INTERACTIONS BETWEEN THE EXTRACELLULAR AND TRANSMEMBRANE DOMAINS OF THE IG-α/β HETERODIMER ARE REQUIRED FOR BCR ASSEMBLY AND CELL SURFACE EXPRESSION

by

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Bachelor of Science, University of British Columbia, 2002

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA

November 2005

ABSTRACT

The B cell antigen receptor (BCR) is expressed on the surface of B-lymphocytes where it binds antigen and transmits signals that regulate B cell activation, growth and differentiation. The BCR is composed of membrane IgM (mIgM) and two signaling proteins, Ig-α and Ig-β. If either of the signaling proteins is not expressed, the incomplete mIgM-containing BCR will not traffic to the cell surface. Our hypothesis is that specific protein:protein interactions between both the extracellular and transmembrane (TM) regions of Ig-α and Ig-β are necessary for receptor assembly, cell surface expression and effective signaling to support the proper development of B cells. While previous work has shown the importance of the TM region in BCR assembly, this study indicates that a heterodimer of the extracellular domains of Ig-α and Ig-β are also required for proper association with mIgM. Cell lines expressing mutated Ig-α proteins that did not heterodimerize with Ig-β in the extracellular and TM domains were unable to properly assemble the BCR. Conversely, an Ig-α mutant with an Ig-β cytoplasmic tail (Cβ (α/α/β)) was able to assemble with the rest of the BCR and traffic to the cell surface. Thus, both the extracellular and TM regions if the Ig-α/Ig-β must be properly associated in order for the BCR to assemble. Additionally, an Ig-α mutant with a truncated cytoplasmic domain (ΔαKVK (α/α/0)) was not able to associate with Ig-β, indicating that the cytoplasmic domain may play a role in BCR assembly. Further studies with truncation mutants are required to confirm this result. In the future additional Ig-α/Ig-β mutants will be expressed to better define the regions of protein:protein interactions.

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LIST OF ABBREVIATIONS

APC- antigen presenting cell

BCA- bicinchoninic acid

BCLL- B cell chronic lymphocytic leukemia

BCR- B cell receptor

BLNK- B cell linker adapter protein

C region- constant region

DAG- D-acetyl-glycerol

DMEM- Dulbecco's modified Eagle medium

DNA- deoxyribonucleic acid

DTT- dithiothreitol

ECL- enhanced chemiluminescence

EDTA- ethylenediaminetetraