; 0.5 mg (1 mg/ml) of purified antibody in PBS was dialysed against 2 litres of bicarbonate buffer (pH 9.3) (17.3 g/l NaHCO_3 and 8.6 g/l Na_2CO_3) for 24 hours at 4°C. The

antibody solution was made up to a volume of 0.5 ml and 20 μ l of 10 mg/ml NHS-biotin in DMF added and the mixture incubated for 1 hour at room temperature. The solution was then dialysed against 2 litres of PBS for 48 hours with 4 changes of PBS. The solution was then stored prior to use in the presence of 0.02% sodium azide.

Detection of Proteins on Slide Immobilised Cells by Immunofluorescence

To screen cells for expression of particular proteins, slide fluorescence was used. The 15 well slides (obtained from ICN/FLOW) used for this protocol were prepared as follows: Poly-L-lysine was dropped onto each well and was allowed to remain in place for 20 minutes before being washed off with distilled water. They were then allowed to dry at RT. Cells (at a concentration not greater than approximately 2×10^5 /ml in PBS) that had already been washed once in PBS were dropped on to the wells and left at 37°C in a moist box for 0.5-1 hour. Cells were fixed by placing the slides in -20°C methanol for 20 minutes at -20°C. The slides were then left to dry at -20°C for at least 20 minutes. Staining was performed by dropping 5 μ l of 1% BSA/1% FCS in PBS containing 0.01 mg/ml antibody or FITC-conjugated streptavidin onto the immobilised cells followed by incubation at RT for 20 min before three washes in PBS. Multiple layer staining was performed by repeating this staining procedure as desired. Coverslips were placed onto the slides and were sealed by daubing the edges with nail varnish before viewing under ultraviolet illumination using a Zeiss IIRS transmission microscope.

Treatment of Cells with Phorbol Ester and Peroxide

1 ml of DMEM media containing 20 μ M or 80 μ M of phorbol ester (PMA) was added to cells (3×10^6) incubated at 37°C in 1 ml of DMEM media and the suspension pipetted up and down to ensure uniform mixing. After an appropriate time, 2 ml PBS

containing 0.02% sodium azide was added and the cells pelleted by centrifugation at 4°C at 1000 rpm. The cells were washed once in PBS/0.02% sodium azide prior to addition of 1% BSA/1% FCS/0.02% sodium azide in PBS and analysis by FACS using 0.02% sodium azide in all solutions. This method is adapted from Dietrich et al., 1994. For analysis of IgM expression, FITC-conjugated goat anti- μ antisera was used. For cell lines, CD22 expression was assessed using the TIB163 anti-CD22 mAb followed by washing and application of a secondary layer of FITC-conjugated goat anti-mouse IgG1. For primary cells, CD22 expression was assessed using biotinylated TIB163 anti-CD22 mAb followed by washing and application of a secondary layer of FITC-conjugated streptavidin. Cells were treated with hydrogen peroxide by adding 1.3 μ l of H₂O₂ to 5 x 10⁷ cells in 1 ml DMEM medium at 37°C for 10 minutes. Cells were then pelleted at 1000 rpm for 5 minutes, washed once at 4°C in ice cold PBS containing 1 mM Na₂VO₄, lysed and immunoprecipitates prepared (see previously).

Preparation of Selective Media

2 mg/ml G418 sulfate selective medium was prepared by adding to 500 ml DMEM/10% FCS/5 mM β -mercaptoethanol; 3 pasteur pipette drops of 12.5N NaOH, 1 g G418 sulfate (Geneticin) and 100 ml of 1 M Hepes buffer. Mycophenolic acid selective media was prepared by adding to 500 mls DMEM/10% FCS/5 mM β -mercaptoethanol; 5 ml 0.5 M xanthine, 25 ml 0.5 M hypoxanthine, 3 ml 0.5 M NaOH and 100 ml 0.5 M mycophenolic acid. Hygromycin selective medium was made by simply adding the appropriate quantity of hygromycin powder to 500 mls DMEM/10% FCS/5 mM β -mercaptoethanol. All solutions were filtered through 0.45 micrometre membrane prior to use.

Internalisation Assay

This protocol is an adaptation of the methods of Bretscher (1989), (1992) and Bretscher and Lutter (1988), with radioiodine labelling of cell surface proteins substituted with surface biotinylation and appropriate alterations to the detection methods used. Cells (5×10^6 - 2×10^7) (matched numbers for each sample per experiment) that had been labelled with NHS-SS-biotin (see previously) were resuspended in 50 μ l of ice cold PBS. To promote internalisation, cells were warmed to 37°C by adding 2 ml of 37°C DMEM medium per 3×10^6 cells and incubated in a 37°C water bath for desired times. Where appropriate PMA was included at a final concentration of 40 μ M and medium containing 400 mM sucrose was used. Ice cold PBS/10% FCS was then added to a total volume of 15 ml and the cells centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was discarded and the cells were then resuspended in 50 μ l of ice cold PBS, placed in a 1.5 ml tube and held on ice. To examine the internal pool of biotinylated protein, cells (in 1.5 ml tubes) were suspended in stripping buffer which cleaves accessible biotin because it contains a reducing agent which hydrolyses the disulphide bond that links the biotin moiety to protein. This stripping was performed by adding stripping buffer to the cells so that, upon closing the 1.5 ml tube, it contained a small air bubble. The tube was then placed in a beaker of iced water that was stirred vigorously for 20 minutes. The stripping buffer was made up as follows; 90 ml of distilled water, 1.5 ml of 5 M NaCl, 0.2 ml of 0.1 M EDTA, 0.1 ml of 1 M MgCl₂ and 0.1 ml of 1 M CaCl₂ were mixed and then bubbled with nitrogen for 5 minutes, before the addition of 1.2 g of cysteine and bubbling continued for a further 5 minutes. The buffer was stored overnight at 4°C in a tightly stoppered container. Immediately prior to use, 400 μ l 12.5 M NaCl and 10 ml FCS were added. After incubation with stripping buffer, cells were centrifuged at 1000 rpm for 5 minutes at 4°C and then washed twice in a total volume of 15 mls of ice cold PBS/10% FCS. Subsequently, the cells were resuspended in 0.8 ml resuspension buffer (0.1 M NaCl, 0.05 M Tris (pH 7.4), 5 mM MgCl, 1 mM CaCl₂, 1 mM PMSF, 5 mg/ml

iodoacetamide, 1% BSA) and held on ice for 10 minutes. Detergent was then added (0.2 ml 10% Triton X100 or 0.2 ml 5% digitonin), the sample vortexed and left on ice for 10 minutes. The sample was then centrifuged at 13000 rpm for 10 minutes and the membrane immunoglobulin immunoprecipitated from the supernatant as previously (using 10 µg rabbit anti-mouse µ or rabbit anti-human µ with 20 µl protein A-Sepharose to immunoprecipitate IgM and 20 µl protein G-Sepharose to precipitate mIgG from A20 cell lines). Washing was performed 5 times with resuspension buffer containing the appropriate concentration of detergent and the sample boiled in non-reducing sample buffer and stored at -20°C prior to analysis by non-reducing SDS/PAGE and transfer to nitrocellulose (see previously). Biotinylated proteins were detected by probing with HRP-streptavidin and the signal visualised using the Enhanced Chemiluminescence system (see previously).

Orthophosphate Labelling of Cells

2 x 10⁷ WEHI-231 cells (2 x 10⁶/ml) were labelled with radioactive phosphate by incubation for 3.5 hours in phosphate-free DMEM containing 10% dialysed FCS and 40 µCi/ml [³²P] orthophosphate at 37°C in an atmosphere of 10% CO₂. The medium had been prepared by adding L-inositol, glucose, L-leucine, L-glutamine, L-methionine, L-cysteine and L-arginine to DMEM that lacked phosphate and all these substances to essentially produce standard DMEM without phosphate (ingredients from Gibco/BRL). FCS (40 ml) was dialysed against 2 litres of saline for 48 hours, at 4°C with 6 regular changes of saline before use.

Experimentation with Orthophosphate Labelled Cells

PMA treatment: After labelling, 2 x 10⁷ cells were washed once in 50 ml PBS at 37°C by centrifugation at 1000 rpm for 5 minutes. The pellet was then resuspended in 20 ml 37°C DMEM with or without PMA (40 µM) and incubated at 37°C for 4

minutes. The cells were pelleted by centrifugation at 37°C for 5 min at 1000 rpm and then lysed in 1 ml of lysis buffer (1% digitonin, 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM NaVO₄, 1 mM PMSF) by incubation on ice for 10 minutes. Immunoprecipitation was carried out as previously using 2 x 10⁷ cells per immunoprecipitation, concluding with elution of the precipitated protein by boiling in non-reducing SDS-sample buffer. Half of each sample was analysed by 2D SDS/PAGE. For the treatment of orthophosphate labelled cells with anti- μ antisera, 2 x 10⁷ cells were prepared as above except that they were resuspended in 1 ml 37°C DMEM containing 20 μ g of F(ab)₂' rabbit anti- μ or control F(ab')₂ rabbit anti- γ antisera. After 4 minutes, cells were pelleted in ice cold PBS containing 1 mM NaVO₄ and lysed in buffer containing 1% NP40 instead of 1% digitonin and immunoprecipitated as above.

Densitometry

From Raji cell lysates lysed with lysis buffer containing 1% digitonin, IgM was precipitated using anti- μ antisera that had been covalently coupled to protein A-sepharose or CD22 was immunoprecipitated using the anti-CD22 mAb (To15) and protein G-Sepharose. Immunoprecipitation was performed three times for each lysate. Aliquots of each immunoprecipitation were then analysed in parallel by SDS/PAGE, transferred to membrane and probed with rabbit anti- μ antisera and ¹²⁵I-protein A. By densitometry, the intensity of the protein presumed to be μ in the CD22 precipitates was compared to that in the IgM precipitate lanes. In the autoradiograph used, there was no significant protein presumed to be μ evident in the second and third CD22 precipitates and the majority of μ had been isolated in the first and second IgM precipitates. Thus the result obtained of 2% of total μ present in the CD22 precipitates was judged to be a maximum.

RESULTS

3 ASSOCIATION OF CD22 WITH THE B- CELL ANTIGEN RECEPTOR

Introduction

By late 1991, it had been discovered that all five classes of membrane immunoglobulin associate with the α/β sheath (reviewed in Moller, 1993); thus it was apparent that both the B-cell antigen receptor complex and the T-cell antigen receptor consist of ligand binding molecules non-covalently associated with disulphide-linked chains. Despite these similar structures, there are several significant differences between the two receptors which presumably reflects their differing biological roles. It can be easily imagined that the TCR binds monovalently to antigen. Such monovalent binding may be insufficient to efficiently generate signals and to overcome this, the TCR can exploit the co-receptors, CD4 or CD8. Because the B-cell antigen receptor exploits a membrane form of antibody as its ligand binding component, polyvalent antigen alone may be sufficient to efficiently drive transmembrane signalling. It would therefore be reasonable to believe that there is no obvious requirement for co-receptors analogous to CD4 and CD8. However, antigens may not always be sufficiently polyvalent or may exist in too small quantities, to induce sufficient receptor crosslinking. Under these circumstances, co-receptors may be important for antigen receptor function. To identify candidate co-receptors, it was decided to screen for B-cell surface molecules that associate with the B-cell antigen receptor. This chapter demonstrates that the B-cell antigen receptor associates with the transmembrane protein CD22.

Evidence for Surface Proteins Other than the α/β Sheath Associating with the B-Cell Antigen Receptor

In order to detect proteins associated with the B-cell antigen receptor, mIgM was purified from detergent lysates of the human Burkitt lymphoma B-cell line, Daudi and then incubated with [γ -³²]-ATP so that co-purified kinase activity could phosphorylate proteins in the precipitate. This technique will subsequently be called the *in vitro* kinase reaction. Following elution, half of the labelled precipitate was treated

with endoglycosidase F/poly-N-glycosidase F (PNGF) which removes the carbohydrate side chains that are often attached to surface molecules while the other half was incubated in buffer without these deglycosylation enzymes. Analysis by SDS/PAGE and autoradiography revealed several phosphorylated proteins including bands migrating between approximately 35-55 kD (in the undigested fraction) that probably correspond to α and β (van Noesal et al., 1992) (Figure 3.1A, page 88). In the sample treated with PNGF, several proteins, in addition those presumed to be α and β , were reduced in molecular weight suggesting that other transmembrane proteins are indeed associated with membrane immunoglobulin.

CD22 Co-precipitates with IgM in Cell Lines

To identify transmembrane proteins that may interact with the B-cell antigen receptor, products of an IgM *in vitro* kinase reaction prepared from the Burkitt line Raji, were eluted and reprecipitation was attempted with several monoclonal antibodies that recognise B-cell surface proteins (each designated with a CD number). In fact, only an anti-CD22 antibody (To15) obviously immunoprecipitated a labelled protein (Figure 3.1B, page 88). Similar results were obtained with another Burkitt cell line, Daudi (Figure 3.1C, page 88). The change in molecular weight of the reprecipitated band (from 140 kD to 100 kD) after treatment with PNGF is consistent with the values expected for CD22 (Wilson et al., 1991; Torres et al., 1993). This was the first significant evidence of an interaction between IgM and CD22.

The Presence of the α/β Sheath in CD22 Immunoprecipitates

It is possible that CD22 may be a preferred substrate of the co-purified kinase activity which implies that the relative inability to detect an association of IgM with other proteins such as CD19 and CD45 (for example in Figure 3.1) may simply be because they are poorly phosphorylated. It was therefore important to investigate

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further whether these proteins associate with the antigen receptor. Thus, I performed the *in vitro* kinase assay in another manner and asked whether the α/β sheath (which because it is a disulphide-linked heterodimer can be easily seen migrating below the diagonal after 2D non-reducing/reducing SDS-PAGE) could be seen in precipitates prepared using several anti-CD antigen monoclonal antibodies. A heterodimer was present in precipitates generated with four anti-CD22 monoclonal antibodies but was not evident in control anti-CD19 or anti-CD45 precipitates (Figure 3.2A-F, page 89). The identification of this heterodimer as the α/β sheath is supported by two lines of evidence. The deglycosylation of IgM or CD22 *in vitro* kinase assay products using PNGF generates a band of approximately 28 kD from both samples (Figure 3.3, page 90), consistent with the expected sizes for the sheath proteins (Hermanson et al., 1988; Sakaguchi et al., 1988). Additionally, the heterodimer can be reprecipitated from IgM or CD22 immunoprecipitates prepared from Daudi cells using a monoclonal antibody that recognises an epitope in the cytoplasmic tail of β (Figure 3.4, pages 91). Consistent with these reprecipitated proteins being α and β , the majority of the labelled products co-migrated as a single band following deglycosylation with PNGF.

Data demonstrating the presence of these antigens on this cell line and the efficacy of the antisera used will be presented later. Thus to summarise it has been demonstrated that a screen using *in vitro* kinase assays has identified a specific association between CD22 and the IgM class of the B-cell antigen receptor.

CD22 Co-precipitates with IgM in Primary Cells

Utilising B lymphoma cells considerably aided the identification of the interaction of CD22 with the B-cell antigen receptor, however, it was important to determine if this interaction also occurs in normal untransformed cells. To accomplish this, *in vitro* kinase assays were performed on IgM immunoprecipitates prepared from tonsillar B cells. As with Burkitt lymphoma lines, the anti-CD22 monoclonal antibody

To15 specifically reprecipitated a protein of approximately 140 kD from IgM precipitates and a phosphorylated heterodimer could be easily detected in CD22, but not in control CD19 precipitates (Figure 3.5, page 92). Thus a CD22/IgM interaction is also evident in primary cells.

Analysis of the CD22/IgM Interaction by Western Blotting

Whilst the *in vitro* kinase assay results demonstrate that CD22 is associated with the antigen receptor complex, estimations of the stoichiometry of association are not easily deduced from these data. As an alternative means of verification that would allow quantification of the detectable degree of association between IgM and CD22, I attempted to detect μ chain in CD22 precipitates by Western blotting. This involved transfer of CD22 precipitates after SDS/PAGE to membrane and probing with rabbit anti- μ chain antisera and ^{125}I protein-A (Figure 3.6, pages 93). Protein likely to be μ heavy chain could indeed be detected in CD22 precipitates prepared from Raji and Daudi cells but was not evident in control precipitates of CD19 and CD45. The efficacy of the antisera used in this study and the presence of the antigens on the Raji and Daudi cell lines was confirmed by surface iodination and immunoprecipitation (Figure 3.7, page 94). Densitometry of Raji CD22 precipitates indicated that approximately 0.2 - 2% of μ is associated with CD22. This relatively low stoichiometry is consistent with the failure to visualise the interaction easily by conventional radioiodine surface labelling procedures. It is possible that the degree of interaction is more significant and may be disrupted by the isolation conditions used. However, association did not appear to be significantly influenced by the choice of anti-CD22 monoclonal antibody used for precipitation because probing for μ chains in precipitates prepared using several anti-CD22 antibodies seemed to reveal similar quantities of μ chain in each (Figure 3.9A, page 96). Furthermore, an interaction between surface IgM and CD22 was not obviously evident when surface iodination techniques were combined with cell lysis and immunoprecipitation using a variety of different detergents (Figure 3.8, page 95).

Indeed, when several detergents were used to prepare CD22 precipitates, and the presence of μ chain assessed in each by Western blotting, the interaction was apparently disrupted in Triton X100 or NP40 but preserved in 1% digitonin, Brij96, Lubrol, or octyl glucoside (Figure 3.9B, page 96).

CD22 Co-precipitates with IgG and IgD

The presence of IgD and IgG antigen receptors in B cells prepared from mononuclear tonsils made it possible to ask whether CD22 can be co-precipitated with these two classes of antigen receptor. From *in vitro* kinase assay products prepared from IgD or IgG precipitates, it could be shown that a protein of approximately 140 kD can be specifically reprecipitated with the anti-CD22 monoclonal antibody (To15) (Figure 3.10, page 97). In addition, protein of an appropriate molecular weight to be δ heavy chain could be detected in a CD22 precipitate by western blotting and probing with anti- δ antisera and ^{125}I protein-A. These data suggest CD22 can associate with multiple classes of antigen receptor.

Evidence for a Cell Surface Interaction between CD22 and IgM

As yet, from the experiments performed it is not clear whether the CD22/IgM interaction could occur on the cell surface. This is because, as noted above, precipitation of IgM from Daudi or Raji after ^{125}I labelling did not reveal any significant association of CD22 with the antigen receptor, results that are consistent with the interaction being of low stoichiometry *in vitro*. Because it is not possible to distinguish between intracellular and surface associations using conventional *in vitro* kinase assays and western blotting techniques, I required a more sensitive method for detecting surface complexes. Being aware of the method of labelling surface proteins using sulfo-NHS-biotin (Hurley and Finkelstein, 1990), I wondered whether this could be

combined with the *in vitro* kinase assay in a two-stage labelling reaction. The strategy involved preparing biotinylated Daudi cells and then, after lysis, performing *in vitro* kinase assays on purified IgM or CD22. The labelled precipitates were then disrupted by boiling and biotinylated, radiolabelled surface molecules reprecipitated using streptavidin-agarose and analysed by SDS/PAGE and visualised by autoradiography. As can be seen in Figure 3.11, page 98, CD22 precipitates contained biotinylated heterodimers and conversely IgM precipitates apparently contained CD22. This suggests that CD22 and the antigen receptor do indeed interact on the cell surface although the possibility that *in vitro* association after lysis occurs cannot be discounted.

Association of CD22 with IgM in Murine Primary Cells

In order to dissect the CD22/IgM association at the molecular level, it was important to possess a cell line that both exhibits this interaction and can be easily manipulated (for example the capacity to derive stable transfectants would be critical). An assumption would be that mouse B-cell lines would provide a convenient system in which the interaction could be studied. I therefore wished to determine if the CD22/IgM interaction occurs in murine cells. Thus murine IgM was immunoprecipitated from digitonin lysates of splenic cells from a BALB/c mouse and incubated with [γ - 32 P]-ATP so that any co-purifying kinase activity could phosphorylate proteins within the complex. As expected, (Figure 3.12, page 99) several phosphoproteins co-precipitated with IgM including bands probably corresponding to *src*-related tyrosine kinases (around 55 kD), Syk (around 70 kD) and one of approximately 140 kD that is of a size expected for murine CD22 (Torres et al., 1993). To assess whether this protein was CD22, the products of an IgM *in vitro* kinase assay were eluted from the Sepharose and reprecipitation attempted with the anti-murine CD22 IgG1 mAb TIB163 or a control IgG1 mAb. A 140 kD protein was specifically reprecipitated by the TIB163 monoclonal antibody. This suggests that murine CD22 associates with the murine B-cell antigen receptor complex.

Analysis of the CD22/IgM Interaction in Murine Cell Lines

To continue with the use of murine cells, mouse cell lines which express CD22 and mIg were tested to see if they display a CD22/IgM association. To do this, the products of *in vitro* kinase assays on CD22 immunoprecipitates were analysed by 2D SDS/PAGE and the presence of a phosphorylated heterodimer corresponding to the α/β sheath assessed. By this criterion, which has previously been a most convincing means of demonstrating the interaction, it was surprising to find no significant evidence for the interaction in mouse cell lines including WEHI-231 (Figure 3.13, page 100). The inability to use 2D SDS/PAGE and the presence of the phosphorylated α/β heterodimer as an indicator of the CD22/IgM association, the availability of a murine CD22 expression vector and the fact that use of the TIB163 mAb produces a high background of other bands suggesting that this antibody crossreacts with a variety of proteins led me to concentrate on a second strategy to dissect the interaction.

Association of Murine CD22 with Human IgM

The alternative strategy involved deriving stable transfectants of the Raji human Burkitt lymphoma line in which murine CD22 co-precipitates with human IgM. Raji was transfected by electroporation with a murine CD22 expression vector containing a gene that confers resistance to the antibiotic G418 sulphate and resulting clones screened for murine CD22 expression by staining cells with an anti-murine CD22 mAb and a FITC-conjugated secondary antibody. The frequency of stable murine CD22 positive transfectants was very low (approximately 1 per 10^7 electroporated cells). A clone (Raji22) was selected and enriched for murine CD22 surface expression by cell sorting once prior to further analysis (Figure 3.14A, pages 101). In order to address whether transfected murine CD22 associated with the endogenous IgM of Raji, *in vitro* kinase assays were performed on IgM or control precipitates. The presence of CD22

was then assessed by reprecipitation from these samples using the anti-CD22 mAb TIB163. As shown in Figure 3.14B, pages 101, murine CD22 does appear to associate with human IgM.

Having obtained evidence that murine CD22 can interact with human IgM in Raji it seemed feasible to address which parts of CD22 are required for the interaction by introducing plasmids expressing mutant CD22 molecules and assaying for whether they co-precipitate with IgM. I attempted to establish suitably matched clones of Raji expressing murine CD22 and a mutant of murine CD22 (and of mutant IgM molecules) however these constructs transfected at a low frequency and additionally many proved to be too unstable to analyse. I therefore only managed to derive two matched transfectants of Raji; one that expressed a gene encoding murine CD22 (RajiCD22) (expressing significantly less murine CD22 than the Raji22 transfectant analysed above) and another expressing a murine CD22 gene which lacks sequence corresponding to the natural cytoplasmic tail of CD22 (RajiMutCD22) and has it replaced with the sequence of the α sheath protein gene (see appendix for structure). Both cell lines had similar expression of the murine CD22 antigen by judged by FACS (Figure 3.15A, page 102) and expressed molecules of appropriate molecular weights as determined by surface iodination of Raji transfectants and analysis by SDS/PAGE of murine CD22 precipitates (Figure 3.15B, page 102). The ability of human IgM to interact with murine CD22 was assessed by immunoprecipitation using the anti-murine CD22 monoclonal antibody (TIB163) from these cell lines, and then analysing the precipitates for the presence of IgM by SDS/PAGE, Western blotting and probing with rabbit anti- μ antisera and ^{125}I -protein A. Figure 3.15C, page 102 shows that whilst protein of the appropriate molecular weight for μ chain (approximately 70 kD) was apparently present in murine CD22 precipitates, there was no evidence of μ associating with the mutant CD22. Unfortunately, the weakness of the signal produced does not allow firm conclusions to be drawn.

Conclusions

This chapter demonstrates that CD22 associates with the B-cell antigen receptor. Although initially characterised in human Burkitt lymphoma cell lines, the interaction is also present in primary B cells. Furthermore, it seems to be evident with multiple immunoglobulin isotypes (IgM, IgD and IgG). Analysis of the interaction shows that, whilst it is specific, stable and can apparently occur on the cell surface, it is of a relatively low stoichiometry *in vitro* with 0.2-2% of μ heavy chain associated with CD22.

Figure 3.1

Evidence for Surface Proteins Other than the α/β Sheath Associating with the B-cell Antigen Receptor

(A) Analysis by SDS/PAGE of the products of *in vitro* kinase reactions performed on an IgM or control precipitate. Lysed Daudi cells were incubated with control rabbit serum (lanes 1 and 2) or with rabbit anti- μ antisera (lanes 3 and 4) and kinase assays performed on immune complexes immobilised on protein A-Sepharose. For lanes 2 and 4, following elution by boiling from the Sepharose, the eluted proteins were treated with endoglycosidase F and poly-N-glycosidase F (PNGF) to remove carbohydrate side chains.

CD22 Co-precipitates with IgM in Cell Lines

(B) Analysis by SDS/PAGE of the products of an *in vitro* kinase assay performed on an IgM immunoprecipitate prepared from Raji. Lane 1, total products of the kinase assay eluted from the protein A-Sepharose by boiling. Lanes 2 - 5, analyses of material prepared by reprecipitation with the indicated anti-CD antibodies from the products eluted off protein A-Sepharose. The α and β components presumably run close to the dye front under these conditions. The presumed position of CD22 is indicated by an arrowhead.

(C) Analysis by SDS/PAGE of the products of *in vitro* kinase assays performed on immunoprecipitates prepared from Daudi. Cell lysates prepared from Daudi cells were incubated with control rabbit serum (lane 6) or with rabbit anti- μ antisera (lane 7) and kinase assays performed on immune complexes immobilised on protein A-Sepharose. Before analysis by SDS/PAGE, to prepare the samples in lanes 8 and 9, the products of the reaction were eluted from the Sepharose by boiling and were then reprecipitated with the anti-CD22 mAb as before. They were then either treated with endoglycosidase F and poly-N-glycosidase F (PNGF) to remove carbohydrates (+) or control incubated with digestion buffer alone (-) as indicated. The presumed position of CD22 is indicated by an arrowhead. The bands migrating at around 46 kD are likely to derive from the various forms of the α/β sheath (van Noesal et al., 1992) (The lower right hand panel 46 kD marker should be approximately 1 cm further up).

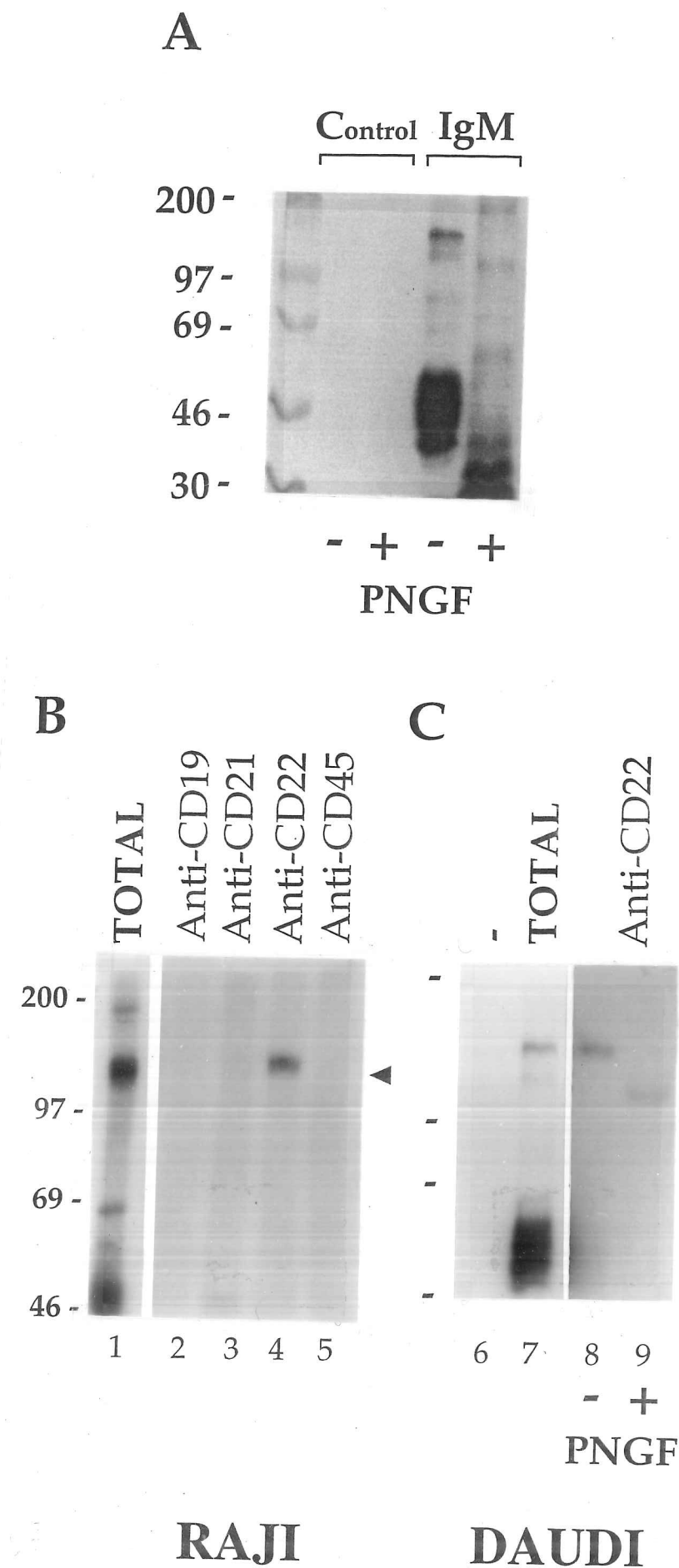


Figure 3.2

Presence of a Phosphorylated Heterodimer in CD22 Immunoprecipitates

(A to E) Analysis by 2D SDS/PAGE of the products of *in vitro* kinase assays performed on precipitates prepared using four different anti-CD22 mAbs or anti-CD19 and anti-CD45 mAbs as indicated. The assays was performed as for Figure 3.1B except protein G-Sepharose was used for preclearing and immunoprecipitation. The anti-CD22 antibodies recognise different epitopes (Knapp et al., 1989). Arrowheads indicate the probable positions of CD22, CD19 and the chains of the α/β heterodimer. All the panels are photos taken from autoradiographs exposed for the same length of time.

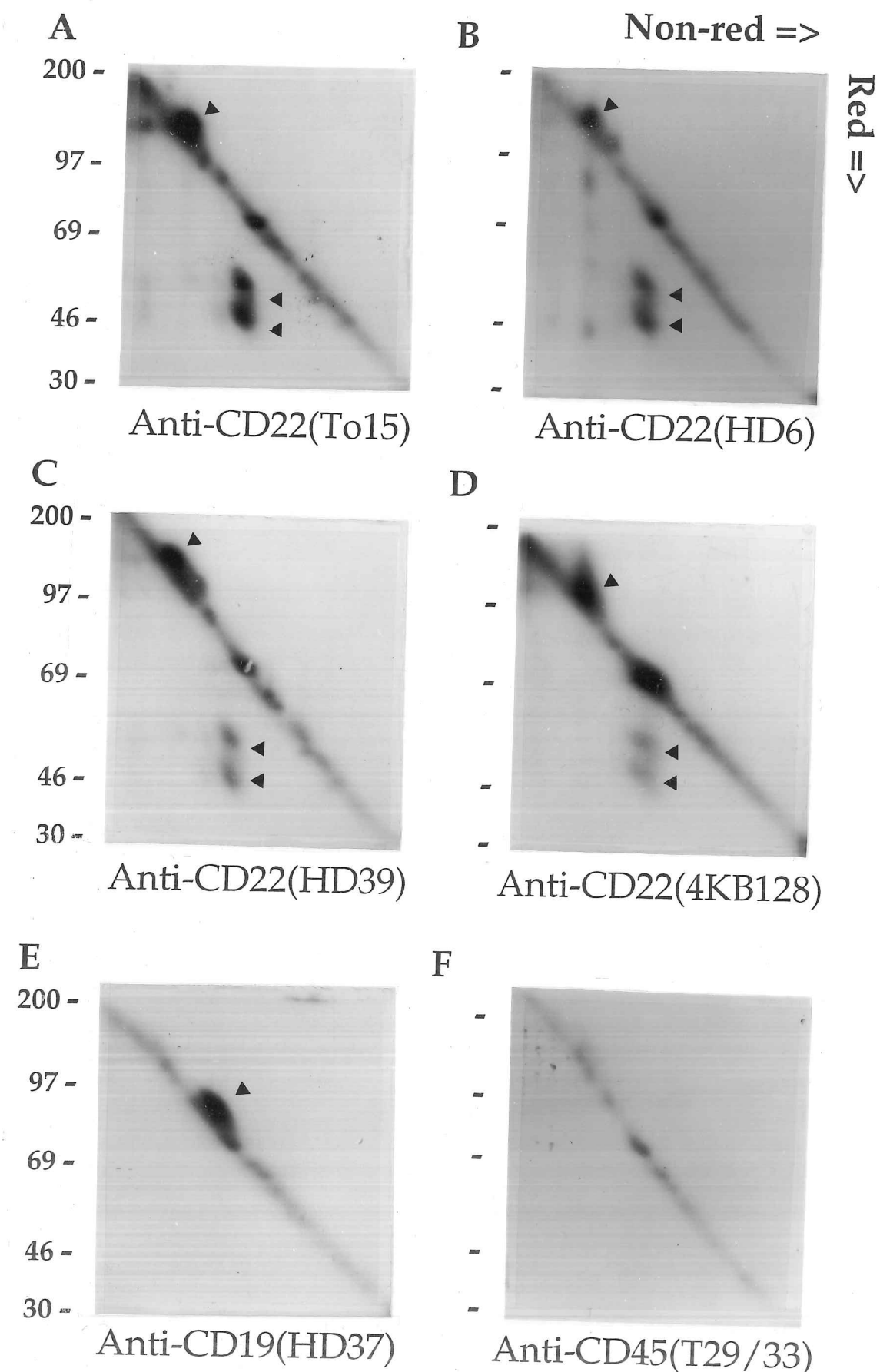


Figure 3.3

The α/β Sheath Associates with CD22

I

Analysis by SDS/PAGE of the products of *in vitro* kinase assays performed on either anti- μ (lanes 1 and 2) or anti-CD22 (To15) (lanes 3 and 4) immunoprecipitates. They were incubated with (lanes 2 and 3) or without (lanes 1 and 4) PNGF to confirm that the heterogeneous Mr 35-56 kD bands co-migrate following deglycosylation as expected for the α and β sheath proteins from their amino acid sequences (indicated by a bracket) (Hermanson et al., 1988; Sakaguichi et al., 1988).

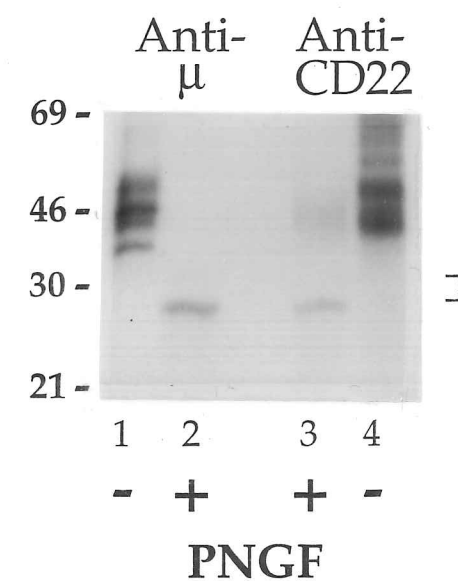


Figure 3.4

The α/β Sheath Associates with CD22

II

(A) SDS/PAGE analysis of the proteins re-precipitated with an anti- β mAb from the products of *in vitro* kinases assays on IgM or CD22 precipitates. Sepharose immobilised precipitates were prepared using control rabbit serum (lane 1), rabbit anti- μ antisera (lane 2), the anti-CD22 mAb (To15) (lane 5) or solely protein G-Sepharose (lane 6). Proteins were reprecipitated using an anti- β mAb from IgM (lanes 3 and 4) or CD22 precipitates (lanes 7 and 8) and were incubated in digestion buffer in the presence (lanes 4 and 8) or the absence (lanes 3 and 7) of PNGF. Probable positions of the sheath proteins are indicated by a bracket.

(B) Analysis by 2D SDS/PAGE of the products of an *in vitro* kinase assay on a CD22 precipitate that had been reprecipitated with an anti- β mAb (as in lane 7). Note the disulphide linked heterodimer.

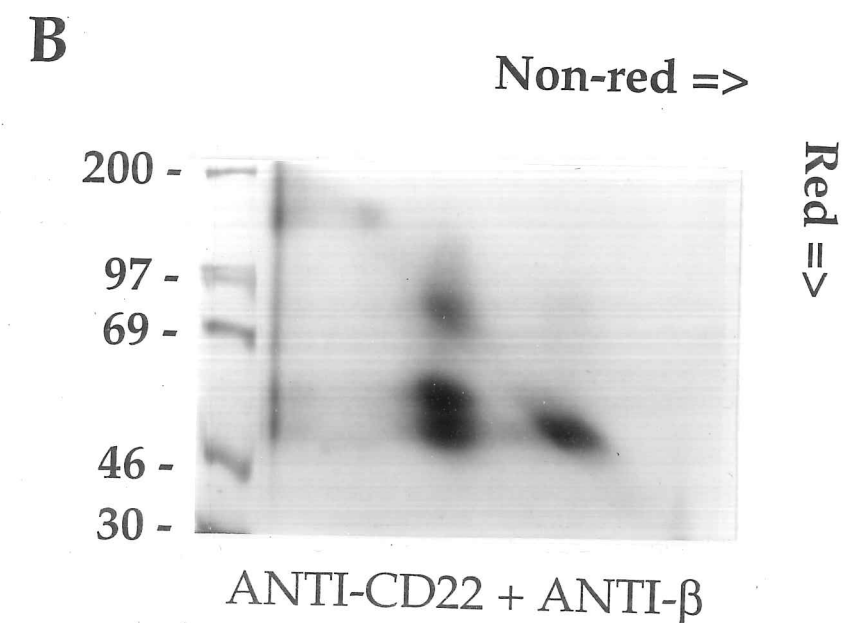
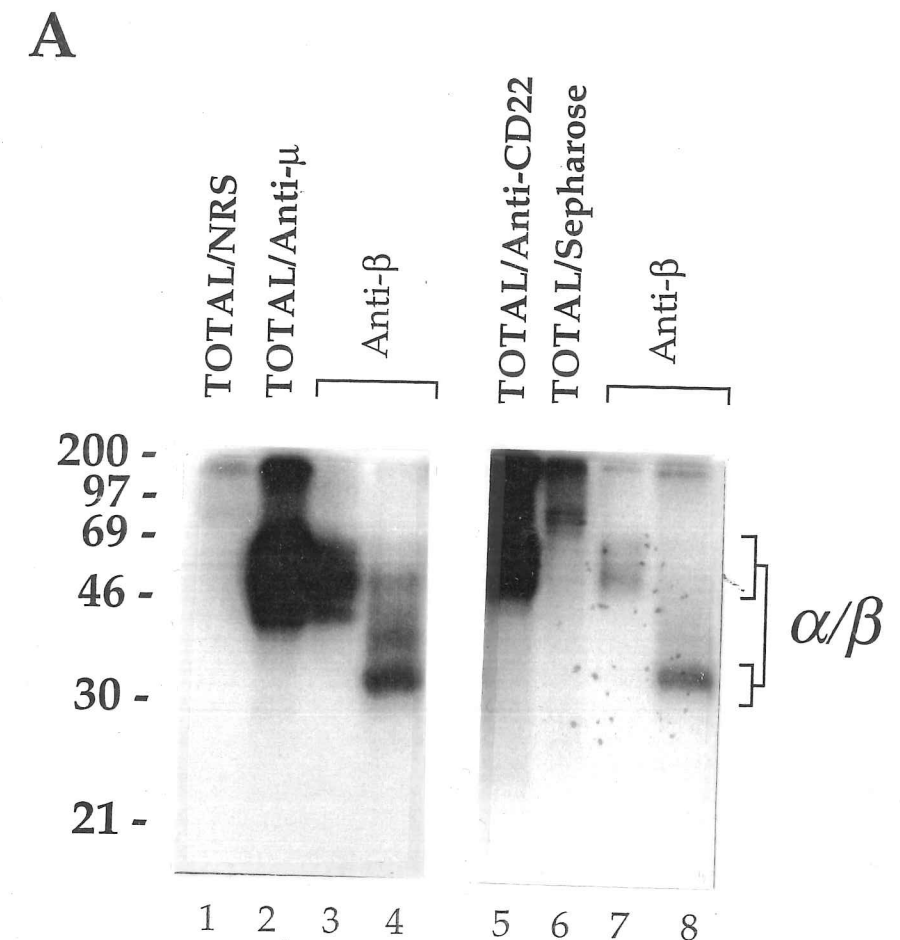


Figure 3.5

CD22 Co-precipitates with IgM in Primary Cells

Human tonsil cells were lysed and immunoprecipitates prepared with anti- μ , anti-CD19 or anti-CD22 antibodies followed by *in vitro* kinase assay, reprecipitation where appropriate and analysis by SDS/PAGE.

(A) The left hand panel shows an analysis by SDS/PAGE of *in vitro* kinase assay products prepared from IgM immune complexes before (**TOTAL**) or after (**Anti-CD22**) reprecipitation with the anti-CD22 antibody (To15) or a control reprecipitation with protein G-Sepharose alone (**Control**). The right hand panel shows a similar analysis of the total products of an *in vitro* kinase reaction on a CD22 or CD19 precipitate as indicated. The presumed position of CD22 is indicated by an arrow head, the presumed location of α and β by brackets. CD19 probably migrates at around 90 kD.

(B, C and D) Analysis by 2D reducing/non-reducing SDS/PAGE of *in vitro* kinase assay products from IgM, CD22 or CD19 immunoprecipitates. Note the presence of the heterodimer in the CD22 but not the CD19 precipitate and the fact that C and D represent autoradiographs exposed for approximately 3X longer than B.

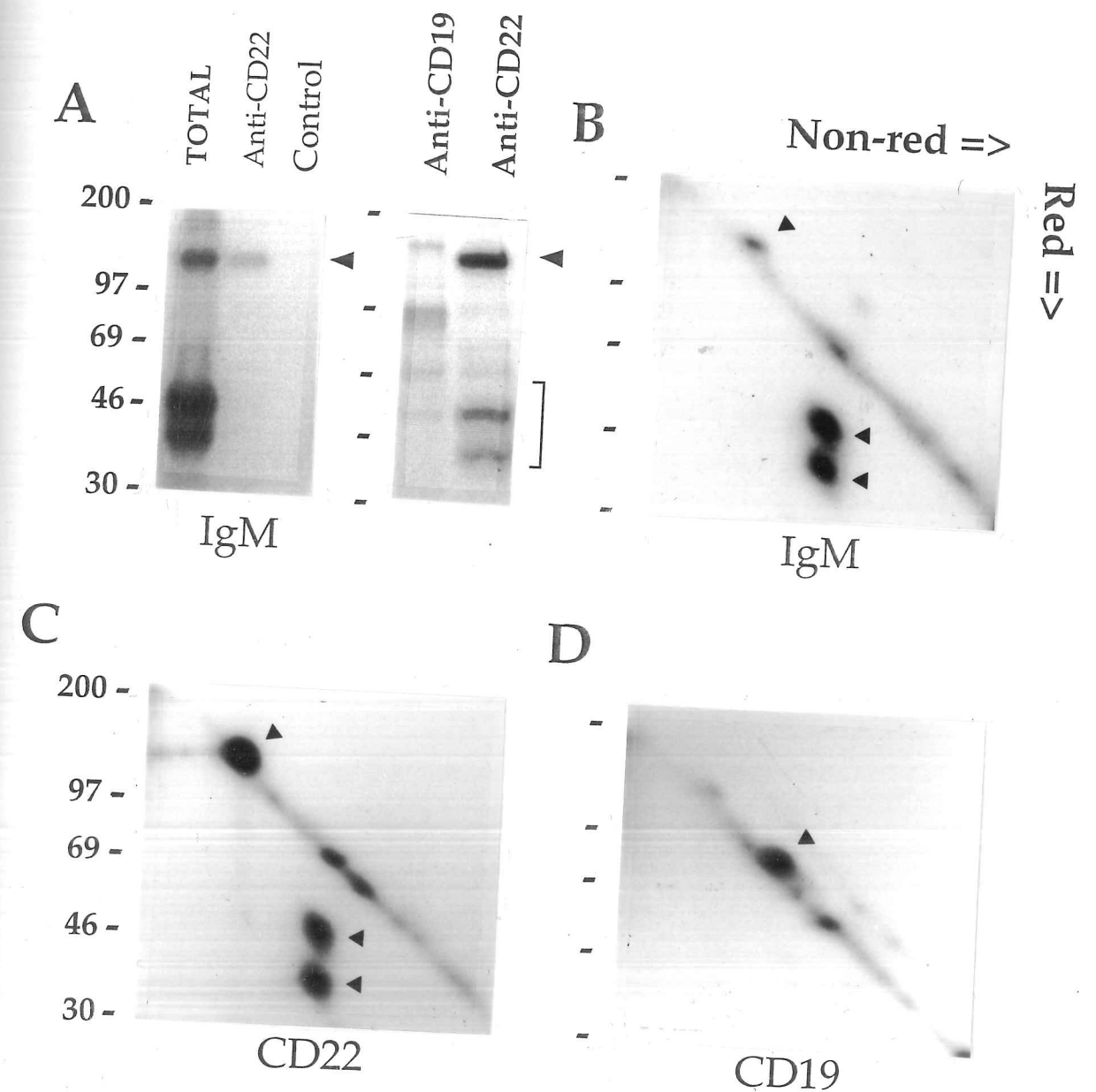


Figure 3.6

Analysis of the CD22/IgM Interaction by Western Blotting

Detection of IgM in CD22 immunoprecipitates by Western blotting. Immunoprecipitates from Daudi (lanes 1, 2 and 3) or Raji (lanes 4 and 5) lysates were prepared using monoclonal antibodies to CD22, CD19 or CD45 and protein G-Sepharose or rabbit anti- μ -Sepharose alone as indicated. Samples were subjected to SDS/PAGE and, following transfer to membrane probed with rabbit anti-human μ antiserum and ^{125}I -protein A. The bands presumed to be human μ chains are indicated by arrowheads. The weaker bands at around 50 kD probably correspond to ^{125}I -protein A interaction with partially renatured heavy chains of the monoclonal mouse anti-CD antibodies used. Raji μ migrates around 70 kD whereas Daudi μ migrates around 85 kD; this difference and the expression of CD19 and CD45 on the Burkitt lines has been confirmed by the analysis by SDS/PAGE of immunoprecipitates prepared after cell surface iodination (Figure 3.7, page 94).

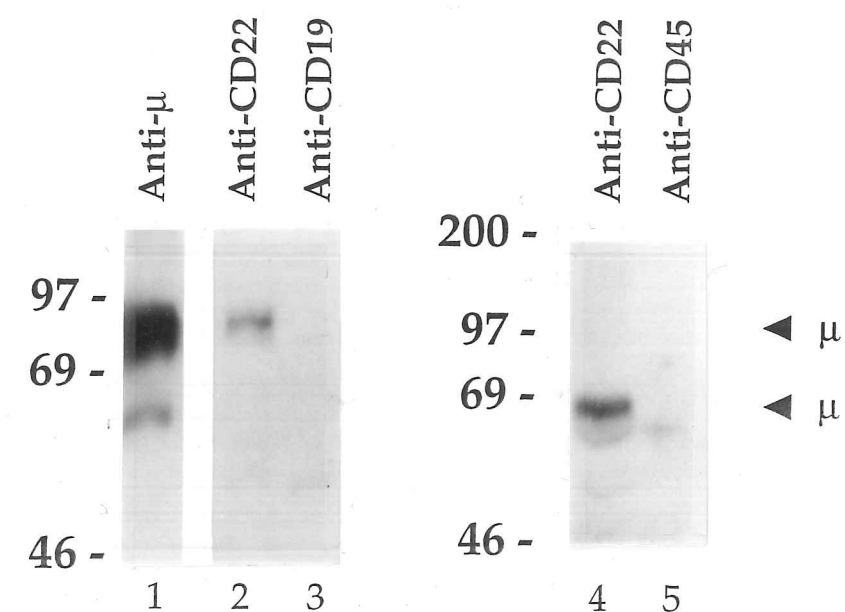


Figure 3.7

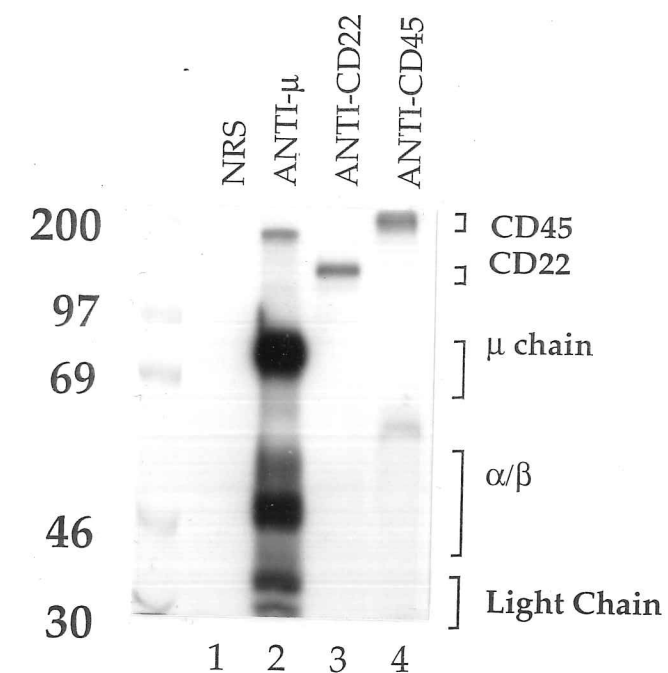
Detection of Surface Antigens on Raji and Daudi Cells

Analysis by SDS/PAGE of immunoprecipitates prepared from digitonin lysates of ^{125}I -surface labelled Daudi cells or Raji cells using the appropriate antisera and protein A- or protein G-Sepharose.

(A) Daudi cell precipitations performed with control rabbit serum (lane 1), rabbit anti- μ antiserum (lane 2), or mouse IgG2b monoclonal antibodies; anti-CD22 (To15) (lane 3) or anti-CD45 (lane 4). The presumed positions of the various proteins are marked.

(B) Raji cell precipitations performed with control rabbit serum (lane 1), rabbit anti- μ (lane 2) or mouse monoclonal antibodies; anti-CD22 (To15) (lane 3), anti- β (lane 4), anti-CD45 (lane 4), control with protein-G Sepharose only (lane 6), anti-CD19 (lane 7) or anti-CD21 (lane 8). The presumed positions of the various proteins are marked.

A



B

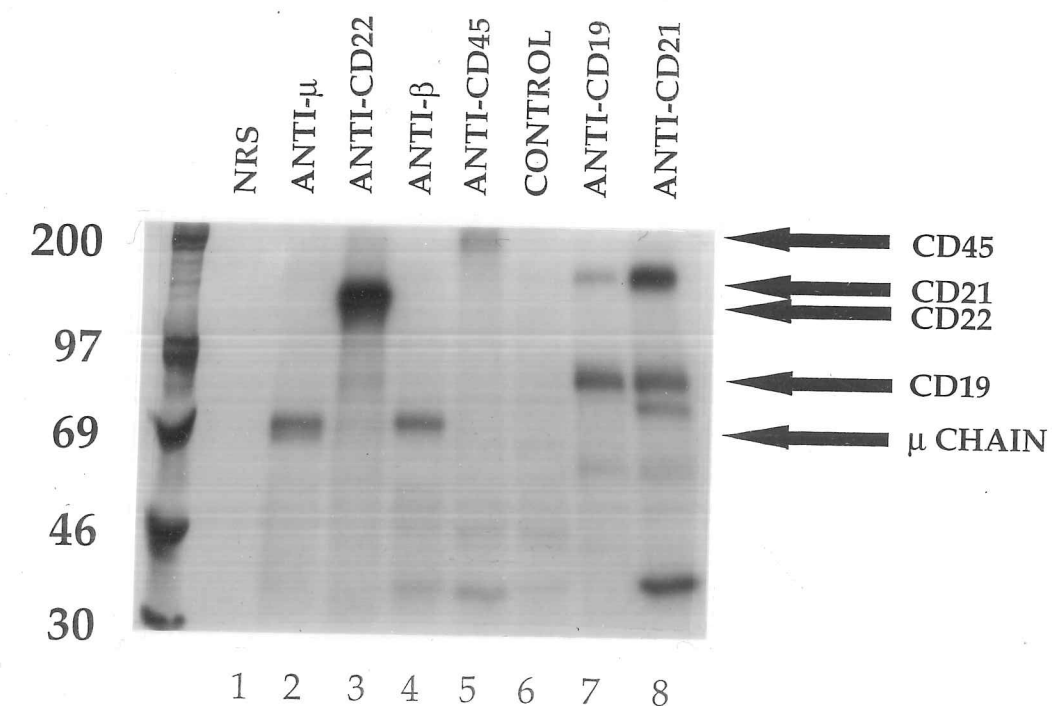


Figure 3.8

Analysis of the Membrane IgM complex after Surface Iodination

SDS/PAGE analysis of ^{125}I -labelled mIgM complexes precipitated from cells lysed with a variety of detergents. Briefly, Daudi cells were surface iodinated and lysed in the detergents noted below. The lysates, following preclearing, were passed down anti- μ -Sepharose columns. Following washing, bound proteins were eluted by boiling and then analysed by SDS/PAGE. The following detergents in lysis buffer were used; 1% Triton X100 (lanes 1 and 2), 1% digitonin (Sigma) (lane 4), 1% digitonin (BDH) (lane 5), 1% Brij 96 (lane 6), 1% NP40 (lane 7), 1% Chaps (lane 8), and 1% octyl glucoside (lane 9). The expected position of CD22 was assessed using an aliquot CD22 immunoprecipitate prepared from ^{125}I -labelled Daudi cells (lane 3). Lane 2 contains the products of a control immunoprecipitation using a rabbit Ig-Sepharose column. The presumed positions of various proteins are indicated.

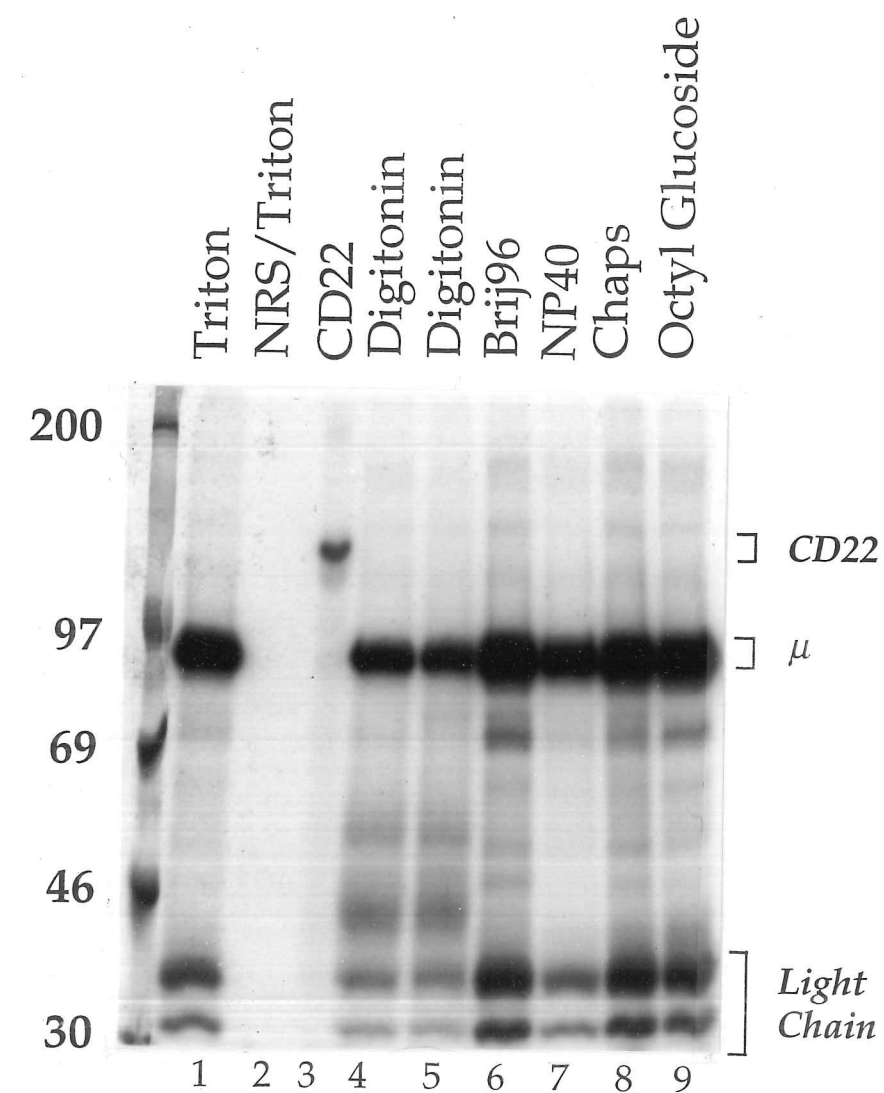


Figure 3.9

Western Blotting Analysis of the CD22/IgM Interaction using Assorted Antibodies and Detergents

Probing for μ heavy chains in CD22 immunoprecipitates by Western blotting.

(A) Immunoprecipitates from Daudi cell digitonin lysates were prepared using rabbit Ig-Sepharose (lane 2), rabbit anti- μ Sepharose (lane 3), a variety of mouse anti-CD22 antibodies namely; To15 (lane 4), 4KB128 (lane 5), HD6 (lane 6), or HD39 (lane 7); or anti-CD19 (lane 8) and anti-CD45 mAbs (lane 9). Lane 1 refers to a control eluate prepared by boiling anti- μ Sepharose. Samples were subjected to SDS/PAGE and blotted with rabbit anti-human μ antiserum and the blot developed with ^{125}I -protein A. The presumed position of μ is indicated by a bracket.

(B) Immunoprecipitates prepared using the anti-CD22 mAb (To15) from Daudi cell lysates made using a variety of detergents in the lysis buffer, as follows; 1% digitonin (lane 1), Triton X100 (lane 2), 1% NP40 (lane 3), 1% octyl glucoside (lane 4), 1% Lubrol (lane 5), 1% Brij96 (lane 6) and 1% NP40/0.1% SDS (lane 7). The blots were probed for μ in the same way as in (A). Its presumed position is indicated by a bracket.

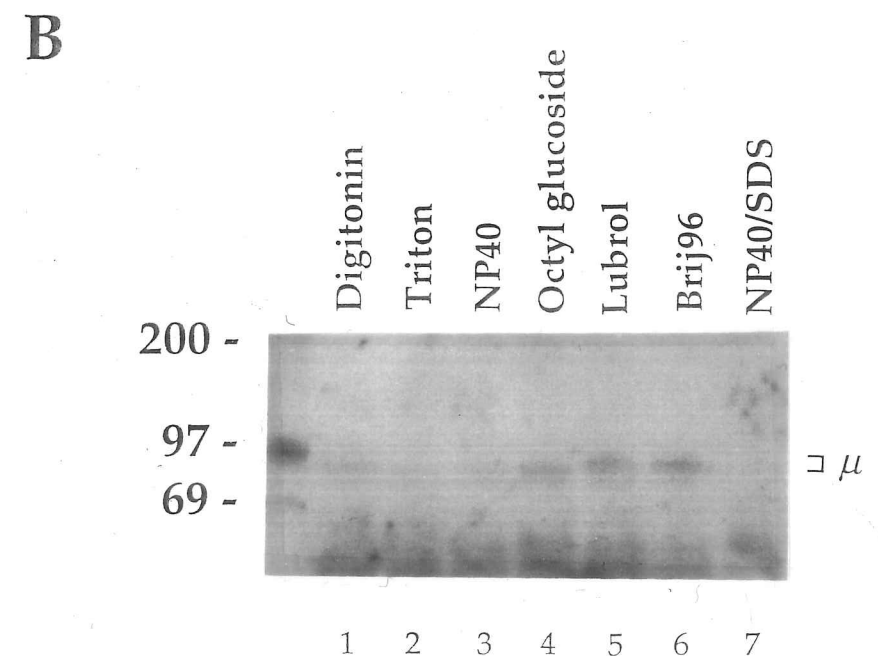
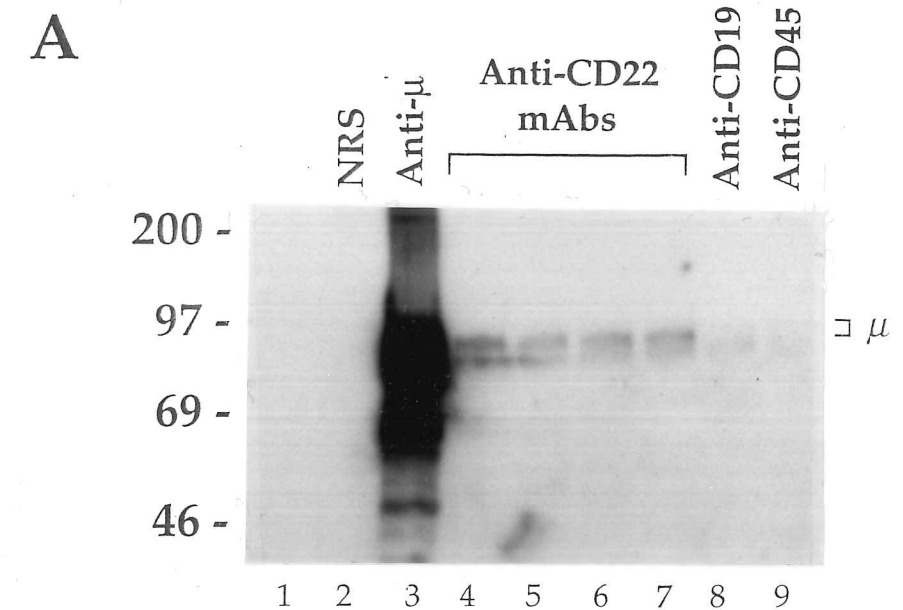


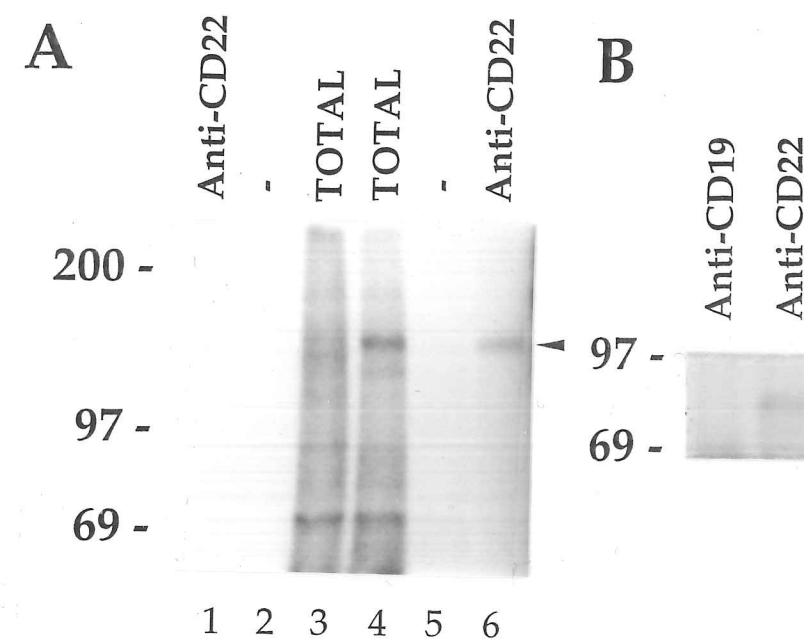
Figure 3.10

CD22 Co-precipitates with IgG and IgD

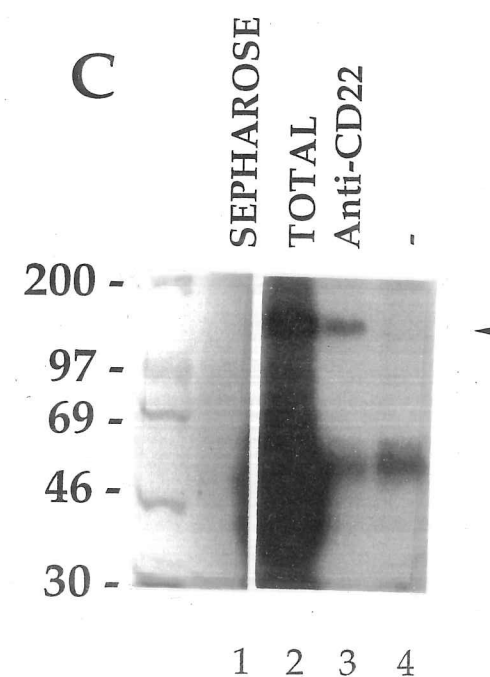
(A) SDS/PAGE analysis of the products of *in vitro* kinase reactions performed on precipitates prepared from human tonsil cells. Precipitates were prepared (denoted by **TOTAL**) and the presence of CD22 assessed by boiling these samples and re-precipitating with the IgG2b anti-CD22 mAb (To15) (denoted by **Anti-CD22**) or control IgG2b mAb (AG10) (denoted by -) and protein G-Sepharose. Precipitates are as follows; control goat Ig-Sepharose (lane 3) with re-precipitations in lanes 1 and 2, Anti- δ -Sepharose (lane 4) with reprecipitations in lanes 5 and 6. All of the lanes contain equivalent proportions of the products. The presumed positions of CD22 are denoted by arrowheads.

(B) Western blot of precipitates probed for δ chain. Precipitates were prepared from tonsil cells using the anti-CD22 mAb (To15) or an anti-CD19 mAb (as indicated). Following resolution by SDS/PAGE, probing was performed with rabbit anti- δ chain antisera and 125 I-protein A.

(C) SDS/PAGE analysis of the products of *in vitro* kinase reactions performed on precipitates presumed to contain IgG. Precipitates were prepared (denoted by **TOTAL**) and the presence of CD22 assessed by boiling these samples and re-precipitating with the IgG2b anti-CD22 mAb (To15) (denoted by **Anti-CD22**) or control IgG2b mAb (AG10) (denoted by -) and protein G-Sepharose. Precipitates are as follows; control Sepharose (lane 1), protein A-Sepharose (lane 2) with re-precipitations in lanes 3 and 4. All of the SDS/PAGE lanes contain equivalent proportions of the products. The presumed positions of CD22 are denoted by arrowheads.



IgD



IgG

Figure 3.11

Evidence for a Surface Interaction between CD22 and IgM

(A) Analysis by SDS/PAGE of the products of *in vitro* kinase assays performed on anti-CD22 (lanes 1, 2, 5 and 6) or anti- μ (lanes 3, 4, 7 and 8) precipitates from Daudi cells that had (**Biotinylated**; lanes 5, 6, 7 and 8) or had not (Control); lanes 1, 2, 3 and 4) been biotinylated on the surface prior to lysis. A sample of each kinase reaction was run directly on the gel in lanes 1, 3, 5 and 7 (**TOTAL**) whereas, for lanes 2, 4, 6 and 8, proteins were eluted from the kinase reaction complex by boiling and then reprecipitated with streptavidin-agarose before SDS/PAGE. Thus bands appearing in lane 6 and 8 presumably represent proteins that had been biotinylated on the surface. The presumed positions of CD22 and the sheath proteins are indicated by arrows or brackets. The *in vitro* kinase assays were performed as previously; with a 10% aliquot of the dissociated complexes (**TOTAL**) or 50% of the samples purified on streptavidin-agarose analysed by SDS/PAGE. For further details, see materials and methods.

(B) Analysis by 2D SDS/PAGE of the products of *in vitro* kinase reactions on anti-CD22 or anti- μ precipitates from biotinylated cells that had been reprecipitated using streptavidin-agarose. The samples are the same as those in lanes 6 and 8 of panel A.

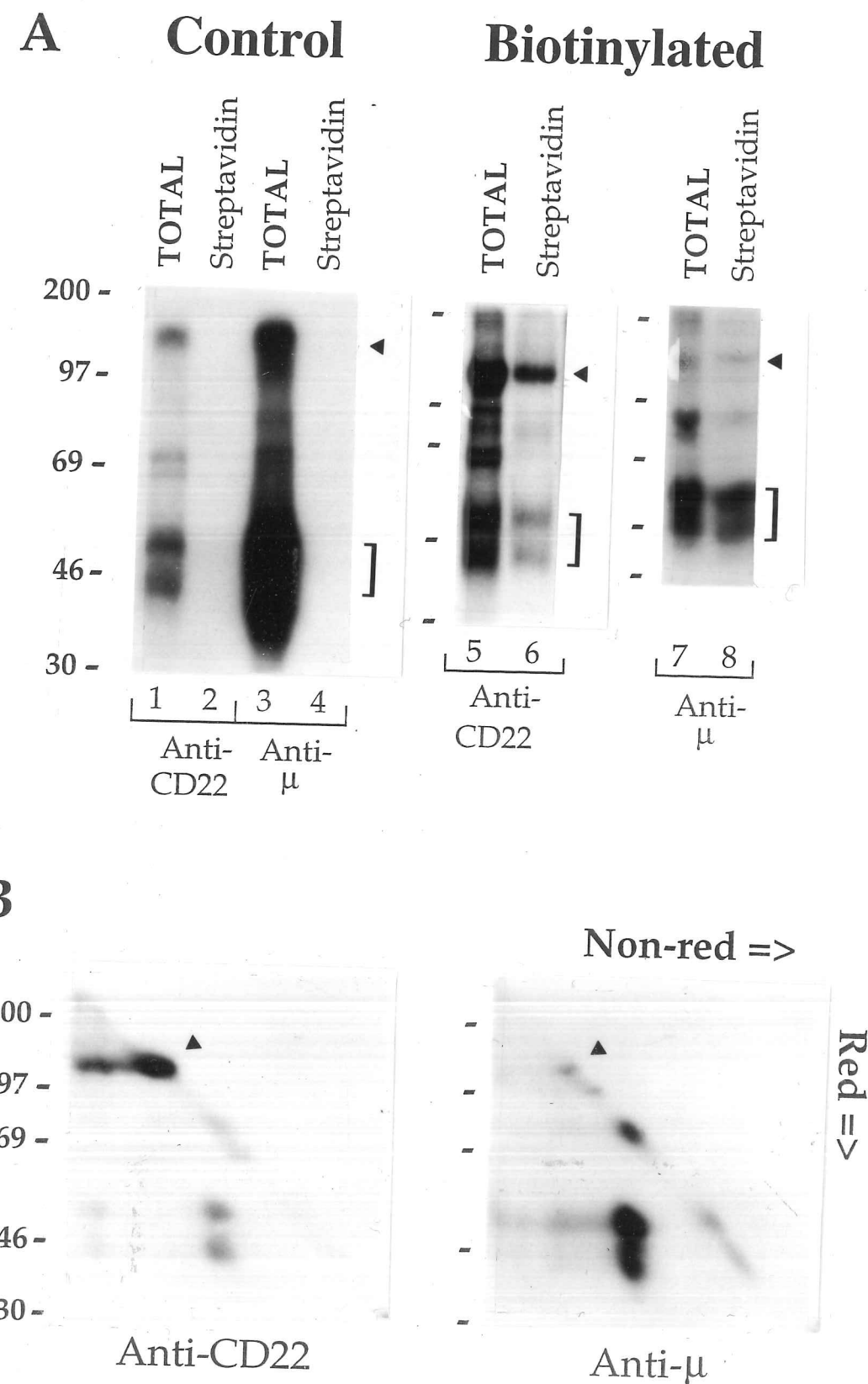


Figure 3.12

The CD22/IgM Interaction occurs in Murine Primary Cells

Analysis by SDS/PAGE of the products of *in vitro* kinase assays performed on IgM immunoprecipitates from spleen cells. Lysates of splenic cells from BALB/c mice lysed in digitonin lysis buffer were incubated with rabbit anti- μ antisera (lane 1) or control rabbit serum (lane 4) and *in vitro* kinase assays performed on the immune complexes that had been immobilised on protein A-Sepharose. For the remaining lanes, the products were eluted from the Sepharose and re-precipitated with the IgG1 anti-murine CD22 mAb (TIB163) (lanes 2 and 5) (**Anti-CD22**) or a control IgG1 mAb (lanes 3 and 6) (**Control**) and protein G-Sepharose. The presumed migration position of murine CD22 is indicated by an arrowhead. This figure was prepared from one photo of a gel with intervening lanes removed.

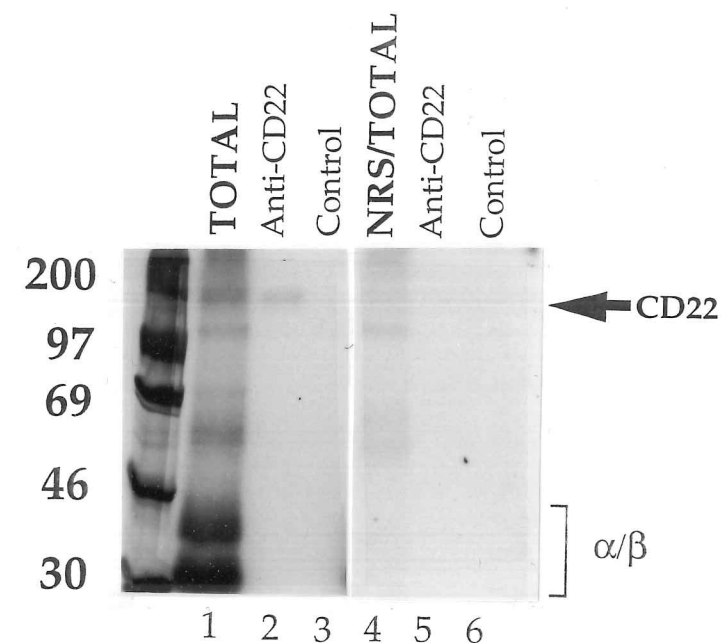


Figure 3.13

Analysis of the CD22/IgM Interaction in Murine Cell Lines

Analysis by 2D SDS/PAGE of the products of *in vitro* kinase reactions performed on immunoprecipitates prepared from digitonin lysates of the mIgM⁺ cell lines WEHI-231 (A), myeloma transfectants J558L/IgM (B) and J558L/IgM/CD22 (C) which expresses mIgM and CD22 (see next chapter for further details). An α/β sheath that migrates as a distinct phosphorylated heterodimer is apparently not evident in CD22 precipitates (Anti-CD22), control precipitates prepared with a control IgG1 mAb (IgG Control), or a precipitate prepared with rabbit serum (NRS), but a heterodimer likely to be the sheath is obvious in the IgM precipitate (IgM).

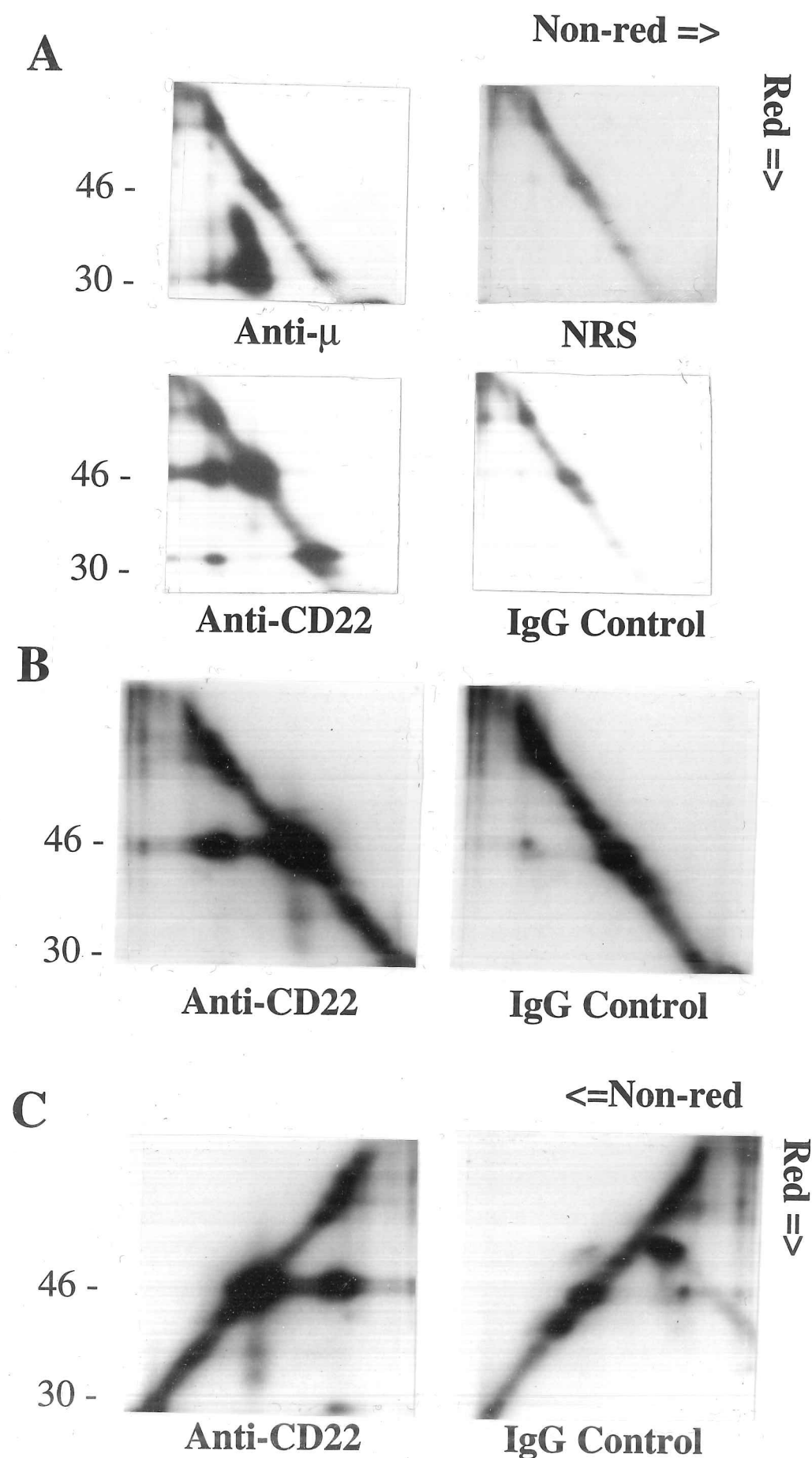


Figure 3.14

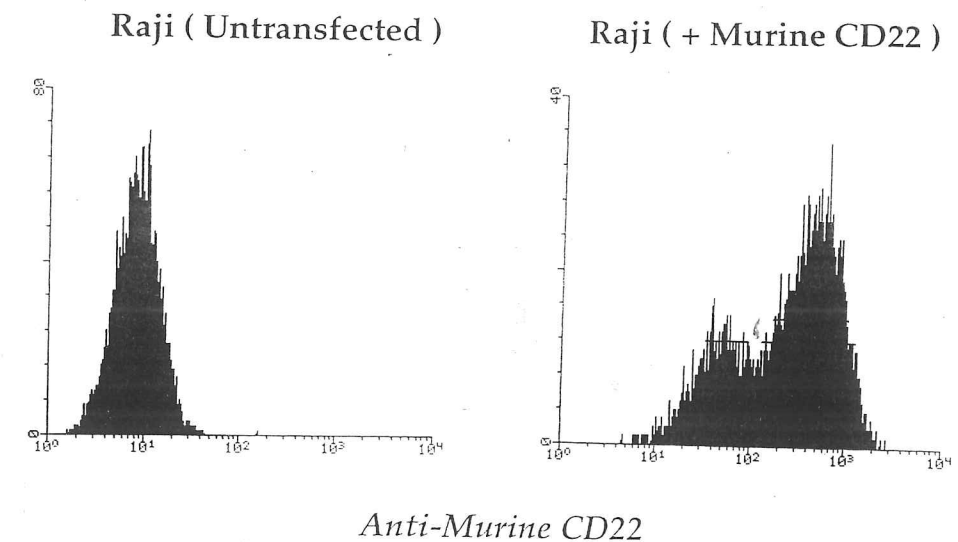
Association of Murine CD22 with Human IgM

I

(A) The right hand panel shows a FACS analysis of a clone of the human Burkitt lymphoma line Raji (Raji22) that has been stably transfected with a murine CD22 expression vector. The left hand panel is an analysis of untransfected Raji cells stained in the same manner. Vertical axes reflect the number of cells, horizontal axes reflect the log (fluorescence).

(B) SDS/PAGE analysis of *in vitro* kinase assays reveal an association of murine CD22 with human IgM in a Raji transfectant (Raji22) that expresses murine CD22. Digitonin lysed Raji or Raji22 cells (as indicated) were incubated with rabbit anti- μ antisera (lanes 1-8) or control rabbit serum (lane 9) and *in vitro* kinases assays performed on the precipitates immobilised on protein A-Sepharose. To assess for the presence of CD22, the products of the reactions were eluted from the Sepharose and were then re-precipitated with anti-human CD22 (To15) (lanes 3 and 7), IgG1 anti-murine CD22 (TIB 163) (lanes 2, 6 and 10) or IgG1 control mAbs (lanes 4 and 8). The presumed positions of CD22 are indicated.

A



B

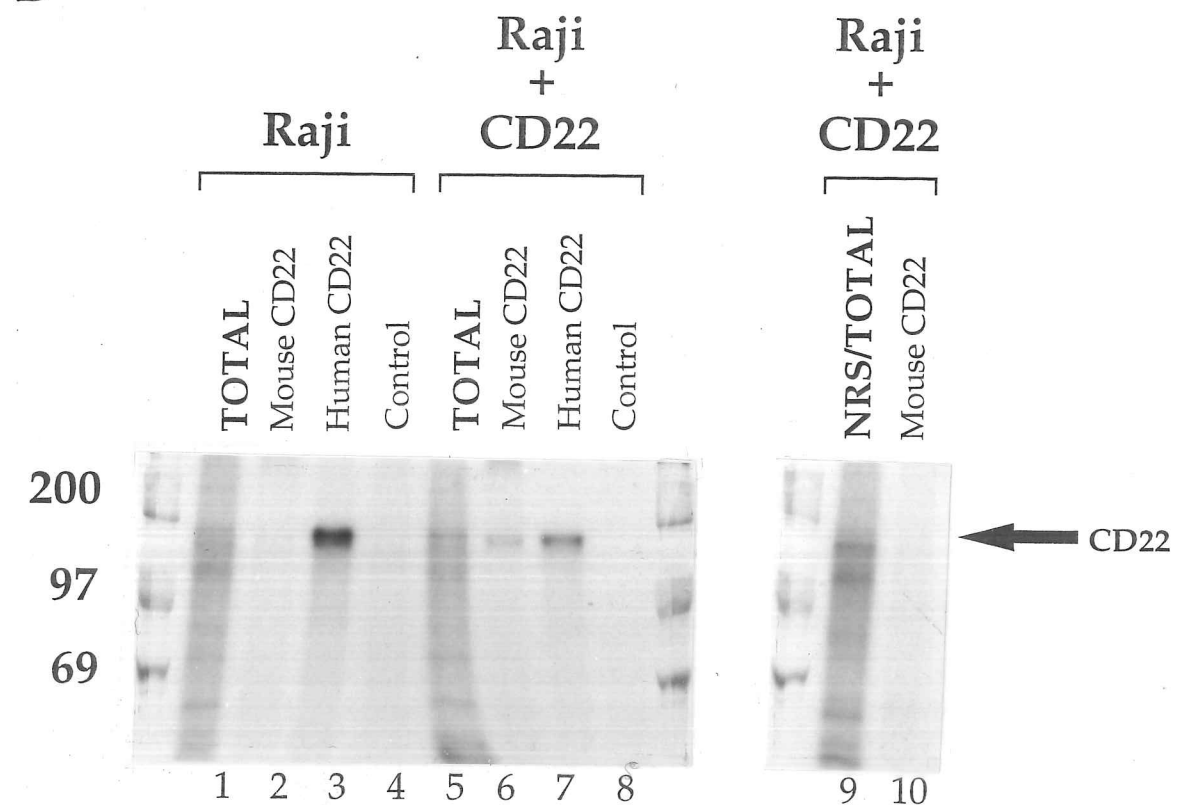


Figure 3.15

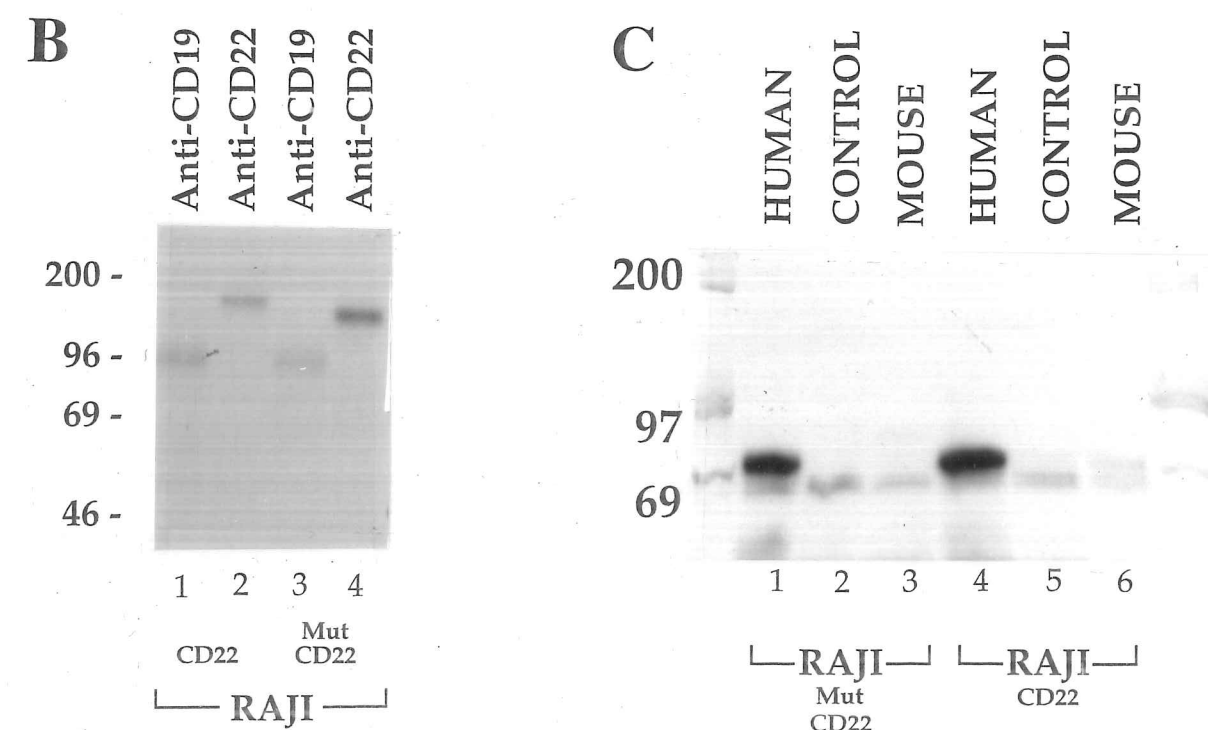
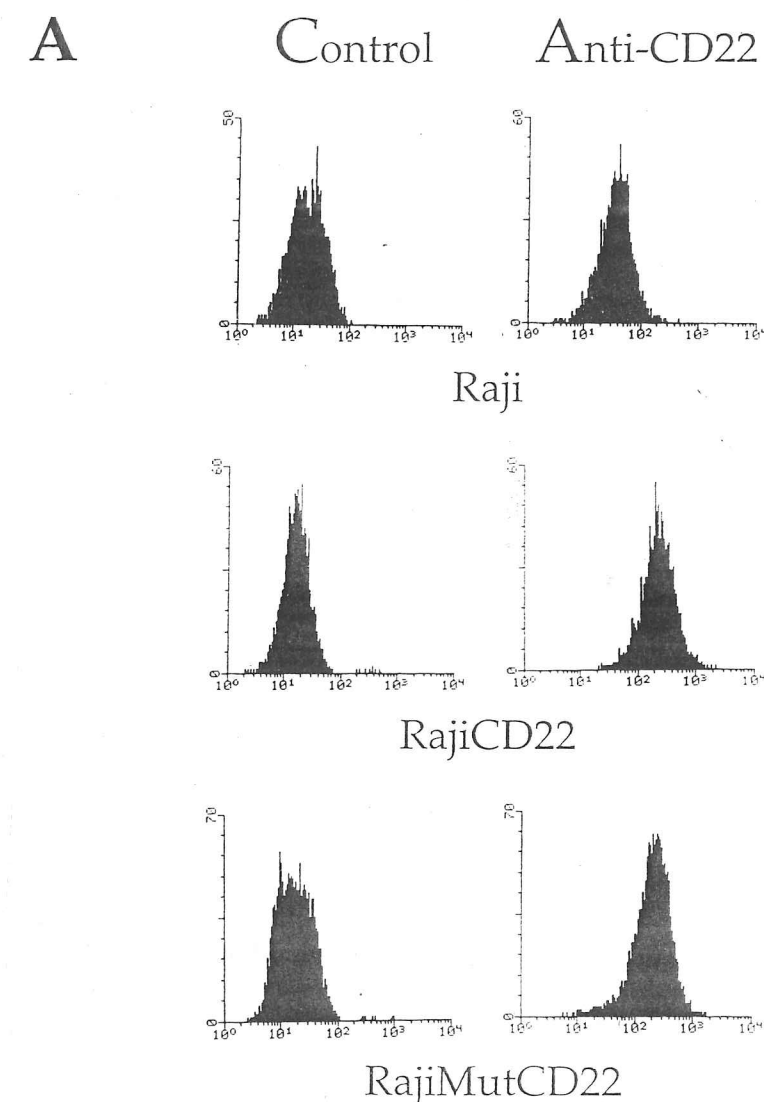
Association of Murine CD22 with Human IgM

II

(A) FACS analysis of three cell lines, stained for murine CD22 using the anti-CD22 mAb TIB163 and a secondary label of FITC-conjugated goat anti-mouse IgG₁; untransfected Raji (**Raji**), a Raji clone expressing murine CD22 (**RajiCD22**) and a Raji clone expressing mutant CD22 (**RajiMutCD22**). The left hand panel in each case corresponds to a control staining using the secondary antibody only. Vertical axes reflect the number of cells, horizontal axes reflect the log (fluorescence).

(B) Analysis by SDS/PAGE of precipitates prepared using the IgG1 anti-murine CD22 mAb (TIB163) or a control IgG1 anti-human CD19 mAb (as indicated) prepared from ¹²⁵I-labelled RajiCD22 or RajiMutCD22 cells lysed in Triton X100 lysis buffer.

(C) Western blotting of precipitates prepared using the IgG2b anti-human CD22 (To15) mAb (**HUMAN**), the IgG1 anti-murine CD22 mAb (TIB163) (**MOUSE**) or a control IgG1 anti-CD19 mAb (**CONTROL**). RajiCD22 or RajiMutCD22 cells were lysed in digitonin lysis buffer, and precipitates were analysed by SDS/PAGE, transferred to membrane and probed with rabbit anti- μ antisera and ¹²⁵I-protein A. The dark band at approximately 70 kD in the human CD22 precipitates is highly likely to be μ . A protein band of an appropriate molecular weight to be human μ can also be seen in the murine CD22 precipitate from RajiCD22 but is not evident in the MutCD22 precipitate. To ensure matched cell lines, a different RajiCD22 transfectant was used for this experiment than was used in Figure 3.14, page 101.



RESULTS

4 TYROSINE PHOSPHORYLATION OF CD22 AFTER ANTIGEN RECEPTOR CROSSLINKING

Introduction

The specific interaction between CD22 and the antigen receptor suggests that CD22 functions during antigen receptor-mediated signal transduction. Consistent with this suggestion, examination of the CD22 cytoplasmic tail reveals that it contains several tyrosines which are present in an arrangement with significant homology to the tyrosine-based motifs present in the antigen receptor sheath tails (Reth, 1989) (Figure 4.1, page 109). I therefore wondered if CD22, like α and β , is tyrosine phosphorylated after antigen receptor stimulation. This chapter demonstrates that CD22 is, indeed, tyrosine phosphorylated after receptor crosslinking and investigates which components of the antigen receptor are responsible.

Tyrosine Phosphorylation of CD22 After Antigen Receptor Crosslinking

The following experiment was performed in order to determine if CD22 may be tyrosine phosphorylated after antigen receptor stimulation. Daudi cells were incubated with rabbit anti- μ antisera which binds to mIgM and probably triggers transmembrane signalling by crosslinking the receptor on the cell surface. CD22 was then immunoprecipitated using the anti-CD22 mAb To15 from detergent lysates of the cells and the immunoprecipitates analysed by SDS/PAGE and then electroblotted onto membrane. The phosphorylation of tyrosines in CD22 was assessed by probing the blot with rabbit anti-phosphotyrosine antisera and ^{125}I -protein A. The results (Figure 4.2, page 110) suggest that CD22 is tyrosine phosphorylated after antigen receptor crosslinking. This effect was rapid, and like the increase in the tyrosine phosphorylation of the α/β sheath, was evident after incubation with antisera for 1 minute. I repeated this experiment, but instead used whole goat anti- μ antisera and F(ab) $_2$ ' goat anti- μ antisera for crosslinking (Figure 4.3A and B, page 111). The fact that goat antisera could apparently induce CD22 tyrosine phosphorylation suggests that the change is not a specific property of rabbit antisera. The ability of F(ab) $_2$ ' antisera to

mediate phosphorylation implies that it is not dependent on the interaction of the constant regions of the crosslinking antisera with Fc receptors.

Can CD22 Mediate Tyrosine Phosphorylation?

Having established that CD22 can be tyrosine phosphorylated, I wondered if CD22 itself, may possess another characteristic of Reth motif containing proteins and have the capacity to induce tyrosine phosphorylation. In order to address this issue, Daudi cells were incubated with anti- μ , anti-CD22, anti-CD19 or control antisera together with secondary crosslinking antisera where appropriate and the change in the tyrosine phosphorylation of cellular proteins assessed. This was done by probing whole cell lysates, that had been analysed by SDS/PAGE and electroblotted onto membrane, with anti-phosphotyrosine antisera and ^{125}I -protein A. As is evident in Figure 4.4A, page 112, extensive tyrosine phosphorylation was induced by crosslinking mIgM, however little change was induced by the other treatments. Thus whilst CD22 apparently does not mediate extensive tyrosine phosphorylation under the conditions above, it clearly may have less obvious effects on cellular tyrosine phosphorylation. I therefore assessed the effect of incubating Daudi cells with anti-CD22 antisera on the tyrosine phosphorylation of the α/β sheath proteins and CD22 itself. Significant changes in the amount of tyrosine phosphorylation of these proteins were not evident (Figure 4.4B, page 112). These data imply that mIgM is a more potent inducer of tyrosine phosphorylation than CD22 and argue that stimulation of the antigen receptor (as opposed to stimulation of CD22) is an important mechanism for inducing CD22 tyrosine phosphorylation.

Murine CD22 is Tyrosine Phosphorylated after Antigen Receptor

Crosslinking

I intended to study this pathway further using murine cells and therefore addressed whether CD22 is tyrosine phosphorylated following antigen receptor crosslinking in murine cell lines. Thus the B-cell lymphoma WEHI-231, which expresses mIgM, was incubated with anti- μ antisera, murine CD22 immunoprecipitated and the presence of phosphotyrosines in CD22 immunoprecipitates assessed using anti-phosphotyrosine antisera similarly to before, except that the blots were developed with peroxidase anti-rabbit Ig antisera and a luminescent substrate. As shown in Figure 4.5A, pages 113, murine CD22 appears to be tyrosine phosphorylated after mIgM crosslinking despite the fact that I was unable to demonstrate an association between IgM and CD22 in this cell line (see Chapter 3). This property does not appear to be limited to B cells which express mIgM because a similar experiment performed by incubating the mIgG⁺ B-cell lymphoma A20 with anti- γ antisera also seems to result in the tyrosine phosphorylation of CD22 (Figure 4.5B, pages 113). Further evidence that murine CD22 is tyrosine phosphorylated after antigen receptor crosslinking will be presented in the next chapter. It can therefore be concluded that the ability of the antigen receptor to mediate CD22 tyrosine phosphorylation is neither a species- nor isotype-specific phenomenon.

The Role of the α/β Sheath in CD22 Tyrosine Phosphorylation

How does the antigen receptor mediate the tyrosine phosphorylation of transmembrane proteins like CD22? To address which components of the B-cell antigen receptor are responsible, matched transfectants of the A20 cell line expressing various mutant antigen receptors were used. These transfectants expressed either mIgM (A20/IgM), a mutant antigen receptor which contains the extracellular domain of IgM heavy chain, the transmembrane of MHC class I H2^K and the cytoplasmic tail of the β sheath protein (A20/IgM-Mut β) or variants of this that contain either one or both of the tyrosines within the β tail mutated (A20/IgM-Mut β Y¹⁹⁵>L and A20/IgM-Mut β Y^{195,206}>L respectively). They were all derived by transfection of an expression

plasmid encoding a lambda light chain and the appropriate heavy chain with combined binding specificity for the hapten NIP (originally described and derived by Patel and Neuberger, 1993). The cell lines were sorted and analysed by FACS to ensure comparable degrees of IgM expression (Figure 4.6, page 114).

The A20 transfectants, together with untransfected A20 cells as a control, were each incubated with anti- μ or anti- γ antisera and the tyrosine phosphorylation of CD22 assessed. Figure 4.7A, page 115 shows that both the A20/IgM and A20/IgM-Mut β cell lines could efficiently induce CD22 tyrosine phosphorylation under these conditions but, in contrast, there was no evidence that untransfected A20 could do so when incubated with anti- μ antisera. Thus it can be concluded that both the IgM and Mut β antigen receptors can induce CD22 tyrosine phosphorylation. Both of the transfectants that express mutant IgM molecules with a β tail that has one or both tyrosines mutated appeared to be unable to induce significant phosphorylation of CD22 after incubation with anti- μ antisera (Figure 4.7B, page 115) even though their endogenous mIgG antigen receptors could efficiently do so. Taken together these results indicate that the ability to phosphorylate CD22 can be ascribed to the α/β sheath in the B-cell antigen receptor, and provision of the β tail alone is sufficient to allow CD22 tyrosine phosphorylation after crosslinking. In addition, the failure of the tyrosine mutated antigen receptors to induce detectable CD22 phosphorylation suggests that the tyrosines within the tail of β are essential for this activity.

Conclusions

CD22 contains a sequence with significant homology to the Reth motif in its cytoplasmic tail and is tyrosine phosphorylated after antigen receptor crosslinking. The ability of both mIgM and mIgG to mediate this phosphorylation implies that components common to these isotypes are responsible. Consistent with this notion, analyses of the signalling capabilities of mutant antigen receptors suggests that the

ability of the receptor to elicit the phosphorylation can be ascribed to the presence of α/β sheath. Indeed, the cytoplasmic tail of β is sufficient for this signal to be generated. Mutagenesis of the tyrosines within the β tail appears to abolish its ability to mediate significant CD22 phosphorylation upon crosslinking suggesting that these sheath tyrosines and by implication SH2-domain containing proteins are important for inducing the tyrosine phosphorylation of CD22.

Figure 4.1

A Putative Signalling Motif in the Cytoplasmic Tail of Human CD22

(The complete human CD22 sequence can be found in Wilson et al., 1991; a similar sequence is also present in murine CD22, see Torres et al., 1993)

Motif

D/E - X(7) - D/E - X(2) - Y - X(2) - L/I - X(7) - Y - X(2) - L/I

D	- X(7) -	D - X(2) - Y - X(2) - L	- X(7)	- Y - X(2) - L	α
E	- X(7) -	E - X(2) - Y - X(2) - L	- X(7)	- Y - X(2) - L	β
D	- X(9) -	E - X(3) - Y - X(2) - L	- X(16)	- Y - X(2) - L	CD22

Tail Sequences

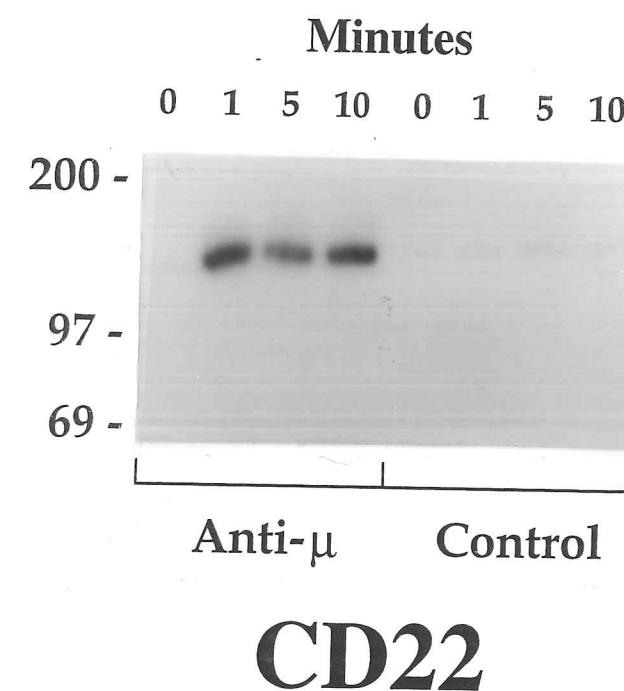
Figure 4.2

Tyrosine Phosphorylation of CD22 After Antigen Receptor Crosslinking

I

Anti-phosphotyrosine Western blots of CD22 immunoprecipitates prepared following antigen receptor crosslinking. Daudi cells were incubated with rabbit anti- μ antisera or control rabbit serum for the times indicated and immunoprecipitates prepared from cell lysates using the anti-CD22 mAb (To15) (A) or the anti- β mAb (HM47) (B). Following SDS/PAGE and transfer to membrane, blots were probed for phosphotyrosine using rabbit anti-phosphotyrosine antisera and ^{125}I -Protein A.

A



B

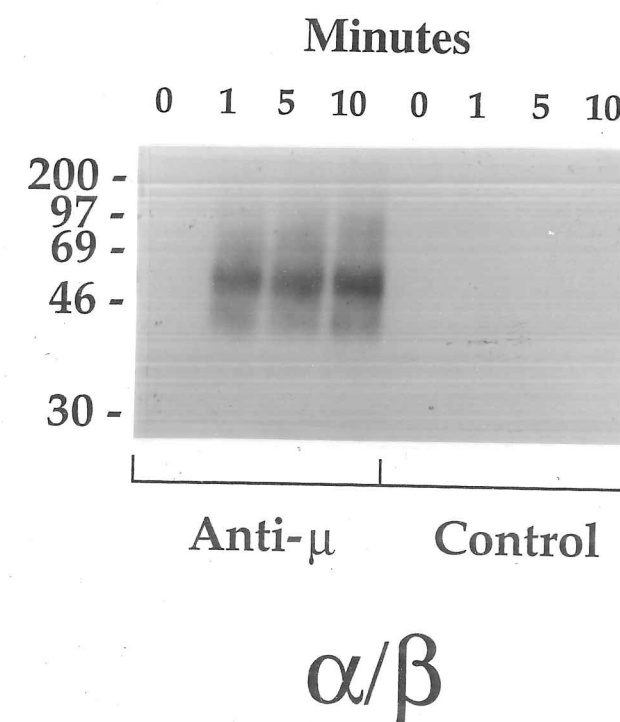


Figure 4.3

Tyrosine Phosphorylation of CD22 After Antigen Receptor Crosslinking II

Anti-phosphotyrosine Western blots of CD22 immunoprecipitates prepared following antigen receptor crosslinking. Daudi cells were incubated with F(ab)2' goat anti- μ antisera (A), whole goat anti- μ antisera (B) or control goat immunoglobulin (Control) for the times indicated and immunoprecipitates prepared from cell lysates using the anti-CD22 mAb (To15). Following SDS/PAGE and transfer to membrane, blots were probed for phosphotyrosine using rabbit anti-phosphotyrosine antisera and ^{125}I -Protein A.

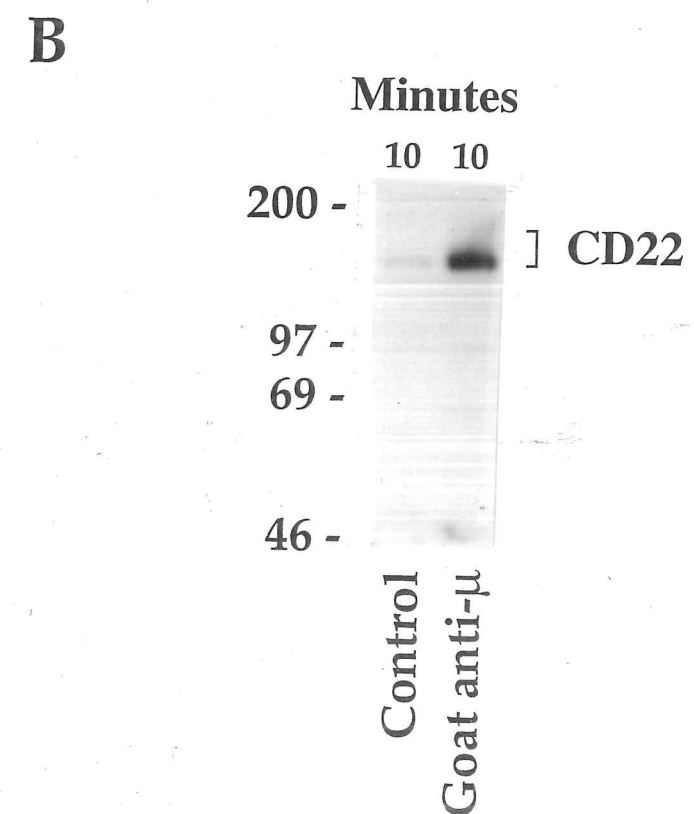
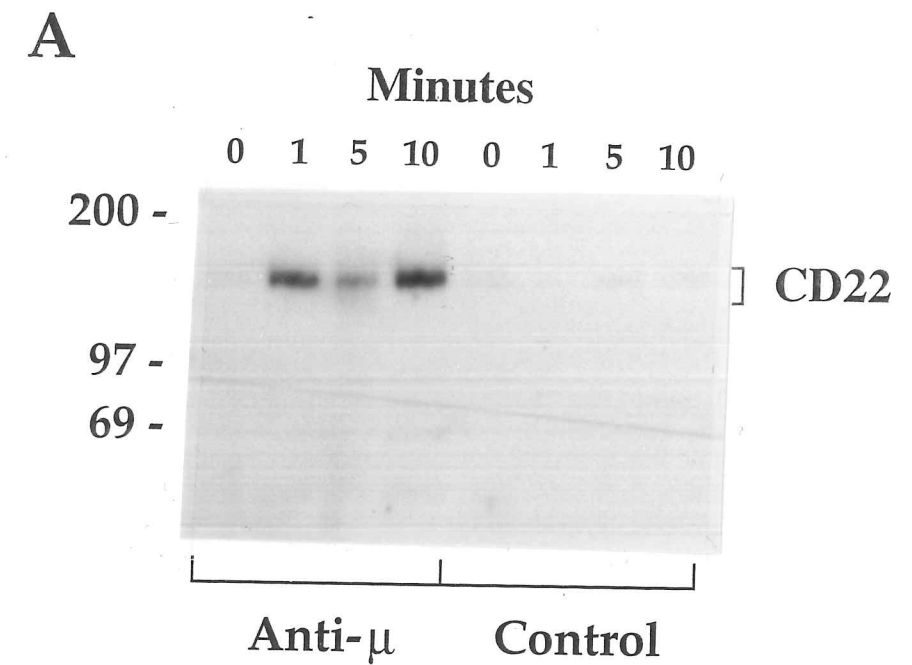


Figure 4.4

Can CD22 Induce Tyrosine Phosphorylation?

(A) Anti-phosphotyrosine Western blot of whole cell lysates prepared from Daudi cells that had been incubated with medium alone (lane 1), rabbit anti- μ antisera (lane 2), control rabbit serum (lane 3), IgG2b anti-CD22 (To15) mAb (lane 4), anti-CD19 mAb (lane 5), two anti-CD22 (HD6 and HD39) mAbs that recognise separate epitopes and do not inhibit each others binding (Knapp et al., 1989) (lane 6) or control mouse IgG2b mAb (lane 7). For the monoclonal antibody treatments, a secondary layer of (Fab')₂ goat anti-mouse IgG was used, see materials and methods. Following cell lysis, whole cellular lysates, from which the cell membranes had been removed, were resolved by SDS/PAGE, blotted on membrane and then probed with anti-phosphotyrosine antisera and ¹²⁵I-protein A.

(B) Anti-phosphotyrosine Western blot of precipitates prepared from Daudi cells that had been incubated with medium alone (lanes 1 and 6), IgG2b anti-CD22 (To15) mAb (lanes 2 and 7), anti-CD19 mAb (lane 3), two anti-CD22 (HD6 and HD39) mAbs that recognise separate epitopes and do not inhibit each others binding (Knapp et al., 1989) (lane 4), control IgG2b mAb (lanes 5 and 8), or rabbit anti- μ antisera. For the monoclonal antibody treatments, a secondary layer of (Fab')₂ goat anti-mouse IgG was used, see materials and methods. Following cell lysis, the α/β sheath was precipitated (lanes 1-5 and 9) using the anti- β mAb or CD22 was immunoprecipitated using the anti-CD22 mAb (To15) (lanes 6-8). Precipitates were resolved by SDS/PAGE, blotted on membrane and then probed with anti-phosphotyrosine antisera and ¹²⁵I-protein A. Note the extensive tyrosine phosphorylation of the α/β sheath induced by anti- μ antisera in lane 9. The bands at approximately 40-55 kD may correspond to binding of ¹²⁵I-protein A to the heavy chain of the mAb heavy chain or reflect tyrosine phosphorylation of sheath proteins.

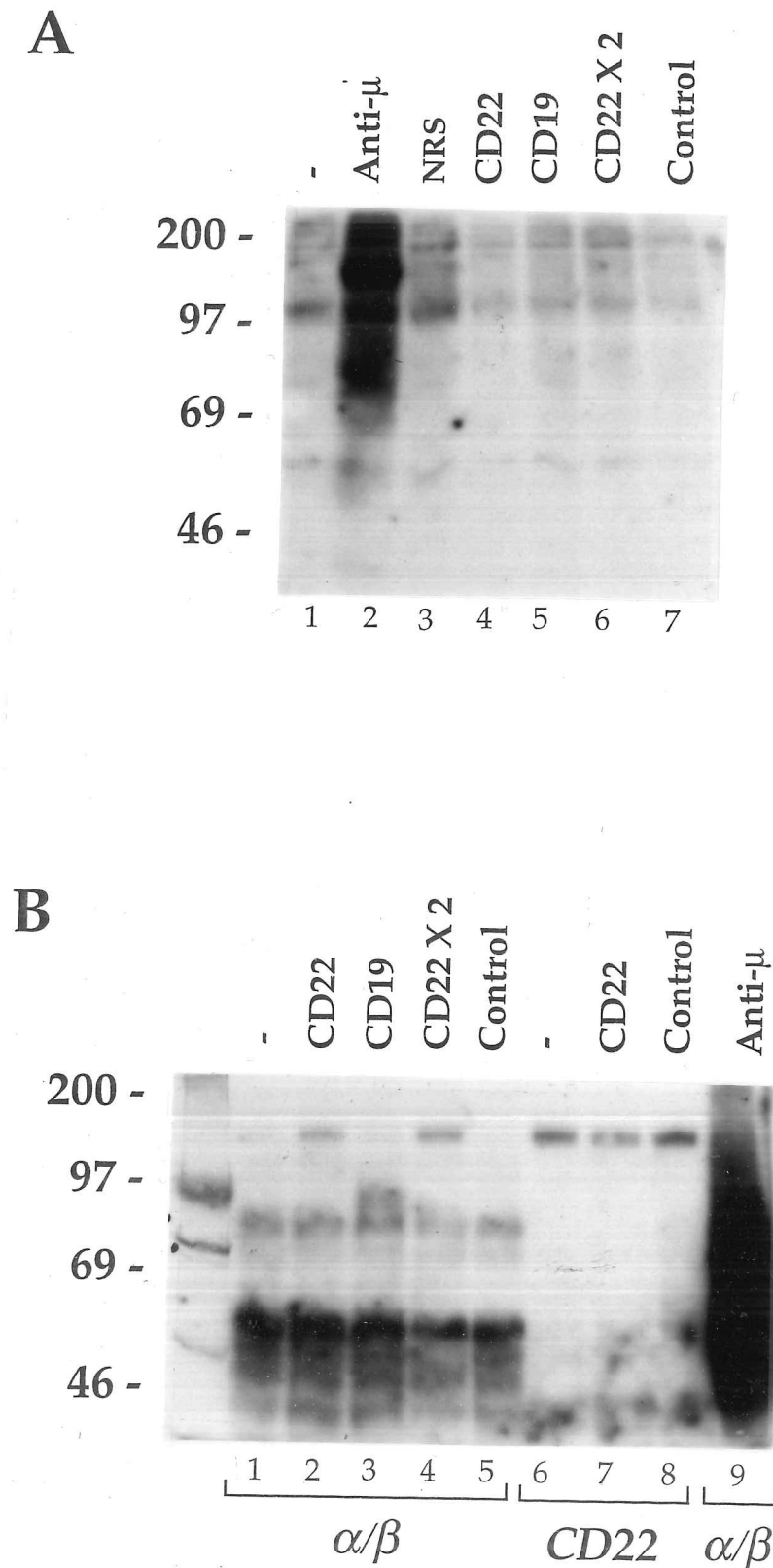


Figure 4.5

Murine CD22 is Tyrosine Phosphorylated after Antigen Receptor Crosslinking

Anti-phosphotyrosine Western blots of CD22 immunoprecipitates prepared from (A) WEHI-231 cells or (B) A20 cells. Prior to precipitation the cells had been incubated with medium alone (-) and (0), (Fab')₂ goat anti- μ or (Fab')₂ anti- γ antisera. With WEHI-231, a control immunoprecipitation was performed with a control IgG1 mAb (Control). With the A20 cells, incubation with crosslinking antisera was performed for either 5 or 10 minutes as indicated.

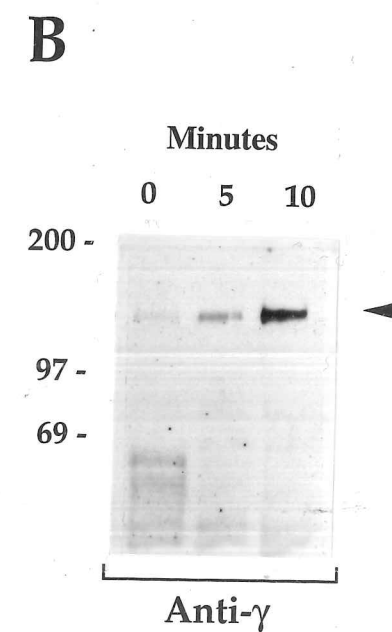
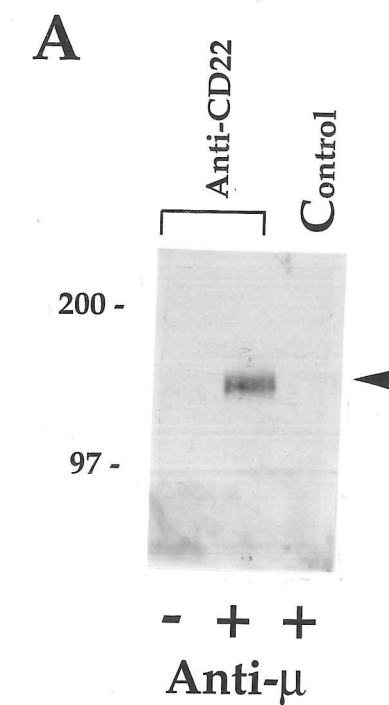
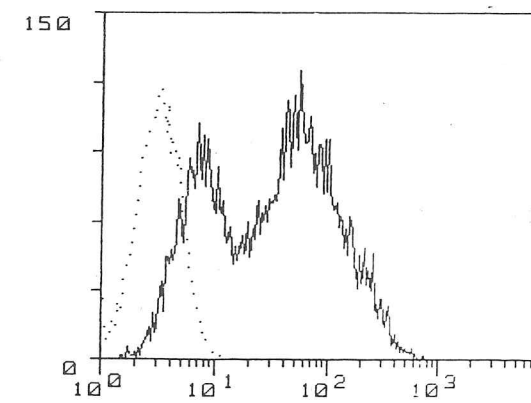


Figure 4.6

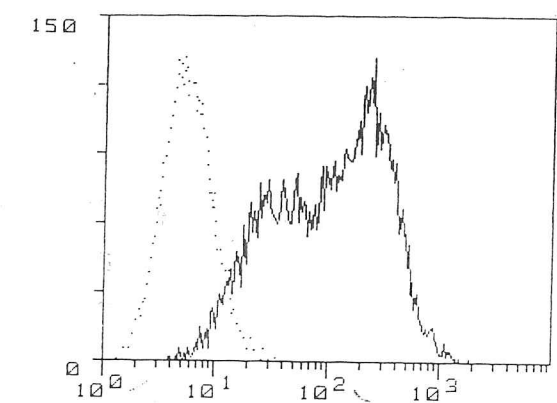
Role of the α/β Sheath in the Tyrosine Phosphorylation of CD22

I

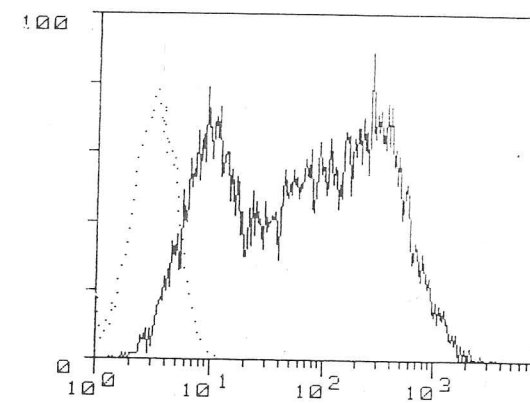
FACS analyses of A20 transfectants expressing normal IgM or mutant IgM molecules, stained with FITC-conjugated goat anti-mouse IgM, as indicated. All exhibit comparable expression. Horizontal axes reflect log of fluorescence, vertical axes correspond to the number of cells. The dotted lines reflect the FACS profile of unstained cells.



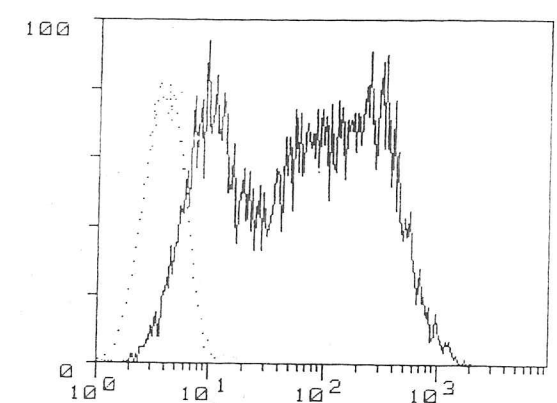
IgM



IgM/Mut β



IgM/Mut β
Y195,206=>L



IgM/Mut β
Y195=>L

Figure 4.7

Role of the α/β Sheath in the Tyrosine Phosphorylation of CD22

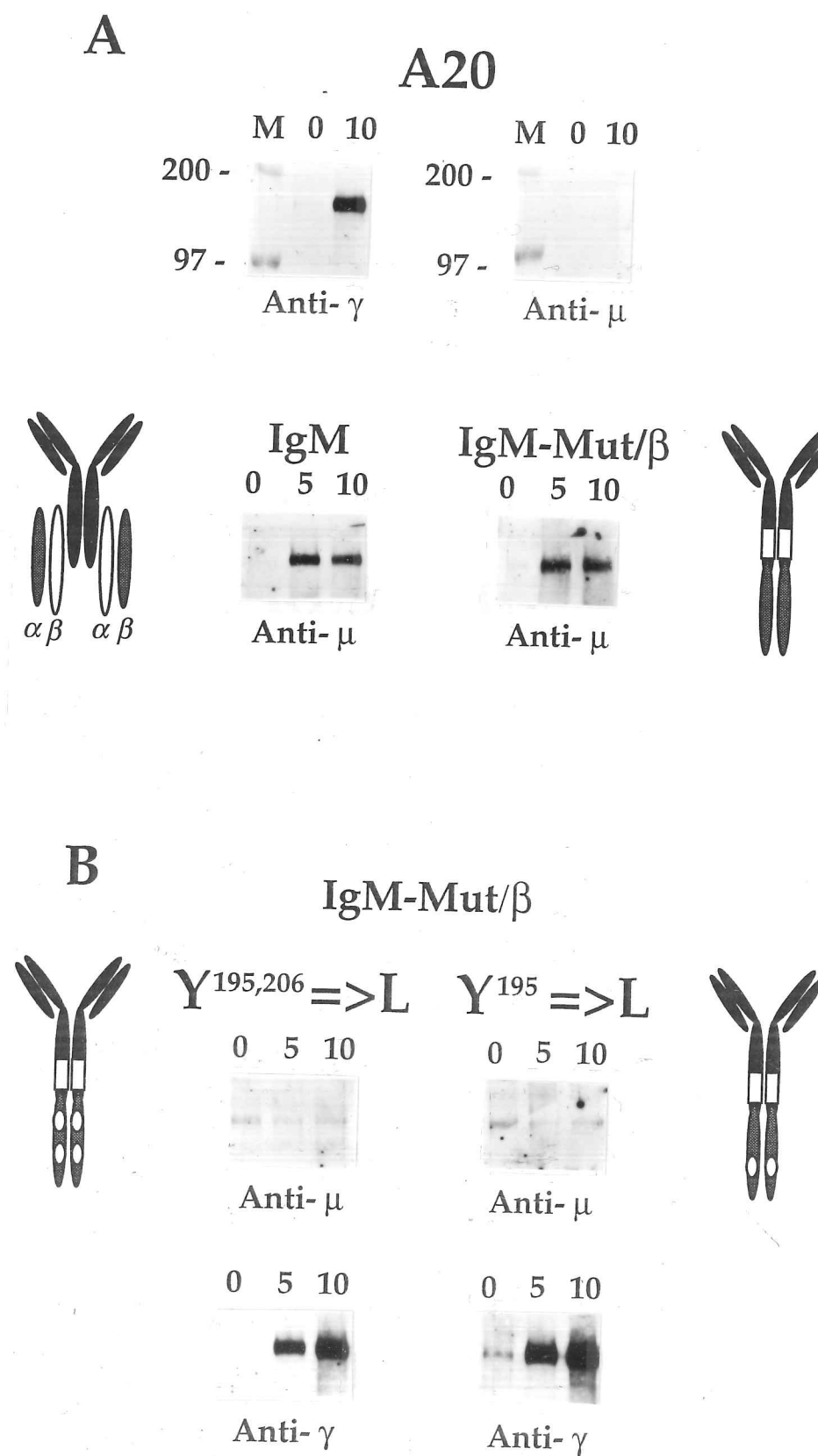
II

Anti-phosphotyrosine Western blots of CD22 immunoprecipitates prepared from cells incubated in medium alone or medium containing anti- μ or anti- γ antisera. All samples were prepared and analysed in parallel.

(A) Top left and right; As a control, untransfected A20 cells were incubated with F(ab)₂' goat anti- γ or F(ab)₂' goat anti- μ antisera for 10 minutes (as indicated) and the phosphorylation status of CD22 assessed by immunoprecipitation with the anti-murine CD22 mAb (TIB163), SDS/PAGE, transfer to membrane and probing for phosphotyrosines.

Lower left and right; A20 cells expressing a wildtype mIgM (IgM) or a mutant IgM containing the transmembrane of MHC class I H2^K and the cytoplasmic tail of β (IgM-Mut β) were incubated with F(ab)₂' goat anti- μ antisera for the times indicated and the tyrosine phosphorylation of CD22 assessed.

(B) A20 cells expressing IgM-Mut β variants with one or both tyrosines of the β tail mutated (as indicated) were incubated with F(ab)₂' goat anti- μ antisera (upper panels) or F(ab)₂' goat anti- γ antisera (that crosslinks the endogenous IgG) (lower panels) and the tyrosine phosphorylation of CD22 assessed.



RESULTS

5 SIGNAL TRANSDUCTION BY THE B-CELL ANTIGEN RECEPTOR

Introduction

The previous chapter attributed the ability of the antigen receptor to mediate CD22 tyrosine phosphorylation to the α/β sheath and tyrosines within β . These findings are consistent with the model whereby signalling involves Src-related kinases phosphorylating the tyrosines in the α and β tails and the recruitment of Syk. I wished to test this model and determine how the antigen receptor actually transduces signals by studying CD22 tyrosine phosphorylation. This chapter concentrates on two strategies (outlined below) that were designed to determine the mechanism(s) by which the sheath mediates CD22 phosphorylation.

CD22 Tyrosine Phosphorylation is Induced by Hydrogen Peroxide

One line of investigation was inspired by the publication of a paper that demonstrated that treatment of B cells with hydrogen peroxide (H_2O_2) activates the tyrosine kinase, Syk but apparently has no significant effect on the *in vitro* activity of the Src-related kinase, Lyn (Schieven et al, 1993). Thus I postulated (but as will be evident later, naively) that if H_2O_2 induced CD22 tyrosine phosphorylation in a B-cell line this would suggest that Syk is involved in phosphorylating CD22. I therefore asked whether the treatment of Daudi or A20 cells with H_2O_2 induces increased CD22 tyrosine phosphorylation. The results (Figure 5.1A and 5.1B lanes 1-3, page 123) suggest that this is the case.

The discovery that H_2O_2 can mediate the tyrosine phosphorylation of CD22 suggested that, by using a cell which expresses CD22 but does not induce its tyrosine phosphorylation in response to H_2O_2 , it may be possible to identify the factors involved (including Syk) by transfection of expression vectors encoding various lymphoid proteins, and determining whether they can potentiate H_2O_2 -mediated tyrosine phosphorylation. To this end, I established a stable transfectant of the murine fibroblast

cell line NIH3T3 that expresses murine CD22. However, incubation of this line with H_2O_2 induced significant tyrosine phosphorylation of CD22 (Figure 5.1B, pages 123). This result demonstrates that this effect of H_2O_2 is not lymphoid-specific, and therefore cannot be easily exploited to identify lymphoid factors that may be involved in the process. H_2O_2 has been shown to inhibit phosphatase activity (Hecht and Zick, 1992) and thus its ability to mediate CD22 phosphorylation can be attributed to its ability to inhibit phosphatase activity in NIH3T3 cells. These findings argue that antigen receptor crosslinking may induce CD22 tyrosine phosphorylation by inhibiting phosphatase action (in addition to activating tyrosine kinases).

CD22 is Tyrosine Phosphorylated after Antigen Receptor Crosslinking in the J558L Plasmacytoma

There is much evidence implicating cytoplasmic proteins such as Src-related tyrosine kinases and Syk in signalling by the B cell antigen receptor. In order to identify if these and other proteins are involved, a second line of investigation involved deriving a cell line which expresses CD22 and mIg but upon crosslinking does not phosphorylate CD22. Thus Src kinases and other proteins could be expressed by gene transfection and their role in signalling determined by measuring their ability to potentiate crosslinking-mediated CD22 phosphorylation. One candidate was the J558L plasmacytoma line which lacks expression of several proteins normally expressed in B cells including CD19 and CD21 (Hombach et al., 1988; Kim et al., 1993). I therefore asked if incubating a J558L transfectant expressing IgM and CD22 (J558L/IgM/CD22), with anti- μ antisera can induce CD22 tyrosine phosphorylation. J558L/IgM/CD22 was derived by transfection of genes encoding μ heavy chain and α (J558L already expresses λ light chain and β) and CD22 (T. O'Keefe and M. Neuberger, unpublished). J558L/IgM which expresses mIgM was used as a control and is the parental cell line from which J558L/IgM/CD22 was derived (Venkitaraman et al., 1991). FACS analyses of these cells stained for IgM and CD22 together with

untransfected J558L as a control are shown Figure 5.2, page 124. Although, no evidence of CD22 tyrosine phosphorylation was seen in the control J558L/IgM cell line, incubation of J558L/IgM/CD22 with anti- μ antisera but not control anti- γ antisera could induce significant CD22 tyrosine phosphorylation even though I was not able to detect a CD22/IgM interaction in this cell line in Chapter 3 (Figure 5.3A, page 125).

Until now, polyclonal antisera has been used to induce the receptor to signal and induce CD22 tyrosine phosphorylation. It is generally assumed that this mimics the natural binding of antigens to the B-cell antigen receptor and mediates effects that are representative of physiological signal generation. I wished to determine, if instead, a substance more representative of antigen could also induce CD22 tyrosine phosphorylation. To do this, I used the J558L/IgM/CD22 transfectant which expresses λ and membrane μ chain with combined specificity for the hapten NIP. This cell line was incubated with a NIP-BSA conjugate, (produced by covalently binding NIP to BSA in the ratio of 16:1) that can bind to the membrane immunoglobulin variable regions of the antigen receptors expressed by this cell, and the tyrosine phosphorylation of CD22 assessed. Figure 5.3B, pages 125 suggests that treatment of this transfectant with the conjugate can induce CD22 tyrosine phosphorylation.

These results demonstrate that the ability of the antigen receptor in J558L to induce CD22 tyrosine phosphorylation can be conferred by expression of CD22 itself, μ heavy chain and the α sheath protein. This finding implies that it would be difficult to identify additional factors which are essential for this pathway using J558L transfectants expressing other proteins.

Reconstitution of IgM and CD22 Expression in NIH3T3 Cells

Having discounted J558L as a suitable cell line, and because α and β can signal efficiently in at least one T cell line (Burkhardt et al, 1994), it appeared that that a non-

untransfected J558L as a control are shown Figure 5.2, page 124. Although, no evidence of CD22 tyrosine phosphorylation was seen in the control J558L/IgM cell line, incubation of J558L/IgM/CD22 with anti- μ antisera but not control anti- γ antisera could induce significant CD22 tyrosine phosphorylation even though I was not able to detect a CD22/IgM interaction in this cell line in Chapter 3 (Figure 5.3A, page 125).

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Having discounted J558L as a suitable cell line, and because α and β can signal efficiently in at least one T cell line (Burkhardt et al, 1994), it appeared that that a non-

haematopoietic cell line would need to be used for the reconstitution of antigen receptor-mediated CD22 phosphorylation. Thus the mouse fibroblast cell line NIH3T3 was used for further analyses. By calcium phosphate-mediated gene transfection, NIH3T3 cells were established which expressed the mutant form of IgM that consists of the extracellular domain of IgM, the transmembrane of MHC class I H-2K and the cytoplasmic tail of β (IgM-Mut β ; this has been shown to signal in A20 cells, see Chapter 4), CD22 and the transmembrane phosphatase CD45 (B-cell isoform). This was achieved by a sequential transfection protocol during which resistant transfectants were screened for IgM expression by staining with FITC-conjugated anti- μ antisera, for CD22 expression by staining with the anti-CD22 mAb (TIB163) and a secondary layer of FITC-conjugated anti-IgG1, and for CD45 expression by analysing cells stained with a phycoerythrin-conjugated anti-CD45 mAb (16A) by UV microscopy and FACS. Firstly, NIH3T3 cells expressing λ and heavy chain genes (G.T. Williams and M.S. Neuberger, unpublished) were transfected with a murine CD22 expression vector and then propagated in medium containing G418 sulfate. A population expressing IgM and CD22 was isolated and then transfected with a CD45 expression vector together with an expression plasmid conferring resistance to the antibiotic hygromycin. This latter vector also contained Lyn and Syk cDNA under heterologous promoters. An IgM/CD22/CD45 expressing population was selected for further analysis. FACS profiles of these cells and untransfected NIH3T3 cells stained for CD22, IgM and CD45 are in Figure 5.4, page 126. For details of vectors see the appendix.

These cells were incubated with anti- μ antisera and the tyrosine phosphorylation of CD22 assessed. Significant tyrosine phosphorylation of CD22 after incubation of these cells with anti- μ antisera was not detected (Figure 5.5, pages 127) even though H_2O_2 treatment reveals that there are significant quantities of CD22 present. I did not have available appropriate reagents to assess whether Syk and Lyn proteins were expressed and since examination of the ability of IgM-Mut β receptor to signal in other

cell lines was proving technically difficult, no further progress was made with line of investigation.

Detection of a Novel Phosphoprotein in CD22 Precipitates

To identify proteins that may interact with CD22 following antigen receptor crosslinking, Daudi cells were incubated with anti- μ antisera and CD22, CD19 or CD72 precipitates were prepared. Monoclonal antibodies that had been covalently coupled to protein G-Sepharose were used for precipitation to minimise obscuring of protein bands during western blotting and probing due to crossreaction with the immunoprecipitating mAb. The precipitates were analysed by SDS/PAGE, transferred to membrane and probed with anti-phosphotyrosine antisera and ^{125}I -protein A. The result provided an indication that a 65-70 kD unidentified phosphoprotein is specifically detected in CD22 precipitates after crosslinking (Figure 5.6, page 128).

Conclusions

Analysis of the signalling capability of a J558L transfectant that expressed mIgM and CD22 demonstrated that it can efficiently phosphorylate CD22 after receptor crosslinking. It was hoped that this cell line would be defective in this signalling process thus allowing the components of the signaling pathway to be determined by transfection of appropriate expression vectors and assaying for the tyrosine phosphorylation of CD22. The unsuitability of this line led to attempts to reconstitute antigen receptor signalling in the murine fibroblast cell line NIH3T3. Transfection experiments demonstrated that it is possible to obtain coexpression of mIgM, CD22 and CD45 antigens. However, I was unable to detect significant tyrosine phosphorylation of CD22 after incubation with anti- μ antisera.

Crosslinking of the antigen receptor using antisera is not essential for CD22 tyrosine phosphorylation, because treatment of cells with H_2O_2 could also induce CD22 tyrosine phosphorylation. This property of H_2O_2 was not lymphoid-specific and thus could not be easily exploited to allow lymphoid components of the H_2O_2 -mediated phosphorylation pathway to be determined. With regard to the situation after crosslinking, the chapter concludes with evidence that CD22 associates with an unidentified phosphoprotein.

Figure 5.1

CD22 Tyrosine Phosphorylation is induced by Hydrogen Peroxide

Anti-phosphotyrosine Western blots of CD22 precipitates prepared from cells incubated in the presence or absence of hydrogen peroxide.

(A) Daudi cells were incubated with medium alone (lanes 1 and 3), (Fab')₂ goat anti- μ antisera (lane 2) or hydrogen peroxide (H₂O₂) (lane 4) for 10 minutes. Tyrosine phosphorylation of CD22 was assessed by immunoprecipitation using the anti-CD22 mAb (To15), SDS/PAGE, transfer to membrane followed by probing with rabbit anti-phosphotyrosine antisera and ¹²⁵I-protein A. The presumed positions of CD22 are indicated by an arrow.

(B) Cells; A20 (lanes 1-3), NIH3T3 cells stably expressing murine CD22 (lanes 4-6) or control untransfected NIH3T3 cells (lanes 7-9) were treated with medium alone (-) or hydrogen peroxide (+) and then immunoprecipitates prepared using the IgG1 anti-CD22 mAb (TIB163) or a control IgG1 mAb followed by SDS/PAGE, transfer to membrane and probing for anti-phosphotyrosine. The presumed positions of CD22 are indicated by an arrow.

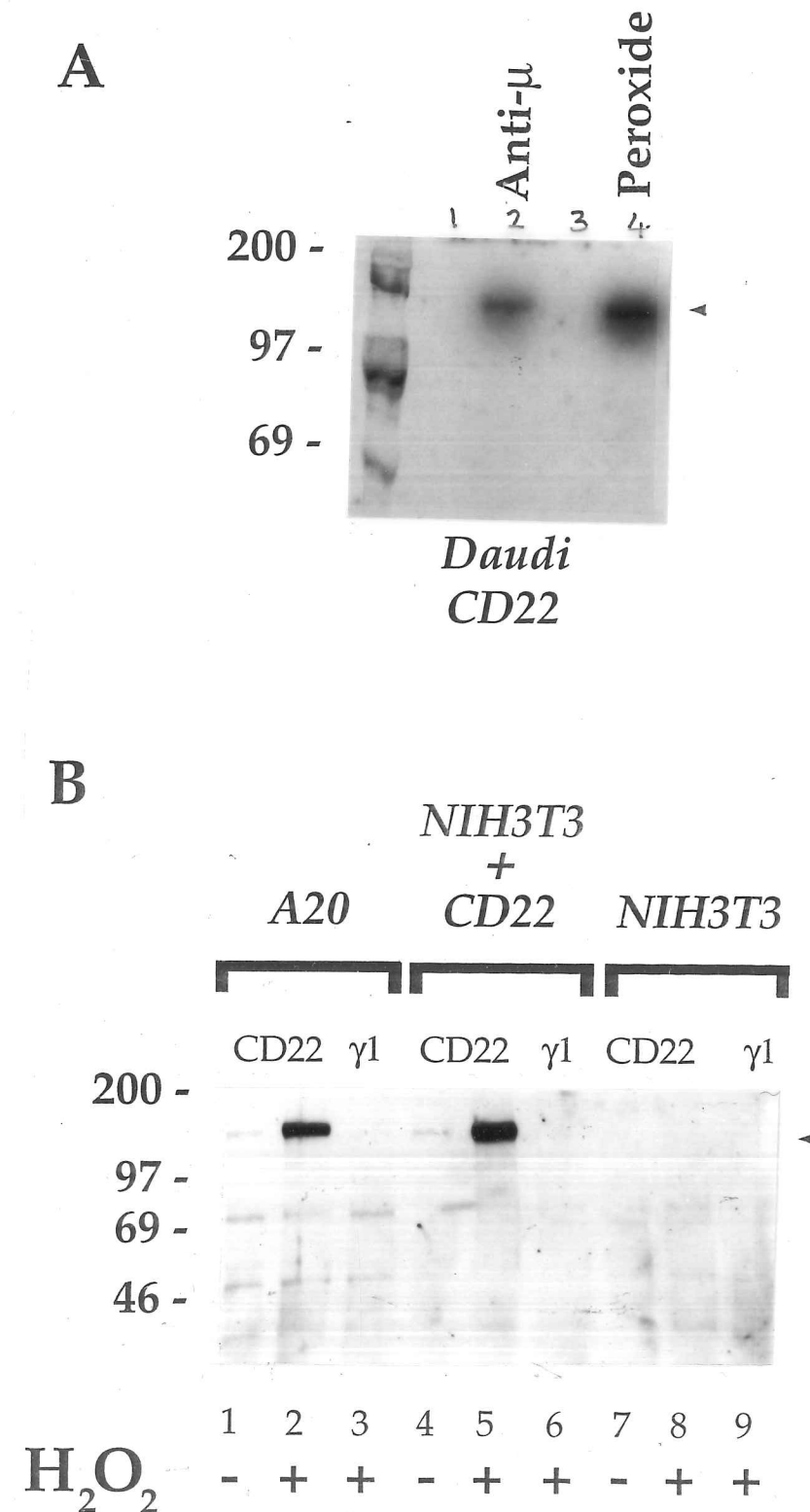


Figure 5.2

CD22 is Tyrosine Phosphorylated after Antigen Receptor Crosslinking
in J558L

I

FACS profiles of J558L, J558L/IgM and J558L/IgM/CD22 cell lines either unstained or stained for CD22 and IgM antigen expression. Vertical axes indicate number of cells, horizontal axes reflect the log (fluorescence).

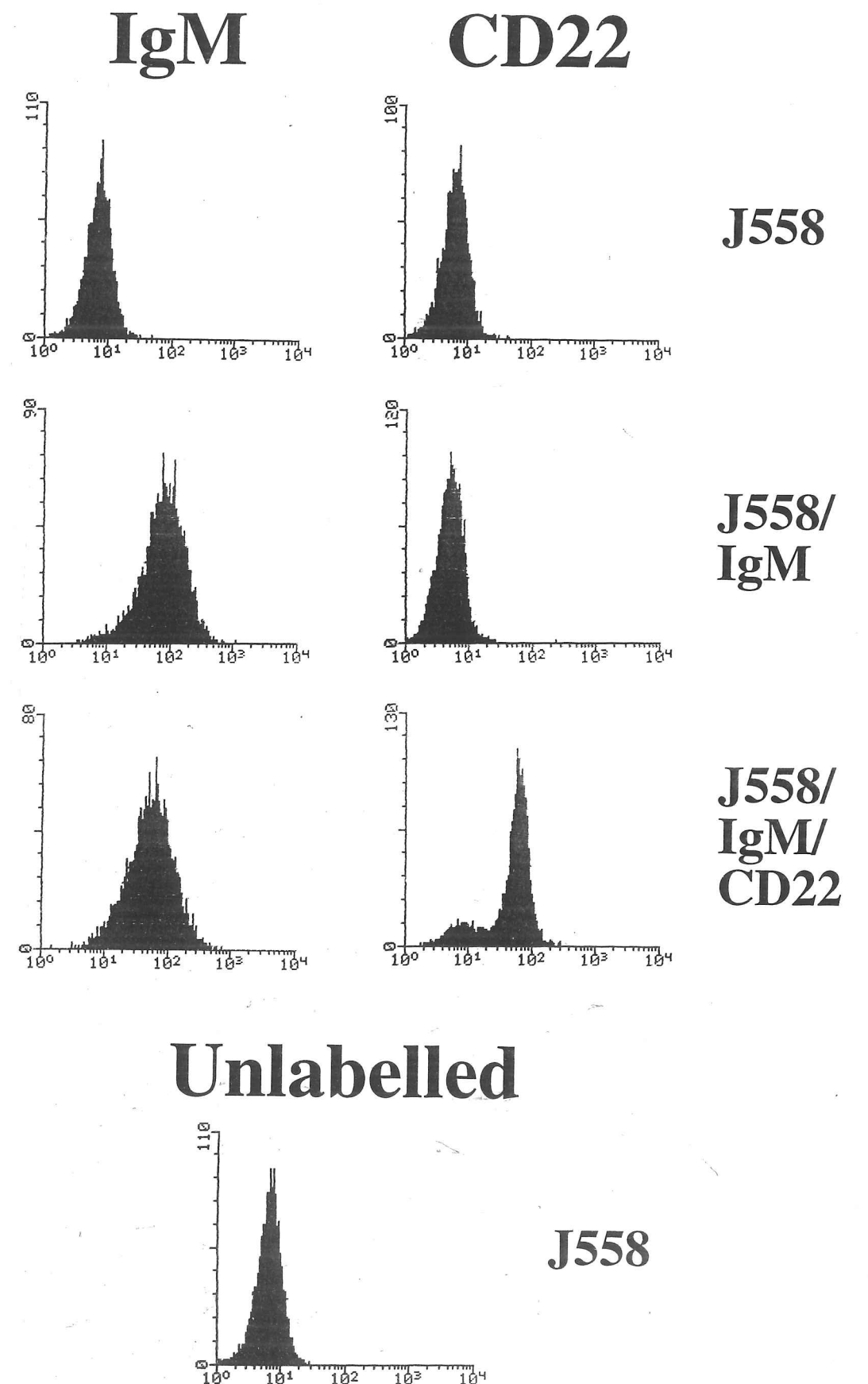


Figure 5.3

CD22 is Tyrosine Phosphorylated after Antigen Receptor Crosslinking in J558L

II

(A) Anti-phosphotyrosine Western blots of immunoprecipitates prepared from J558L cells expressing mIgM and CD22 (J558L/IgM/CD22) or mIgM (J558L/IgM) (as indicated) that had been incubated with medium alone (-) (lane 1), (Fab')₂ goat anti- μ antisera (+) (lanes 2, 3, 7 and 8) or (Fab')₂ goat anti- γ antisera (γ) (lanes 4-6). Precipitates were prepared using the IgG1 anti-CD22 mAb (TIB163) or a control IgG1 mAb. The presumed positions of CD22 are indicated by an arrow. The small amount of CD22 present in lane 1 may have arisen through carry over from the adjacent lane.

(B) Anti-phosphotyrosine Western blot of immunoprecipitates prepared from J558L/IgM/CD22 that had been incubated with medium (M), NIP-BSA (NIPBSA) or anti- μ antisera (Anti- μ) and the phosphorylation of CD22 assessed as above. The presumed positions of CD22 are indicated by an arrow

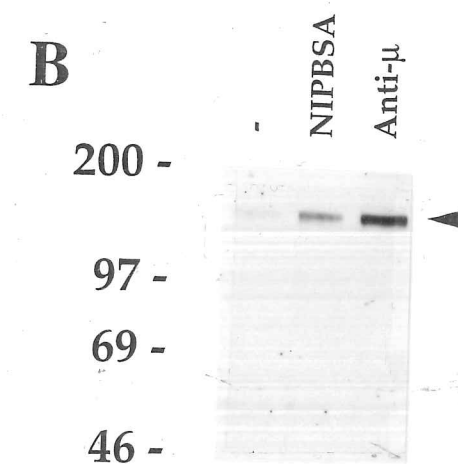
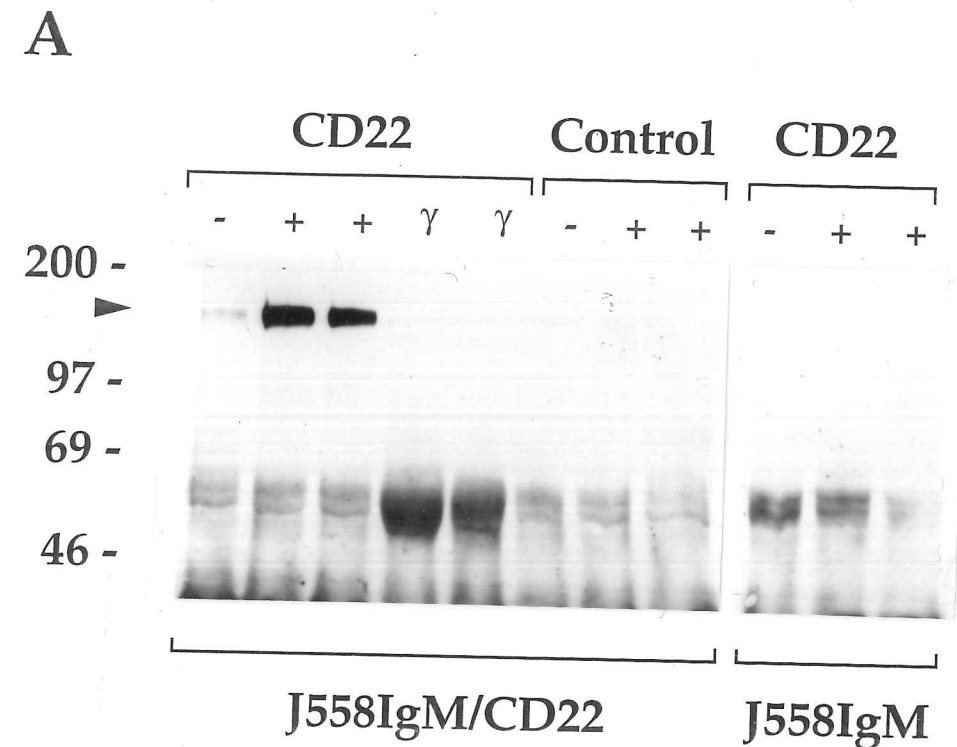


Figure 5.4

Reconstitution of IgM and CD22 Expression in NIH3T3 Cells

I

FACS Analysis of NIH3T3 expressing IgM-Mut β , CD45 and CD22 (as indicated). The left hand panels correspond to untransfected NIH3T3 cells stained for the three markers. For IgM staining, a FITC-conjugated goat anti-mouse IgM was used. For CD45, a phycoerythrin-conjugated rat anti-mouse CD45 that recognises the B cell isoform was used and finally for detection of CD22, staining was performed with the anti-murine CD22 mAb (TIB163) followed by a secondary FITC-conjugated goat anti-mouse IgG1. Vertical axes indicate number of cells, horizontal axes reflect the log (fluorescence).

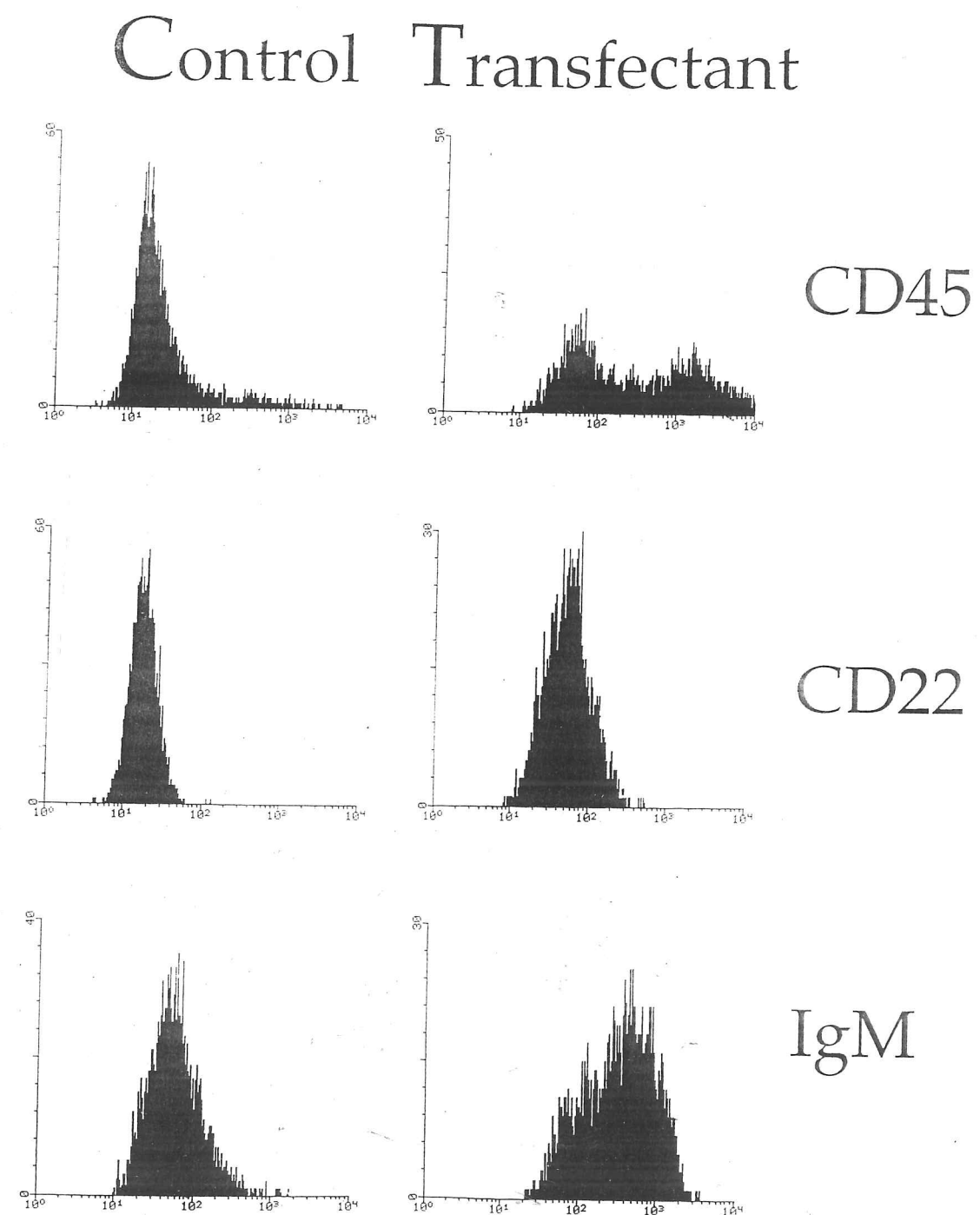


Figure 5.5

Reconstitution of IgM and CD22 Expression in NIH3T3 Cells

II

Anti-phosphotyrosine Western blot of CD22 precipitates prepared from 2×10^8 NIH3T3 cells expressing (as determined by FACS analysis), mutant μ (IgM-Mut β) (that can signal in A20 cells), CD22 and CD45 (see previous page) that had been incubated with medium alone (-), (Fab')₂ goat anti- μ antisera (+), or hydrogen peroxide (H). The phosphorylation of CD22 was assessed by immunoprecipitation using the anti-CD22 mAb (TIB163) followed by SDS/PAGE, western transfer and probing for phosphotyrosine. As a control, J558L/IgM/CD22 cells were incubated with (+) or without (-) (Fab')₂ goat anti- μ antisera, in parallel (**Control**). The presumed positions of CD22 are indicated by an arrow.

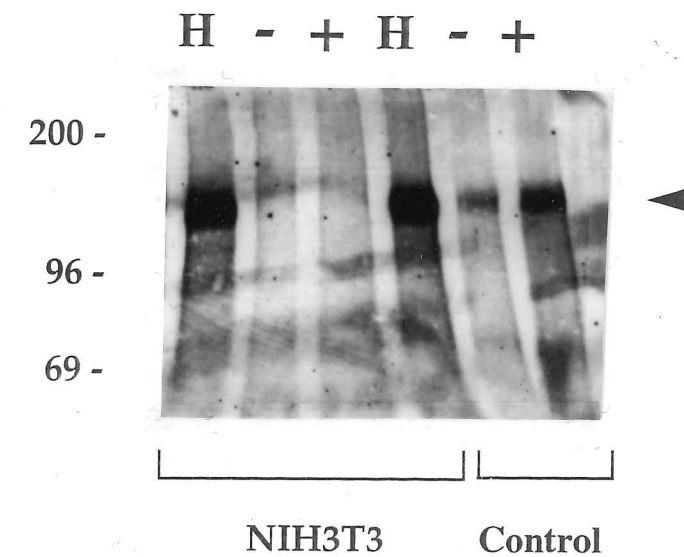
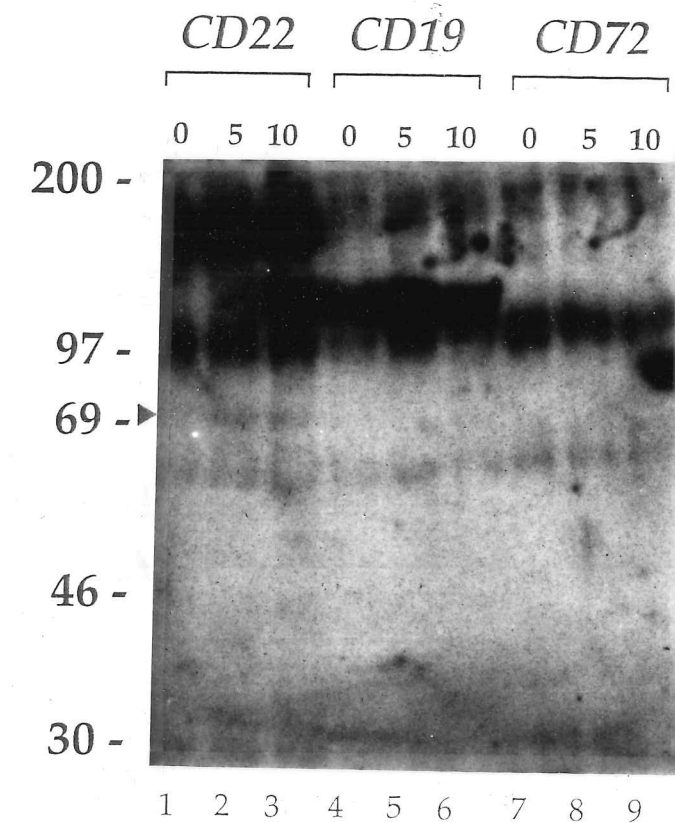


Figure 5.6

Detection of a Phosphoprotein in CD22 Precipitates following Antigen Receptor Crosslinking

Anti-phosphotyrosine Western blot of precipitates prepared from Daudi cells that had been incubated with (Fab')₂ goat anti- μ antisera for 5 or 10 minutes as indicated. Precipitates were prepared from lysates containing 1% NP40 using anti-CD22 (IgG2b)-Sepharose (lanes 1-3), anti-CD19 (IgG1)-Sepharose (lanes 4-6) or control anti-CD72 (IgG2b)-Sepharose (lanes 7-9). Following elution from the Sepharose, precipitates were analysed by SDS/PAGE, transferred to membrane and probed with anti-phosphotyrosine antisera and ¹²⁵I-protein A. The position of the 65-70 kD protein is indicated by an arrow.



RESULTS

6 DOWNREGULATION OF THE B-CELL ANTIGEN RECEPTOR

Introduction

In the absence of the α/β sheath, mIgM is not stably expressed on the cell surface and is retained intracellularly (Sitia et al., 1987; Hombach et al., 1988). This retention can be overcome by replacement of the polar areas in the transmembrane region of IgM with non-polar residues which results in the surface expression of a *sheathless* mIgM (Williams et al., 1990). This finding has led to the suggestion that the α/β sheath potentiates surface expression by masking the transmembrane polar charges thus allowing IgM to be stably incorporated into the lipid bilayer (Williams et al., 1990; Neuberger et al., 1993). By analogy with this phenomenon, the CD22/mIgM interaction highlights the possibility that mIgM and CD22 may also directly influence each others surface expression. Thus the CD22/IgM association and the potential importance of the regulated surface expression of CD22 and mIgM for the generation of immune responses (for example by virtue of the ability of CD22 to bind ligands on other cell types and of the antigen receptor to signal, internalise and potentiate the presentation of antigen) provided the impetus to look for a relationship between the surface expression of these molecules and to examine how CD22 and mIg surface expression is controlled. As a consequence of this approach, this chapter demonstrates that phorbol ester, an agent that activates protein kinase C, can mediate the downregulation of the B-cell antigen receptor complex.

Co-expression of mIgM and CD22 in J558L

Many studies of IgM surface expression have exploited the J558L plasmacytoma as a model cell (Hombach et al., 1988; Hombach et al., 1990; Williams et al., 1990; Venkitaraman et al., 1991; Wienands and Reth, 1992; Williams et al., 1993). This cell line was therefore used to assess whether surface expression of mIgM influences the expression of CD22. This was assessed by supertransfecting a J558L cell expressing CD22 with an expression vector encoding μ heavy chain and the sheath

protein, α . These two proteins are sufficient to reconstitute mIgM antigen receptor expression because J558L already expresses λ light chain and the β sheath protein (Hombach et al., 1990; Venkitaraman et al., 1991). Ten clones were randomly selected and analysed for surface expression of IgM and CD22 by labelling with the appropriate antisera and quantifying the fluorescence by FACS (Figure 6.1, page 138). No significant correlation in the effect of IgM expression on CD22 expression is evident. This conclusion is exemplified by comparison of clones 2 and 10 which both apparently share similar expression levels of CD22 but have dramatically different IgM fluorescence levels.

Effect of Phorbol Ester Treatment on mIgM in J558L

It was decided to test the effect of phorbol ester on the surface expression of the B-cell antigen receptor because phorbol ester has been previously shown to mediate the downregulation of several surface receptors including the T-cell receptor (Cantrell et al., 1987; Dietrich et al., 1994). It is believed to mediate many of its effects by acting as an inefficiently hydrolysable diacylglycerol analogue that permeates cell membranes and induces the activation of protein kinase C (PKC) (Kraft et al., 1982; Mori et al., 1982).

The effect of phorbol ester was examined by incubating a J558L transfectant expressing mIgM (J558L/IgM) (Venkitaraman et al., 1991) in DMEM medium at 37°C, in the presence or absence of the phorbol ester, phorbol 12-myristate 13-acetate (PMA) and assessing the change in detectable IgM by FACS analysis on whole cells labelled with FITC-conjugated goat anti-mouse IgM (Figure 6.2, pages 139). Treatment with PMA for 20 minutes resulted in an approximate 30% decrease in fluorescence suggesting that phorbol ester induces a rapid downregulation of mIgM. This result was consistently reproducible because, in further experiments with PMA incubation times varying between 15 minutes and 30 minutes and PMA concentrations varying from 10

μM to 40 μM , the effects of phorbol ester were similar, with between 25%-33% decreases in fluorescence observed (Figure 6.2, page 139).

Effect of Phorbol Ester Treatment on mIgM in WEHI-231

The effect of phorbol ester on the mIgM antigen receptor may be peculiar to J558L and to see if this is the case, I asked whether a similar effect could be seen using the B-cell lymphoma WEHI-231 which also expresses mIgM. WEHI-231 cells were therefore incubated in DMEM medium in the presence or absence of PMA and the anti-IgM fluorescence analysed by FACS as previously. PMA treatment appeared to result in a significant decrease in fluorescence (Figure 6.3, page 140). Indeed after incubation for 5 minutes, the fluorescence was reduced by around 30% and was at a similar level after 20 minutes of incubation. Notably, decreases of approximately 30% were consistently reproduced with PMA concentrations at 10 μM or 40 μM and when a WEHI-231 clone obtained from another laboratory was used. Thus both J558L and WEHI-231 display fluorescence decreases of between 25-36% in response to phorbol ester when labelled with FITC-conjugated anti-IgM.

PMA Increases the Size of an Intracellular Pool of mIgM

The decreases in fluorescence seen with phorbol ester suggests that PMA treatment increases the internal pool size of the antigen receptor. Whether this is the case was assessed as follows; WEHI-231 cells were labelled with NHS-SS-biotin which results in surface proteins being covalently bound via disulphide bonds to biotin moieties. Cells were then incubated in DMEM medium with or without PMA at 37°C (At this temperature, surface proteins can constitutively internalise efficiently). After appropriate time intervals, cells were placed in an isotonic buffer that contains large quantities of the reducing agent cysteine. This cysteine effectively cleaves the biotin moieties from the labelled proteins by reducing the connecting disulphide bond. During

this stage, the reaction tubes were kept in ice cold water to efficiently inhibit further receptor internalisation. However, those proteins that had already been internalised after biotinylation may be protected from reduction and thus remain conjugated to biotin. After washing and cell lysis, mIgM (or control protein) was immunoprecipitated. The immunoprecipitates were resolved by non-reducing SDS/PAGE and then electroblotted onto nitrocellulose membrane. Biotinylated mIgM was detected by probing with HRP-conjugated streptavidin, which binds with a high affinity and specificity to biotin, and use of an Enhanced Chemiluminescence (ECL) kit which generates light in response to peroxidase that can be visualised on film. This method is adapted from Bretscher and Lutter (1988) and Bretscher (1989 and 1992). Thus, the labelled IgM pool size was compared after incubation in DMEM medium in the presence or absence of PMA for 15 and 20 minutes. As a control, transferrin receptor was immunoprecipitated after similar incubation conditions (Figure 6.4A, page 141). As the results show, PMA significantly increased the amount of labelled IgM with the differences evident after 15 and 20 minutes treatment with phorbol ester. Later in this chapter it will be shown that a PMA-mediated difference can be seen with as little as 3 minutes incubation with PMA. These data argue that PMA stimulates the net movement of mIgM from the cell surface into an intracellular pool, possibly by promoting internalisation and inhibiting receptor recycling.

If PMA increases the internal pool size of the antigen receptor, then agents that prevent internalisation should also inhibit the effect of phorbol ester on the intracellular pool size. To assess whether this is the case, I performed an internalisation assay on WEHI-231 cells in the presence of hypertonic medium (DMEM containing 400 mM sucrose) which inhibits the formation of clathrin-coated pits that may mediate antigen receptor internalisation and can reduce constitutive endocytosis by more than 90% (Daukas and Zigmond, 1985; Davidson et al., 1990). Incubation in hypertonic medium did significantly reduce the constitutive and PMA-stimulated labelled IgM pool size (Figure 6.5A, page 142). This effect was reversible because transferring cells that had

been incubated in hypertonic medium into isotonic medium restored both constitutive and PMA-stimulated pool sizes to close to the levels seen after incubation in only isotonic medium. These data suggest the pool size changes attributable to constitutive endocytosis and PMA require clathrin-coated pit internalisation.

The buffer used to lyse cells in the internalisation experiment contained the detergent Triton X100, which strips away the α/β sheath from the immunoglobulin components (Hombach et al., 1988). A similar experiment was performed but instead of Triton, digitonin was used as the detergent for lysis. Once again, PMA incubation increased the total labelled IgM (Figure 6.4B, page 141). Because the PMA-induced change could still be detected under lysis conditions which preserve association with the α/β sheath, it would seem unlikely that PMA mediates this effect by causing the antigen receptor to become immobilised via the sheath on the cytoskeleton resulting in detergent insolubility; a phenomenon that has been reported to occur after the antigen receptor is crosslinked (Braun et al., 1982; Albrecht and Noelle, 1988).

To assess to what extent the ability of PMA to reduce fluorescence correlates with its ability to increase the detectable labelled pool of IgM, I made quantitative estimates of the pool size changes. The results (Figure 6.5B and 6.5C, page 142) suggest that PMA increases the pool size by about 2.5-5 fold from around 5% to 10-15% of the total surface labelled mIgM. This is significantly less than the 30% decreases in fluorescence consistently seen with this cell line. This difference could arise from PMA mediating changes in background staining and because fluorescence is measured after PMA treatment whereas biotin labelling is performed before PMA treatment; thus changes in fluorescence may result from the cumulative effect of more processes. For example PMA may mediate effects other than the net movement of surface IgM into an intracellular pool such as inhibiting the return of internal IgM (that would be internal during biotin labelling) to the cell surface. Indeed, it has been

previously reported that phorbol ester can affect both the internalisation and externalisation of receptors (Cardone et al., 1994 and references therein).

PMA-Mediated Effects on mIgM are not Evident in all B Cells

How general is the PMA-mediated downregulation of the antigen receptor? I analysed other cells to determine if they also show a similar effect on mIgM expression after incubation with PMA. FACS analyses were performed after incubation in medium at 37°C with or without PMA on A20 cells, freshly isolated splenic mouse cells and mouse splenic cells that had been treated with lipopolysaccharide for 48 hours. There was little evidence for significant decreases (of the magnitude seen in WEHI-231 or J558 cells) in IgM fluorescence in these cases (measured by percentage or in fluorescence units) (Figure 6.6-6.8, pages 143-145). I also performed internalisation assays on A20, Daudi and Raji B-cell lines in the presence or absence of PMA. No PMA-mediated effect is obvious in the A20 or Raji lines although there is evidence of an increase in the pool size of Daudi after treatment with phorbol ester for 1 minute but not at a later time point (Figure 6.9, page 146). Thus it can be concluded that the PMA-mediated effects seen with J558L and WEHI-231 are not evident in all B cells.

Effect of PMA on CD22 in J558L

One way in which PMA may exert its effect is by stimulating the general non-specific internalisation of proteins. To address this issue, the response of two proteins expressed in several J558L cell lines to PMA was examined. Firstly, the effect of PMA on CD22 expressed on J558L was assessed. Surprisingly, PMA treatment induced a significant, rapid increase of around 30% in FACS determined fluorescence after staining with the anti-CD22 mAb (TIB163) and FITC-conjugated goat anti-mouse IgG1 (see Figure 6.10, page 147). Similar changes of between 29-36% were evident in three different CD22 expressing J558L clones (one of which co-expressed mIgM) compared

to changes in fluorescence of between 6-10% seen after staining with the secondary antibody alone. I also tested the effect of PMA on a J558L transfectant expressing a mutant CD22 with the normal cytoplasmic tail putatively replaced with a portion of the α sheath protein tail (J558L/MutCD22) speculating that this might also help determine the region responsible for the increase in anti-CD22 fluorescence seen after phorbol ester treatment in the experiments above. As shown in Figure 6.11, pages 148, after PMA treatment, J558L/MutCD22, in contrast to the behaviour of a normal CD22 expressing cell line, decreased in fluorescence by between 23-26%. When this experiment was repeated decreases of 15-20% were recorded. Thus the CD22 tail apparently influences the response of CD22 to PMA in this cell line.

Because PMA treatment seems to mediate decreases or increases in the expression of IgM and CD22 antigens, these data cannot be explained simply by speculating that phorbol ester induces only a general downregulation of J558L surface proteins. Indeed, because not all antigens downregulate like mIgM, these data provide an indication that phorbol ester is an activator of a specific pathway that induces downregulation of the antigen receptor. This is supported by the finding that the pool of the transferrin receptor did not change as significantly as the pool of IgM in response to PMA in Figure 6.4, page 141.

PMA Alters the Phosphorylation of a Disulphide-linked Heterodimer

As noted previously, phorbol esters induce the downregulation of the T-cell receptor. Evidence that this process involves the ability of PMA to stimulate PKC comes from findings which show mutation of a serine residue in the γ chain, that is phosphorylated upon PMA treatment, abrogates downregulation (Dietrich et al., 1994). Like the γ chain of the TCR, both α and β contain serine residues that can be phosphorylated (van Nossal et al., 1991; Clark et al., 1992); thus PMA may mediate its effect on the B-cell antigen receptor in an analogous manner by activating PKC and

inducing phosphorylation of the α/β sheath. To assess if this could be the case, I examined whether the α/β sheath is phosphorylated under conditions that can presumably increase the pool size as follows; WEHI-231 cells were metabolically labelled with [^{32}P] orthophosphate, and then incubated in DMEM medium at 37°C with or without 40 μM PMA for 4 minutes. After cell lysis, mIgM was immunoprecipitated and the precipitates analysed by 2D SDS/PAGE. The results in Figure 6.12, page 149, suggest that PMA treatment increased the phosphorylation of a di-sulphide linked heterodimer. A shorter time exposure which compares the phosphorylation of the lower band (Figure 6.12B, page 149) implies that the upper band of the heterodimer seen in (Figure 6.12A) is phosphorylated ^{to a greater extent} than the lower in response to PMA. The identification of this heterodimer as the α/β sheath is supported by several criteria, namely it is of the appropriate size and molecular weight for the sheath in WEHI-231 (Kim et al., 1993b) and is immunoprecipitated using anti- μ antisera but not with control rabbit serum. Furthermore in a subsequent experiment, incubation of [^{32}P] orthophosphate labelled WEHI-231 cells with anti- μ antisera, immunoprecipitation with an anti- β monoclonal antibody and analysis by 2D SDS/PAGE demonstrated that a similar heterodimer was phosphorylated after crosslinking the antigen receptor (Figure 6.12C, page 149).

Conclusions

This chapter demonstrates that the phorbol ester, PMA mediates the downregulation of the mIgM B-cell antigen receptor in WEHI-231 and J558L/IgM. It also appears to stimulate a net movement of surface antigen receptors into the internal pool of WEHI-231. However, these PMA mediated effects are not seen in all cells. The response of mIgM to PMA may be mediated by the α/β sheath and, consistent with this hypothesis, PMA alters the phosphorylation of a disulphide-linked heterodimer. These data implicate PKC as a regulator of mIgM surface expression and internalisation.

Figure 6.1

Evidence that mIgM and CD22 are Surface Expressed Independently on the Cell Surface in J558L

FACS profiles and mean fluorescences of 10 FITC-conjugated anti- μ antisera or anti-CD22 mAb + FITC-conjugated anti-IgG1 antisera stained, randomly selected J558L/CD22 clonal populations that had been transfected with an expression vector allowing for the reconstitution of mIgM expression. Below is a table of the mean fluorescences together with the percentage of the population used to determine these statistics (IgM or CD22). The FACS profiles are depicted opposite. The populations to the right of the horizontal marker were presumed to be positive for the purpose of deriving the statistics. Vertical axes reflect cell number, horizontal axes are log (fluorescence).

	<u>IgM</u>		<u>CD22</u>	
Clone	Mean	%	Mean	%
1	48	17	30	68
2	43	4	25	84
3	73	74	23	84
4	44	7	21	80
5	48	31	22	54
6	43	5	28	89
7	52	79	13	32
8	54	16	22	71
9	64	72	30	90
10	60	70	25	86

1

2

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10

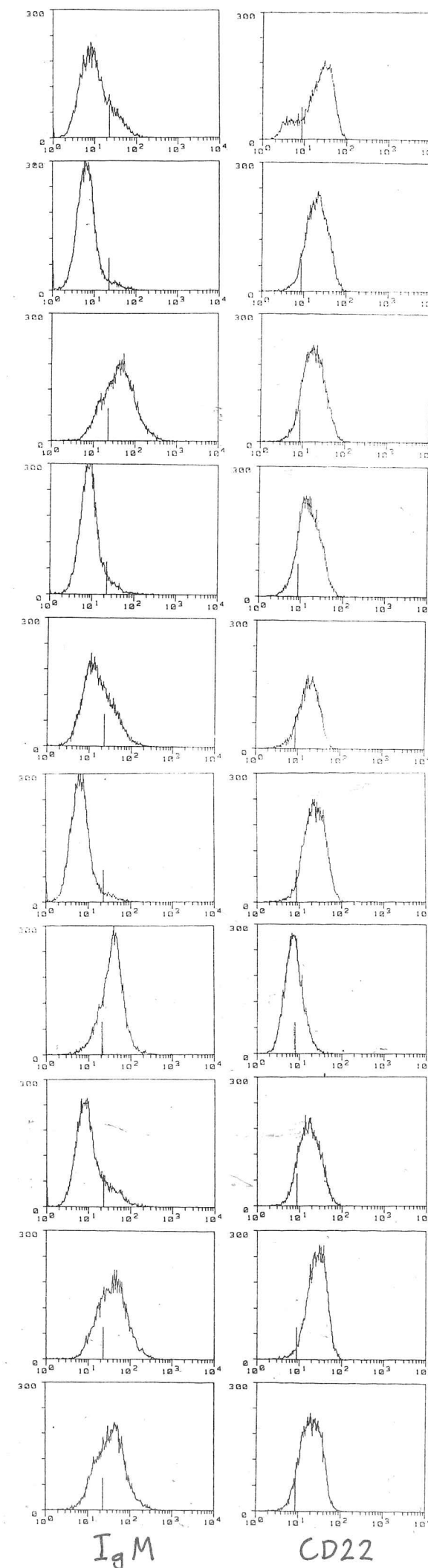


Figure 6.2

Modulation of IgM Fluorescence by Phorbol Ester in J558L

FACS profiles and mean and median fluoresences of FITC-conjugated anti- μ antisera stained J558L transfectants expressing IgM that had been incubated in parallel, with medium alone or medium containing 10 μ M (i and ii) or 40 μ M PMA (iii and iv) for 15 (i and iii) or 30 minutes (ii and iv) (as indicated). The statistical results are presented in the table below and a representative FACS profile (of i) opposite. Vertical axes reflect cell number, horizontal axes are log (fluorescence). Control cells were incubated with PMA but left unstained.

- A Treated with medium + PMA Unlabelled
 B Treated with medium FITC-conjugated anti- μ stained
 C Treated with medium + PMA FITC-conjugated anti- μ stained

J558L/IgM

		Control	-PMA	+PMA	% Change
i	Mean	6.0	69.8	49.4	-29.2
	Median	5.6	57.8	40.7	-29.6
ii	Mean	4.5	60.8	41.1	-32.4
	Median	4.18	45.7	30.8	-32.6
		Control	-PMA	+PMA	% Change
iii	Mean	4.4	86.6	64.2	-25.9
	Median	4.2	69.8	49.4	-29.2
iv	Mean	4.4	96.6	68.0	-29.6
	Median	4.2	81.3	55.7	-31.5

J558/IgM

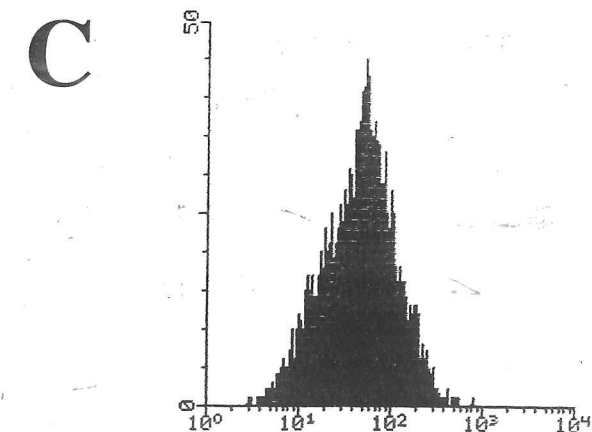
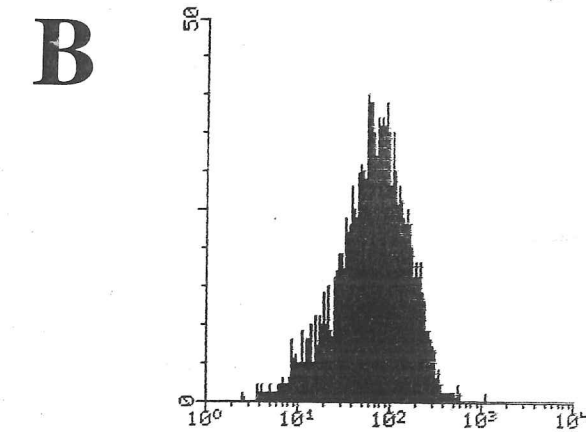
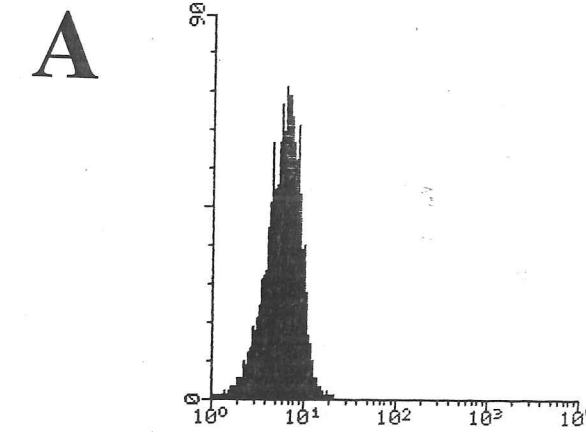


Figure 6.3

Modulation of IgM Expression by Phorbol Ester in WEHI-231

FACS profiles and median and mean fluorescences of FITC-conjugated anti- μ antisera stained WEHI-231 cells that had been incubated, in parallel, with medium alone or medium containing 10 μ M PMA for the indicated times (A-D) or in independent experiments with or without 40 μ M PMA for 30 minutes (E-G). (G) is the result obtained using a WEHI-231 clone from a different laboratory. The FACS profiles of (A-D) are opposite. Vertical axes reflect cell number, horizontal axes are log (fluorescence). The gates M1 and M2 reflect populations considered to be staining negatively or positively for IgM fluorescence respectively for the purposes of generating the statistics below.

WEHI-231/IgM

	A	B	C	D
	Unlabelled	-PMA	5 Minutes	20 Minutes
Mean		1297	924	875
Median		1198	865	820
%Change			-28.8	-32.5
			-27.8	-31.6
E		-PMA	+PMA	% Change
Mean		1150.3	760.9	-33.9
Median		1094.1	697.8	-36.2
F				
Mean		820.7	538.9	-34.3
Median		756.7	487.0	-35.6
G				
Mean		608.7	415.3	-31.8
Median		567.4	375.2	-33.9

WEHI-231

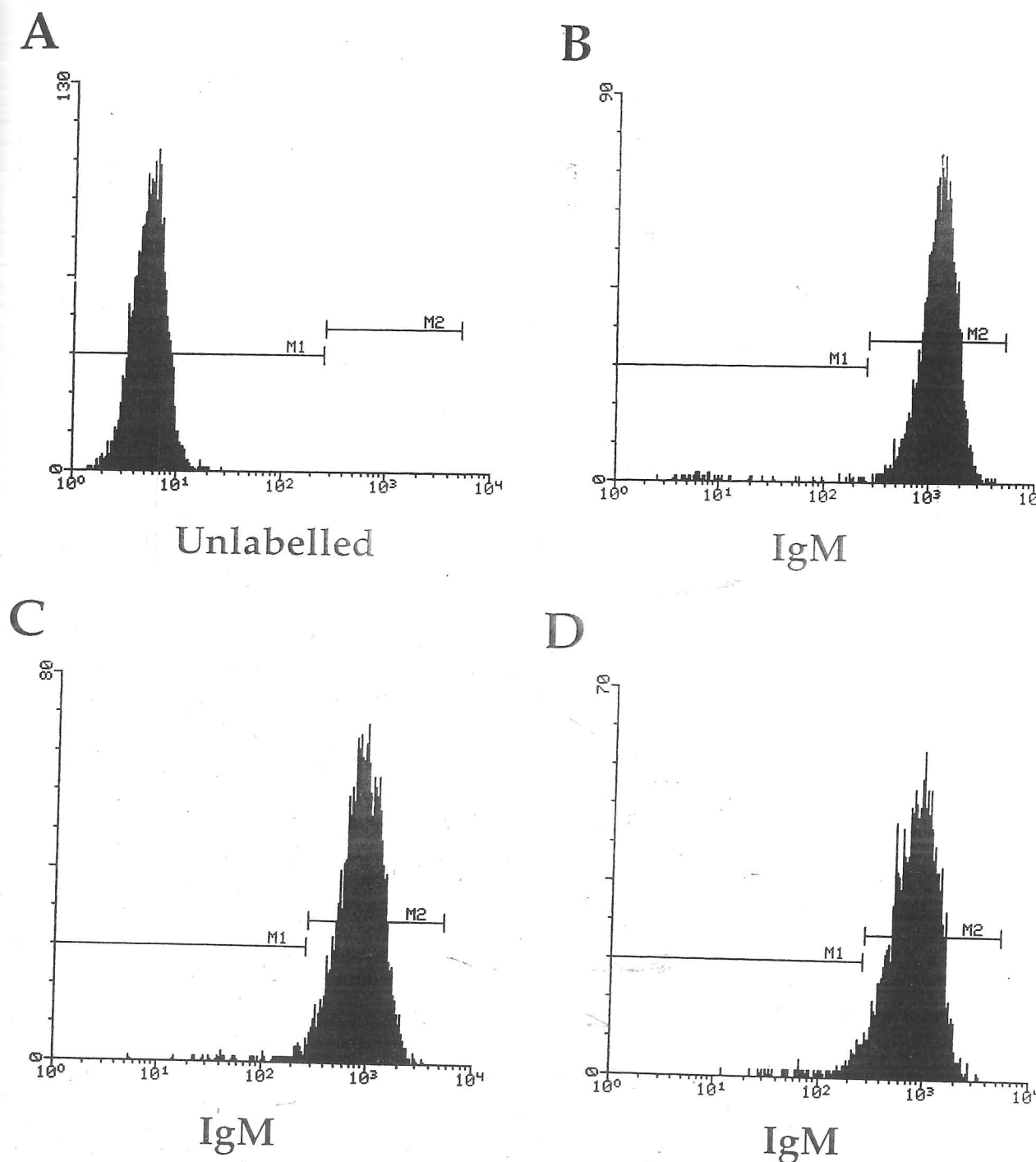


Figure 6.4

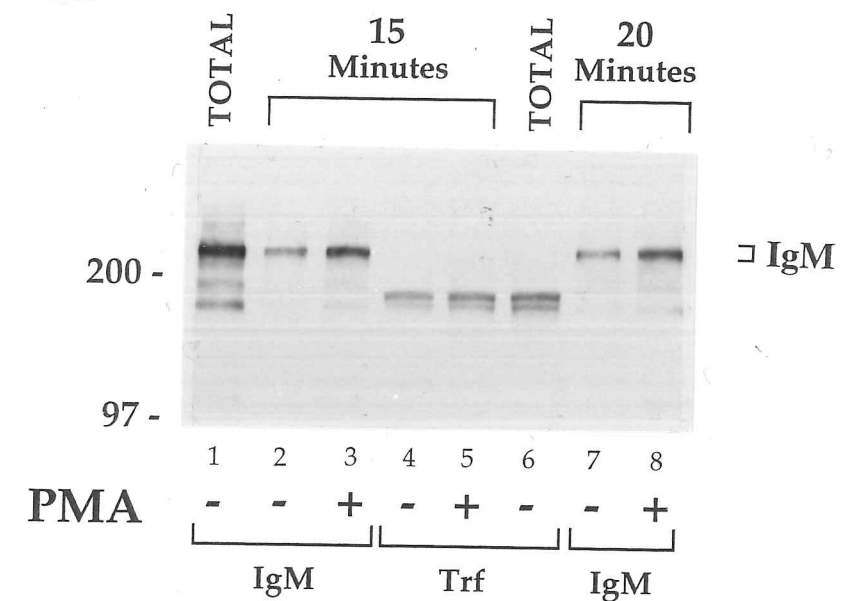
PMA Increases the Size of an Intracellular Pool of mIgM

I

(A) Streptavidin-biotin Western blot of precipitates prepared using 10 μ g of rabbit anti- μ antisera or the anti-transferrin receptor mAb R17 (as indicated), from 1% Triton X100 lysates of biotin labelled WEHI-231 cells that were either incubated on ice (lanes 1 and 6) or in medium with (+) (lanes 3, 5 and 8) or without (-) (lanes 2, 4 and 7) 40 μ M PMA for 15 or 20 minutes (as indicated). Samples were then either kept on ice (**TOTAL**) or appropriately incubated in stripping buffer prior to lysis, precipitation, analysis by non-reducing SDS/PAGE, electroblotting onto nitrocellulose and probing for biotin moieties. The presumed position of IgM is indicated by a bracket.

(B) Streptavidin-biotin Western blot of precipitates prepared using 10 μ g of rabbit anti- μ antisera or control rabbit serum (as indicated), from 1% digitonin lysates of biotin labelled WEHI-231 cells that were either incubated on ice (lanes 1 and 2) or in medium with (+) (lanes 4 and 8) or without (-) (lanes 1-3 and 5-7) 40 μ M PMA for 5 minutes (as indicated). Samples were then either kept on ice (**TOTAL**) or appropriately incubated in stripping buffer prior to lysis, precipitation, analysis by non-reducing SDS/PAGE, electroblotting onto nitrocellulose and probing for biotin moieties. The presumed position of IgM is indicated by a bracket. The diffuse bands migrating around 70 kD may be the sheath proteins, the band migrating at approximately 90 kD may correspond to partially reduced HL dimers.

A



B

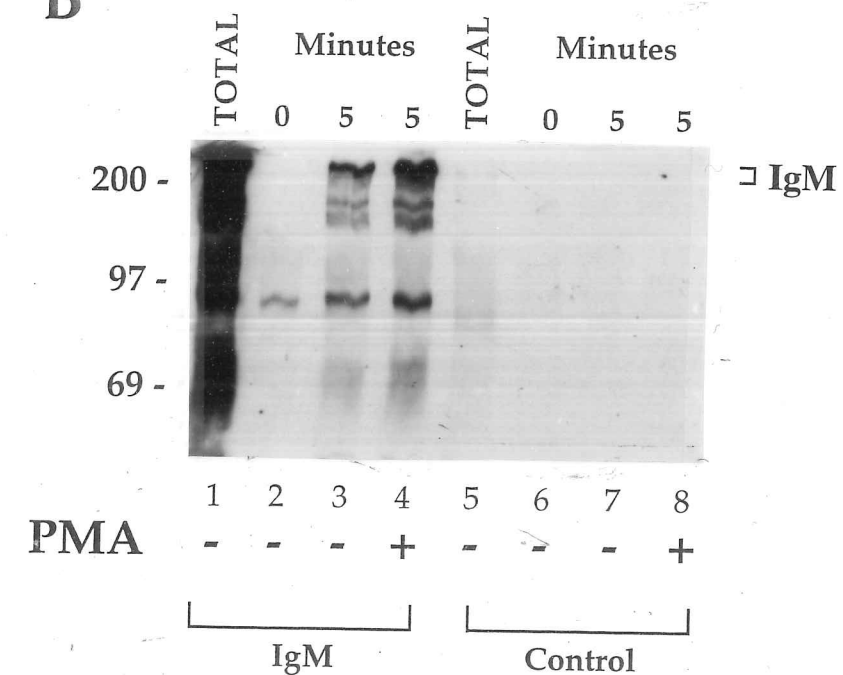


Figure 6.5

PMA Increases the Size of an Intracellular Pool of mIgM II

(A) Streptavidin-biotin Western blot of precipitates prepared using 10 µg of rabbit anti-µ antisera from 1% Triton X100 lysates of biotin labelled WEHI-231 cells that were either incubated on ice (lanes 1 and 2) or in medium with (+) (lanes 3, 5 and 7) or without (-) (lanes 4, 6, and 8) 40 µM PMA (as indicated). Cells were either incubated in normal medium (ISO) for 10 minutes, hypertonic medium containing 400 mM sucrose (HYP) for 10 minutes or normal medium for 10 minutes followed by hypertonic medium for 10 minutes each (ISO+HYP). Samples were then either kept on ice (TOTAL) or appropriately incubated in stripping buffer. Samples were then analysed by non-reducing SDS/PAGE, electroblotted onto nitrocellulose and blots probed for biotin moieties. The presumed position of IgM is indicated by a bracket.

(B) Quantification of the effect of PMA on the pool size of biotin labelled IgM in WEHI-231 cells. Streptavidin-biotin Western blot of precipitates prepared using 10 µg of rabbit anti-µ antisera from 1% Triton X100 lysates of biotin labelled WEHI-231 cells that were

either kept on ice (TOTAL) or appropriately incubated in stripping buffer. Samples were then analysed as above. Lanes 5 and 6, each contain 2×10^6 cell equivalents, lanes 1-4 contain samples equivalent to 15%, 10%, 5% and 1% of this cell number. These data suggest that PMA increases the internal pool size from about 5% to between 10 and 15% of total labelled IgM.

(C) Quantification of the effect of PMA on the pool size of biotin labelled IgM in WEHI-231 cells. This streptavidin-biotin western blot was prepared as in (B) from biotin labelled WEHI-231 cells that were either incubated in medium with (+) or without (-) 40 µM PMA for 5 minutes. Samples were then incubated in stripping buffer. Samples were then analysed as in (A). The far right lane contains 2×10^6 cell equivalents, other lanes contain samples equivalent to 100%, 80%, 60%, 40% and 20% of this cell number. These data suggest that PMA increases the internal pool size by 2.5-5 fold and is consistent with the data in (B) above.

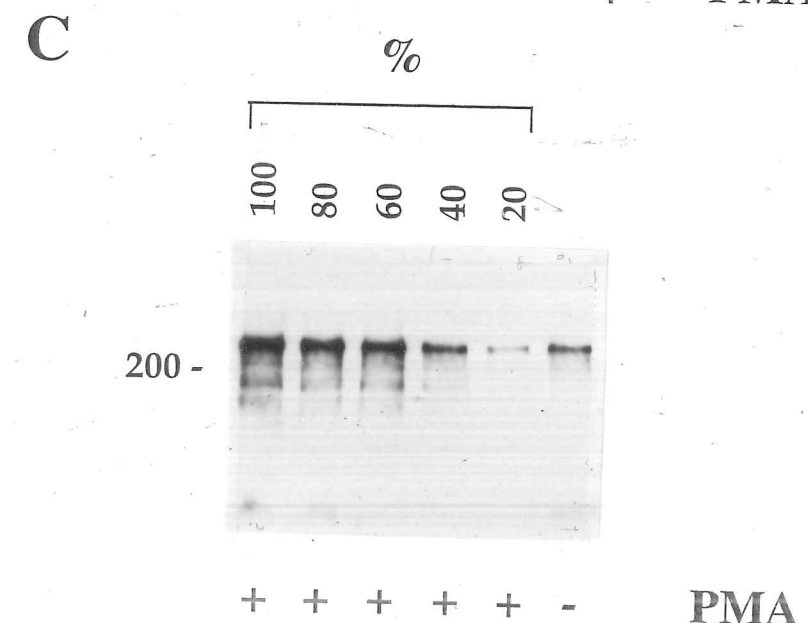
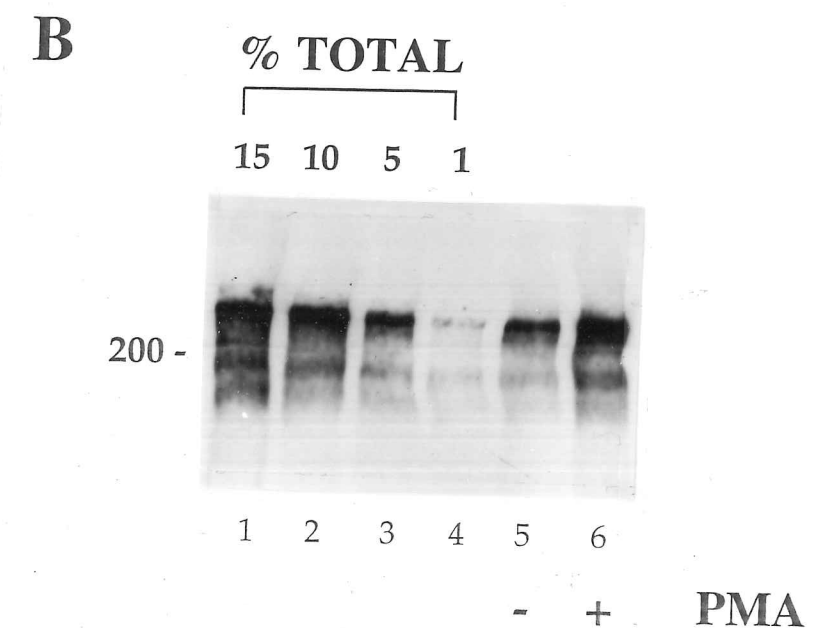
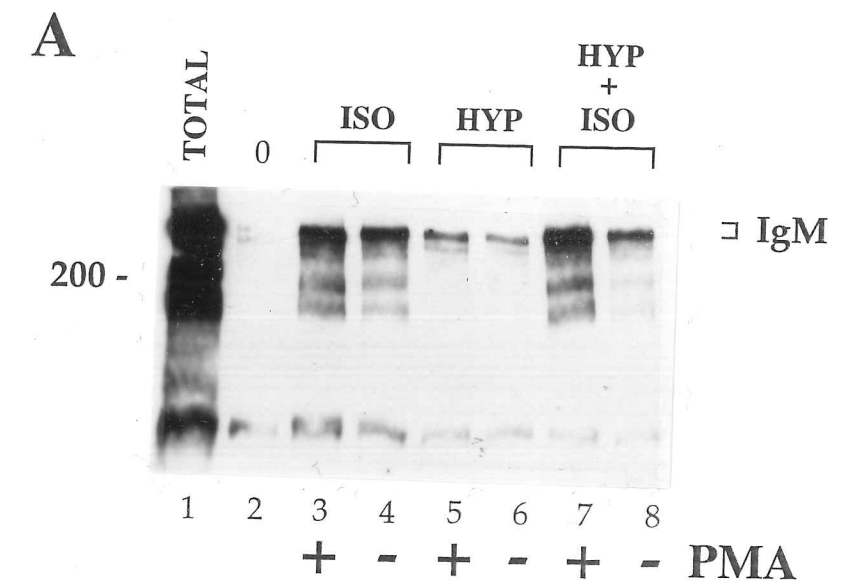


Figure 6.6

Effect of PMA on the IgM and IgG Fluorescence in A20

FACS profiles and mean and median fluorescences of a FITC-conjugated anti- μ antisera stained A20 cell transfectant expressing IgM or a J558L transfectant expressing IgM (J558/IgM) as a control that had all been incubated with medium alone or medium containing 40 μ M PMA for 20 minutes (as indicated). The statistics are presented below and FACS profiles opposite. Cells were analysed for IgG (A) or IgM (B and C) expression following staining with FITC-conjugated anti- γ antisera or FITC-conjugated anti- μ antisera respectively. Vertical axes reflect cell number, horizontal axes are log (fluorescence). The gates M1 and M2 reflect populations considered to be staining negatively or positively respectively for the purposes of generating the statistics below.

A. A20/IgG	-PMA	+PMA	% Change
Mean	100.4	101.6	+1.2
Median	93.1	93.1	0.0
B. A20/IgM			
Mean	221.1	208.4	-5.7
Median	126.4	117.6	-7.0
C. J558/IgM Control			
Mean	82.3	60.3	-26.7
Median	62.6	49.6	-20.8

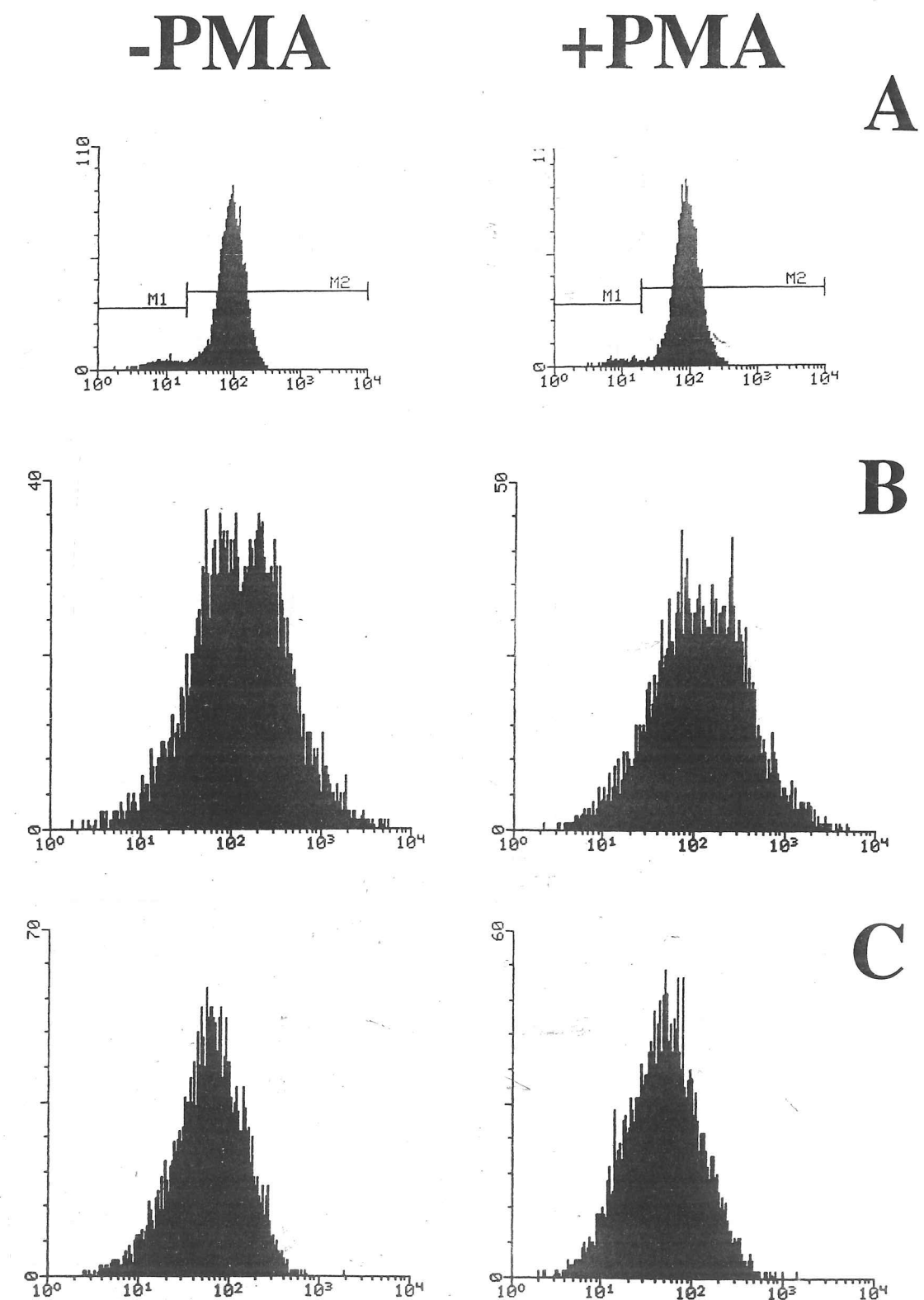


Figure 6.7

Effect of PMA on the IgM and CD22 Fluorescence in Murine Splenic Cells

Mean and median fluorescences of FITC-conjugated antisera stained freshly isolated mouse splenic cells or, as a control, a J558/IgM transfectant that had all been incubated with medium alone or medium containing 40 μ M PMA for 20 minutes and then analysed by FACS. The statistics are presented below. Cells were analysed for IgM (A and C) or CD22 (B) expression following staining with FITC-conjugated anti- μ antisera or biotinylated TIB163 anti-CD22 mAb and FITC-conjugated streptavidin respectively.

Mouse Splenic Cells

A. IgM	-PMA	+PMA	% Change
<i>Mean</i>	1299.7	1336.7	+2.8
<i>Median</i>	956.0	1027.4	+7.1
B. CD22			
<i>Mean</i>	70.9	80.5	+12.0
<i>Median</i>	60.0	68.5	+12.4
C. J558L/IgM Control			
<i>Mean</i>	86.4	57.6	-33.3
<i>Median</i>	71.1	45.7	-35.7

Figure 6.8

Effect of PMA on the IgM and CD22 Fluorescence in LPS-treated Murine Splenic Cells

I

Mean and median fluorescences of FITC-conjugated antisera stained mouse splenic cells that had been incubated with LPS for 48 hrs or, as a control, a J558/IgM transfectant that had been incubated with medium alone or medium containing 40 μ M PMA for 20 minutes and then analysed by FACS. The statistics are presented below. Cells were analysed for IgM (A and B) or CD22 (C and D) expression following staining with FITC-conjugated anti- μ antisera or biotinylated TIB163 anti-CD22 mAb and FITC-conjugated streptavidin respectively. Where the staining was significantly biphasic, statistics were calculated for both peaks

LPS-Treated Mouse Splenic Cells

A. IgM/Area 1	-PMA	+PMA	% Change
<i>Mean</i>	1568.3	1510.8	-3.7
<i>Median</i>	1165.2	1144.4	-1.8
B. IgM/Area 2			
<i>Mean</i>	1632.6	1583.5	-3.0
<i>Median</i>	1229.8	1207.9	-1.8
C. CD22/Area 1			
<i>Mean</i>	18.2	16.7	-8.2
<i>Median</i>	21.0	20.4	-2.9
D. CD22/Area 2			
<i>Mean</i>	116.5	111.9	-3.9
<i>Median</i>	99.1	90.6	-8.6
E. J558L/IgM Control			
<i>Mean</i>	86.4	57.6	-33.3
<i>Median</i>	71.1	45.7	-35.7

Figure 6.9

Effect of PMA on the Labelled Pool Size of Membrane Immunoglobulin Molecules in A20, Raji and Daudi

Streptavidin-biotin Western blots of precipitates prepared using 10 μ g of rabbit anti-mouse μ antisera for murine IgM in A20 and WEHI-231, protein G-Sepharose alone for mIgG from A20 and 10 μ g of rabbit anti-human μ antisera for human IgM from Raji and Daudi, from lysates containing 1% Triton X100 of cells that had been either incubated on ice (**TOTAL** and **0**) or in medium with (+) or without (-) 40 μ M ng/ml PMA for 1 or 3 minutes (as indicated). Samples were then either kept on ice (**TOTAL**) or appropriately incubated in stripping buffer prior to lysis. Samples were then analysed by non-reducing SDS/PAGE, electroblotted onto nitrocellulose and blots then probed for biotin moieties. Panel identities are as follows;

(A) A20/IgG

(B) A20/IgM

(C) A20/IgM-Mut β

(D) WEHI-231/IgM

(E) Daudi/IgM

(F) Raji/IgM

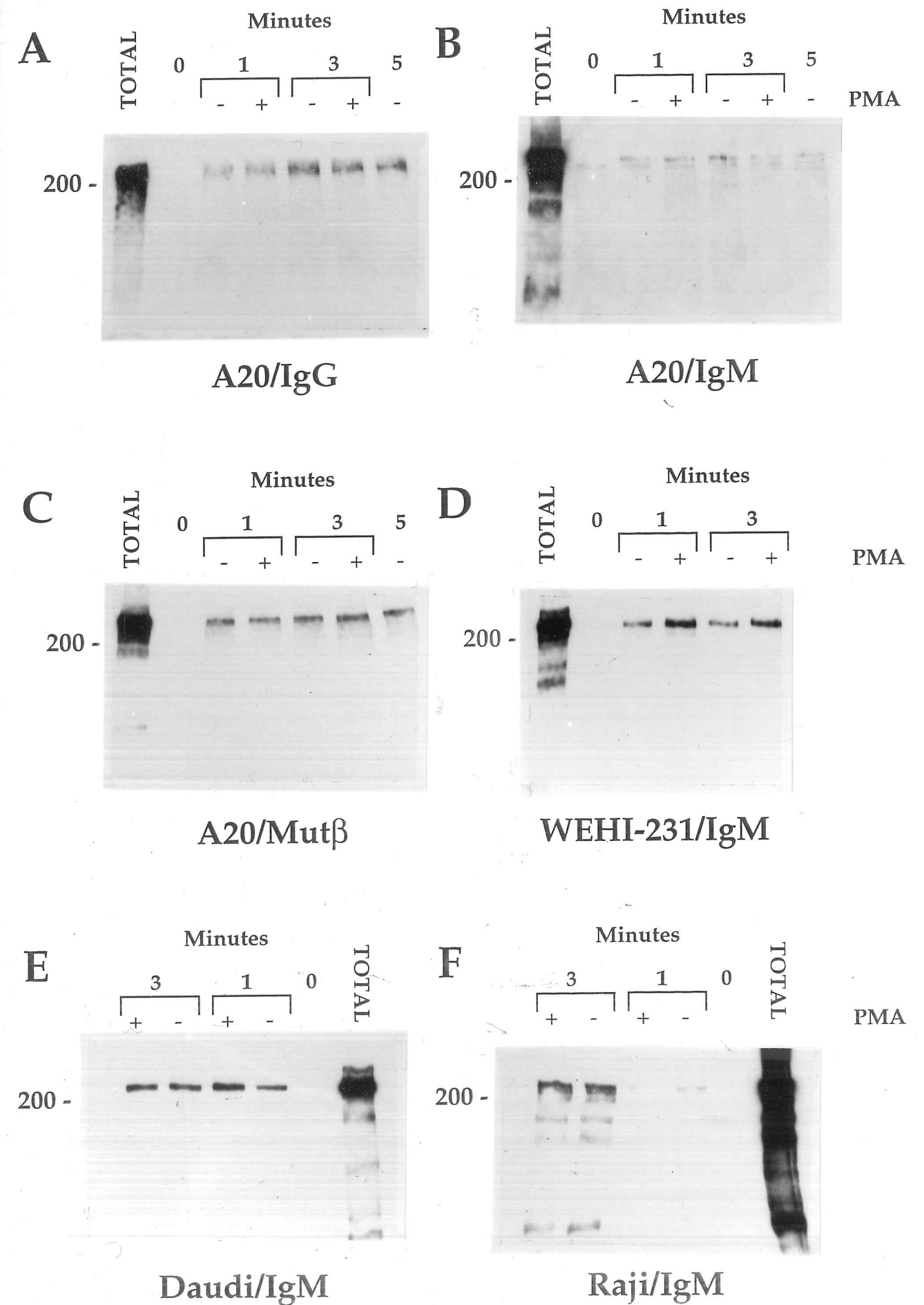


Figure 6.10

Modulation of CD22 Fluorescence by Phorbol Ester in J558L

FACS profiles and mean and median fluorescences of CD22 or IgM stained J558L transfectants either expressing CD22 and IgM (J558L/IgM) or CD22 (J558L/CD22) that had been incubated, in parallel, with medium alone or medium containing 40 μ M PMA for 20 minutes as indicated. Control staining using only the secondary FITC-conjugated anti-mouse IgG1 was also performed. A table of results is featured below, FACS profiles are depicted opposite. Vertical axes reflect cell number, horizontal axes are log (fluorescence). The gates M1 and M2 reflect populations considered to be staining negatively or positively for CD22 fluorescence respectively for the purposes of generating the statistics below.

J558L/IgM/CD22

A. IgM	-PMA	+PMA	% Change
Mean	83.1	59.4	-28.5
Median	59.4	41.1	-30.8

B. CD22

Mean	417.6	607.0	+31.2
Median	388.4	586.5	+33.8

J558L/CD22

C. Clone 1

Mean	104.8	153.5	+31.7
Median	85.8	129.8	+33.9

D. Clone 2

Mean	146.9	208.2	+29.4
Median	126.6	195.9	+35.4

E. Clone 1/Secondary Antibody Only

Mean	36.2	38.7	+6.5
Median	30.2	33.4	+9.6

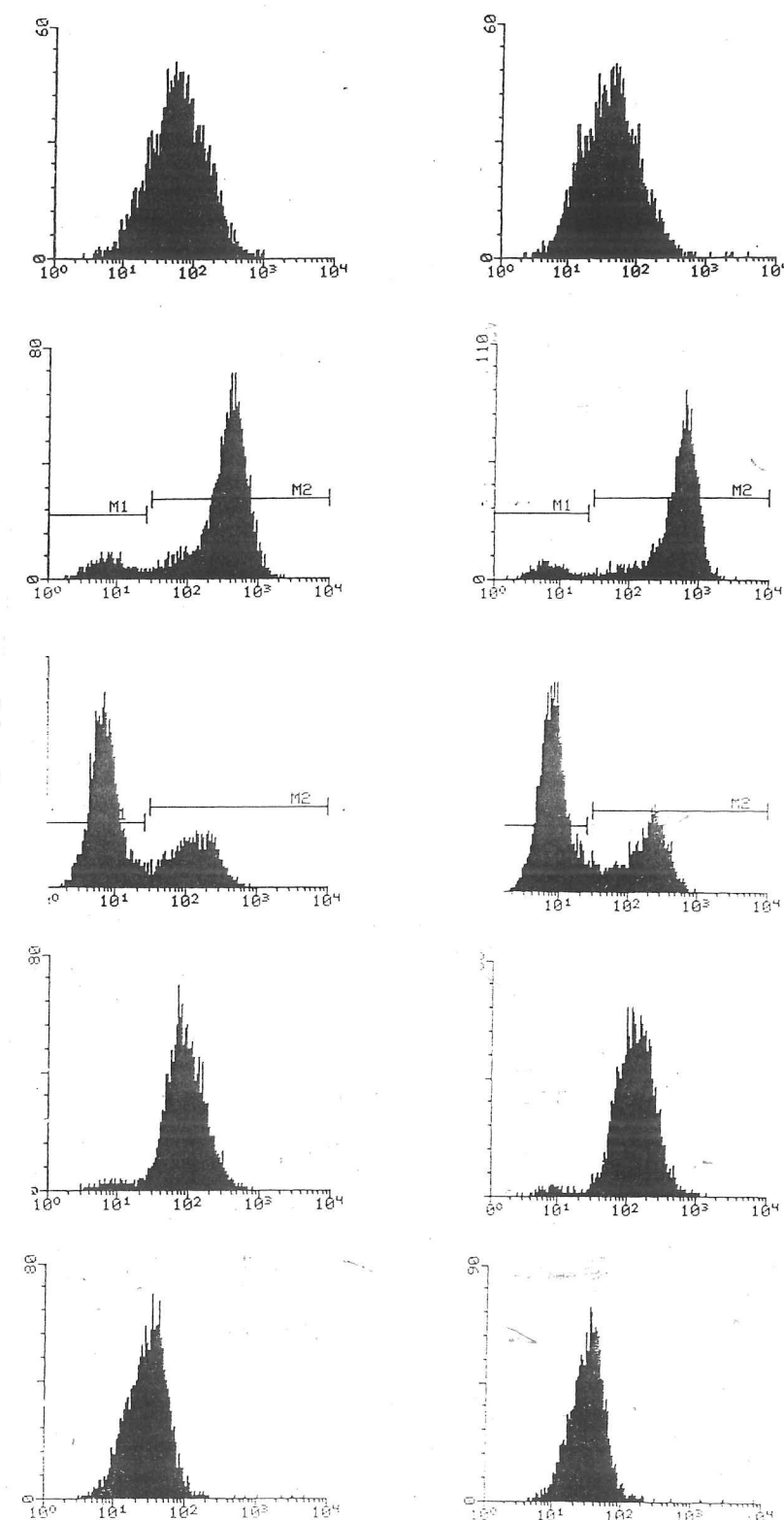


Figure 6.11

The Cytoplasmic Tail Influences the Effect of PMA on CD22 Antigen Fluorescence

FACS profiles and mean and median fluorescences of anti-CD22 stained J558L transfectants expressing CD22 (**J558L/CD22**) or MutCD22 (**J558L/MutCD22**) that had been incubated, in parallel, with medium alone or medium containing 40 μ M PMA for 20 minutes as indicated (A-C). Control staining using only the secondary FITC-conjugated anti-mouse IgG1 was also performed. The results table is below, FACS profiles are opposite. (D) gives the results of a subsequent experiment on J558L/MutCD22 performed under the same conditions. Vertical axes reflect cell number, horizontal axes are log (fluorescence). The gates M1 and M2 reflect populations considered to be staining negatively or positively for CD22 fluorescence respectively for the purposes of generating the statistics below.

J558L/CD22

A. CD22	-PMA	+PMA	% Change
Mean	474.5	619.4	+23.4
Median	437.1	582.9	+25.1

J558L/MutCD22

B. Secondary Antibody Only			
Mean	5.10	5.31	+4.0
Median	4.87	5.05	+3.6
C. MutCD22			
Mean	222.5	175.6	-21.1
Median	187.4	146.3	-21.9
D. MutCD22			
Mean	243.5	206.8	-15.1
Median	214.0	172.7	-19.6

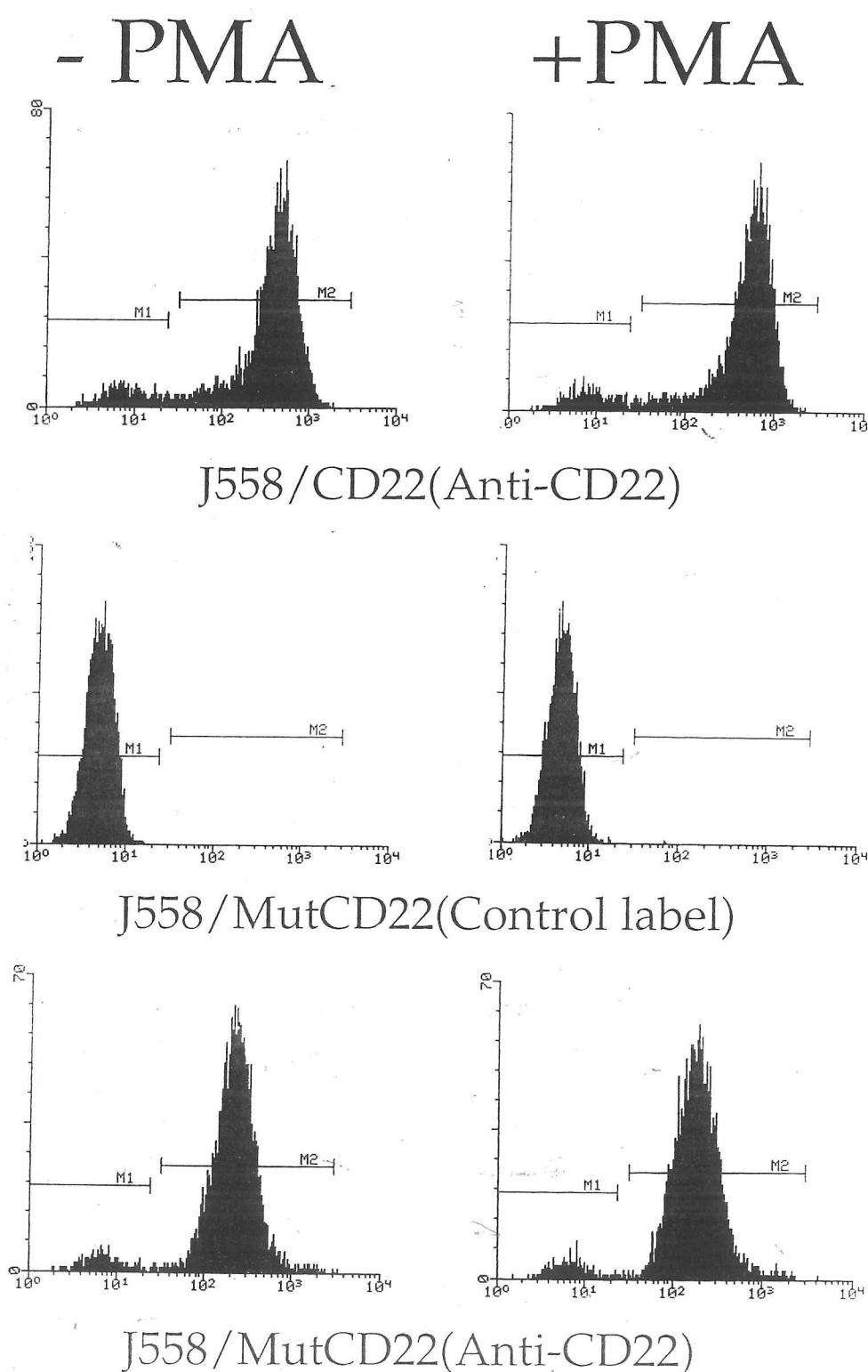


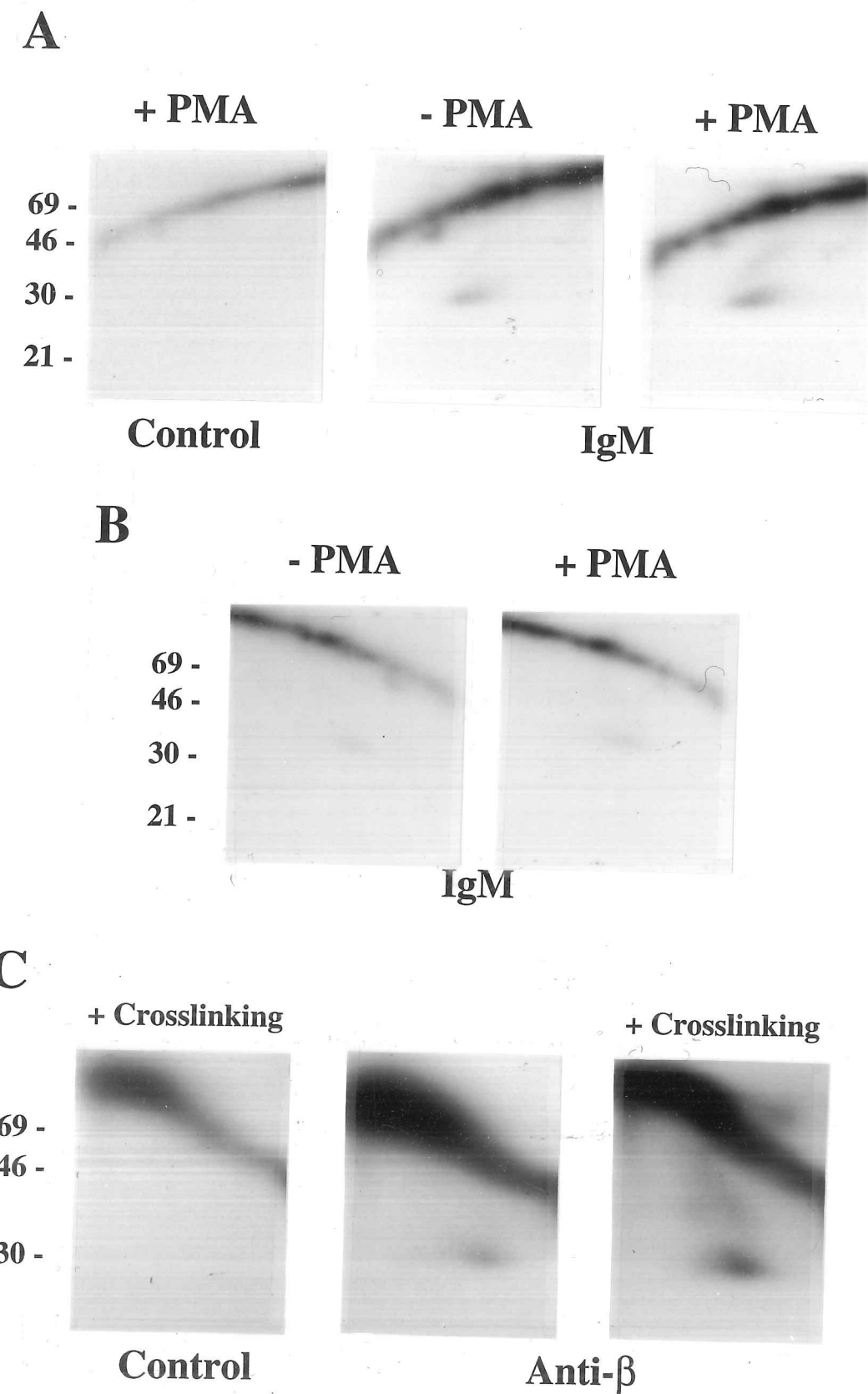
Figure 6.12

PMA Alters the Phosphorylation of a Heterodimer

(A) Analysis by 2D SDS/PAGE of IgM or control precipitates (as indicated) prepared using rabbit anti- μ antisera (10 μ g) or rabbit serum (10 μ g) respectively from digitonin lysates of WEHI-231 cells that had been metabolically labelled with [32 P] orthophosphate and incubated in DMEM medium at 37°C in the presence or absence of 40 μ M PMA (- PMA or +PMA).

(B) Shorter time of exposure than (A)

(C) Analysis by 2D SDS/PAGE of precipitates prepared using the anti- β mAb or a control IgG1 mAb (as indicated) from NP40 lysates of WEHI-231 cells that had been metabolically labelled with [32 P] orthophosphate and incubated in DMEM medium at 37°C in the presence or absence of 20 μ g (Fab')₂ goat anti- μ antisera for 5 minutes (- Crosslinking or +Crosslinking).



7 DISCUSSION

Chapters 3, 4 and 5 demonstrate that in human and mouse cells, the B-cell specific protein CD22 associates with the B-cell antigen receptor complex (this observation for human cells has been independently reported by LePrince et al., 1993) and is tyrosine phosphorylated after antigen receptor crosslinking (observations independently made for human CD22 by Schulte et al., 1992 and LePrince et al., 1993).

What is the Nature of the CD22/Ig Interaction?

Evidence that IgM and CD22 interact at a relatively low stoichiometry comes from the findings that only 0.2-2% of mIgM is detected interacting with CD22 and that the interaction is not evident in surface iodination, co-capping and co-modulation assays (this study; Pesando et al., 1989). Similar results would be generated if all cells displayed a small number of mIg/CD22 complexes or alternatively if the interaction was only present in a small number of cells, but at a high stoichiometry. An alternative hypothesis that could explain these findings is that the interaction, whilst being of a high stoichiometry, may be of a low affinity and be easily disrupted on investigation. This can be considered to be less likely because mIg/CD22 complexes could be consistently detected using techniques involving immunoprecipitation and extensive washing; results which argue that, at least *in vitro*, the association is stable rather than being of a low affinity.

Although the mIg/CD22 association could be driven by a number of mechanisms, I favour a model which supposes that a close interaction of CD22 with IgM is an event that occurs during transmembrane signalling and is evident because techniques involving immunoprecipitation stabilise a subset of mIg molecules (which at the time of lysis are constitutively complexed or colocalised with CD22) as mIg/CD22 complexes. This is my preferred hypothesis because such a model, if it was extended to account for other proteins, would explain why many molecules

involved in a receptor signalling cascade can be co-precipitated (often at a low stoichiometry) with that receptor in the absence of crosslinking. Examples include the ability to co-precipitate tyrosine kinases with the B-cell antigen receptor and the observation that the TCR co-precipitates with CD2, CD5, CD4 and CD8 (Hutchcroft et al., 1992; Yamanashi et al., 1992; Yamada et al., 1993; Campbell and Sefton 1992; Burkhardt et al., 1991; Lin and Justement, 1992; Law et al., 1993; Gold et al., 1994; Beyers et al., 1992b; Brown et al., 1989; Burgess et al., 1992). This model suggests that the molecules which induce CD22 tyrosine phosphorylation are also mediators of the mIg/CD22 association. It is therefore tempting to speculate that the α/β sheath allows CD22 to interact with mIg (although not necessarily by binding directly to CD22) because of its apparent importance for CD22 phosphorylation; a suggestion supported by the finding that multiple isotypes co-precipitate with CD22. As for cells in which the mIg/CD22 interaction is not evident; this may be a consequence of the signalling molecules not possessing the appropriate constitutive activities to generate a sufficiently large enough number of mIg/CD22 complexes to be detected. These ideas do not preclude that CD22 interacts with Ig by other mechanisms such as via extracellular or transmembrane contact. Indeed, the findings which show CD22 can bind to carbohydrate residues on various proteins suggest that carbohydrates present in the antigen receptor complex could serve to recruit CD22 (Stamenkovic et al., 1992; Sgroi et al., 1993; Powel et al., 1993).

What is the Contribution of CD22 to B-cell Function *in vivo*?

The mIg/CD22 association suggests that CD22 acts as a co-receptor for the B-cell antigen receptor complex. When operating in this capacity, CD22 may function by recruiting SH2-domain containing proteins. This conclusion is supported by the findings that CD22 is tyrosine phosphorylated after crosslinking and contains a sequence with similarity to the Reth motif. Further evidence in favour of SH2-domain

recruitment by CD22 derives from the results of Songyang *et al* who have determined phosphotyrosine containing peptide sequences that bind with high affinity to SH2-domains. Extrapolation from this study suggests that CD22 has the capacity, in theory to associate via phosphotyrosines in its cytoplasmic tail with the product of the oncogene *fps/fms* and a protein of unknown function called 3BP2 (Songyang *et al.*, 1994). These two proteins are not, however, of the appropriate molecular weight to be the 65-70 kD protein which was detected in CD22 precipitates in chapter 5 (Ren *et al.*, 1993; Feldman *et al.*, 1987).

Other clues to the function of CD22 come from a variety of sources. Analyses *in vitro* on CD22 have suggested that it is an adhesion molecule that can mediate homotypic B cell - B cell interactions and heterotypic adhesion to other cell types by binding to a variety of carbohydrate-containing proteins (Stamenkovic and Seed, 1990; Wilson *et al.*, 1991; Torres *et al.*, 1993). Additionally, CD22 is most highly expressed in germinal centres (Dorken *et al.*, 1988; Wilson *et al.*, 1991) which are also major sites of expression of a ligand for CD22, the carbohydrate antigen CD75 (Hardie *et al.*, 1993; Knapp *et al.*, 1989). As regards its importance to the B cell, anti-CD22 antisera is co-stimulatory with anti-Ig in mediating proliferation (Pezzutto *et al.*, 1987). Thus taking these findings, together with the observations in this thesis, an attractive hypothesis is that if a cell in the germinal centre expresses a CD22 ligand and also harbours antigen on its surface, this could lead to the co-crosslinking of CD22 and the antigen receptor and a modulation in B cell behaviour; a modulation involving the ability of the sheath to recruit SH2-domain containing proteins and induce the tyrosine phosphorylation of CD22.

The antigen receptor, as well as mediating transmembrane signalling can also bind and internalise antigen; thus potentiating the processing and presentation of antigen to T cells. CD22, by virtue of its close interaction with mIg, may be involved in this pathway. However, the mutant antigen receptors expressed in A20 which could

not mediate CD22 phosphorylation appeared perfectly competent to mediate antigen presentation in a previous study (Patel and Neuberger, 1993). These results imply that, at least in transformed cells, CD22 tyrosine phosphorylation is not essential for the efficient internalisation and processing of antigen.

The Future

It will be particularly interesting to determine the phenotype of CD22 knockout mice and cell lines and to identify molecules that interact with the CD22 tail. Additionally, since CD22 binds to CD75 and both antigens are highly expressed on B cells and in germinal centres it might be worthwhile investigating the effect of CD75 expression in B cell lines (Stamenkovic et al., 1991; Wilson et al., 1990; Knapp et al., 1989; Hardie et al., 1993; Knapp et al., 1989).

Downregulation of the B-Cell Antigen Receptor

Chapter 6 shows that treatment with phorbol ester produces decreases in anti-IgM fluorescence of J558/IgM and WEHI-231 cells and stimulates the movement of IgM from the cell surface into an intracellular pool of WEHI-231 cells.

What is the Effect of Phorbol Ester on mIgM?

I favour the idea that two processes (the PMA-mediated inhibition of receptor externalisation and promotion of internalisation) are predominantly responsible for the changes induced by PMA. This is because firstly, the internalisation assays suggest that PMA stimulates the movement of a significant proportion of IgM into the cell and secondly, because PMA has been shown to affect both the internalisation and externalisation of other receptors (including the polymeric IgA receptor and the transferrin receptor) (Cardone et al., 1994; Rothenburger et al., 1987; Zerial et al.,

1987; McGraw et al., 1988). Quantification of the changes suggest that PMA stimulates an approximately 30% decrease in surface expression and the movement of 10% of the surface antigen receptors into the intracellular pool. Accepting estimates of surface immunoglobulin density to be 2.5×10^5 molecules per cell (Cambier, 1992), these data suggest that an average of at least 2×10^4 molecules are driven inside WEHI-231 in response to PMA. Other factors likely to influence the fluorescence include changes in background staining, in the affinity of the antigen for the labelling antibody, aggregation of mIgM on the cell surface (thus inhibiting access by the labelling antibody) or the shedding of protein into the medium.

Under conditions that would be expected to induce a downregulation of the antigen receptor, PMA altered the phosphorylation of a disulphide-linked heterodimer that is probably the α/β sheath. The fact that this phosphorylation is induced by PMA suggests that the kinase responsible is protein kinase C (PKC). In favour of this hypothesis, α and β do contain potential PKC phosphorylation sites (Hermanson et al., 1988; Sakaguchi et al., 1988), the tails can be serine phosphorylated *in vitro* (Clark et al., 1992) and there is evidence that human β is serine phosphorylated following PMA treatment (van Noesal et al., 1992). However, in this latter study human Daudi cells were incubated for 1 hour with PMA.

Receptor Signalling and Endocytosis

Taking the findings of chapter 6 together with the knowledge that activation of PKC occurs on phorbol ester treatment and also after transmembrane signalling, (Parker et al., 1984; Castagna et al., 1982; reviewed by Nishizuka et al., 1992) it is tempting to speculate that receptor signalling drives the internalisation and intracellular retention of antigen receptors by inducing the activation of PKC and the phosphorylation of the sheath tails. How could this process be beneficial to a B cell? An internalisation mechanism regulated by signalling of this nature could be

important for the uptake of antigens by B cells when antigen is at a low concentration or of a low affinity. This is because an efficient way to internalise such antigen would be for it to be rapidly engulfed before it dissociates from the antigen receptor in response to signals it has induced by binding to the receptor rather than rely on uptake by constitutive endocytosis. Thus the signalling increases the efficiency of internalisation and retention of receptors inside the cell and therefore potentiates ligand breakdown and antigen presentation. This model could be examined by comparing the ability of antigen receptors that are able to signal efficiently with mutant receptors that can not, in respect of their ability to mediate the internalisation and presentation of low affinity antigen.

How Could Receptor Signalling Regulate Endocytosis?

The results of Pure and Tardelli, who showed that tyrosine kinase inhibitors inhibit antigen receptor endocytosis, suggest that tyrosine kinases regulate receptor internalisation (Pure and Tardelli, 1992). How these kinases could effect internalisation is unclear but the finding that PMA stimulates downregulation and pool size changes suggest that they could modulate internalisation by triggering a signalling cascade that results in the activation of PKC. A prediction of this model is that the inhibitory effect of tyrosine kinase inhibitors on receptor endocytosis in primary cells would be diminished by the treatment of cells with PMA. In addition to the antigen receptor and by implication, the α/β sheath stimulating internalisation by signalling and activating PKC, the sheath may also directly mediate the internalisation by binding to internalisation machinery. This issue could be addressed by analysing the ability of mutant antigen receptors expressed in J558L to downregulate in response to PMA and by examining whether the sheath is serine/threonine phosphorylated after crosslinking.

The Future

There are a number of additional experiments suggested from the results obtained during the study of B-cell antigen receptor downregulation. Several cell types did not respond to PMA like J558L and WEHI-231. It might be interesting attempt to understand why this difference arises. One possibility is that they may require signals in addition to those generated by PMA for internalisation. It could therefore be informative to determine whether calcium ionophores together with PMA can stimulate internalisation. Another line of investigation could involve examining the expression of different isoforms of PKC in B cells. Indeed, there is evidence that the isoforms change in expression during B cell differentiation (Michak et al., 1991). It may also be useful to investigate whether the activation of PKC increases, and conversely PKC inhibitors decrease the efficiency of antigen presentation. Furthermore, using the method of biotin labelling, it should be possible to specifically precipitate internalised receptors using streptavidin-agarose. This could facilitate the search for associated proteins that may mediate internalisation and antigen presentation. Of potential significance in this regard, is the finding that a 52 kD protein, which has been reported to interact with α , is phosphorylated after PMA treatment (Kuwahara et al., 1994).

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9 APPENDIX

TRANSFECTANTS

Cell Line	Reference/Source/Origin
A20/IgM	Patel and Neuberger, 1994
A20/IgM-Mut β	Patel and Neuberger, 1994
A20/IgM-Mut β Y ¹⁹⁵ =>L	Patel and Neuberger, 1994
A20/IgM-Mut β Y ¹⁹⁵ , ²⁰⁶ =>L	Patel and Neuberger, 1994
J558/IgM	Venkitaraman et al., 1991
J558/CD22	This study
J558/IgM/CD22	T. O'Keefe/M.S. Neuberger (unpublished)
J558/MutCD22	This study
NIH3T3/IgM	G.T. Williams/M.S. Neuberger (unpublished)
NIH3T3/CD22	This study
NIH3T3/CD22/IgM-Mut β /CD45	This study
Raji22	This study
RajiCD22	This study
RajiMutCD22	This study

PLASMIDS

CD22	T. O'Keefe/M.S. Neuberger (unpublished)
μ heavy chain+ α	Venkitaraman et al., 1991
CD45	G.T. Williams/M.S. Neuberger (unpublished)
MutCD22	G.T.Williams/T. O'Keefe/M.S. Neuberger (unpublished)
Lyn + Syk	G.T.Williams/M.S. Neuberger (unpublished)

MutCD22 was constructed by G.T.Williams and putatively expresses a mutant form of CD22 where all but the three amino acid residues of the cytoplasmic tail proximal to the predicted transmembrane were replaced with the predicted cytoplasmic tail of the murine α sheath protein.

PUBLICATIONS

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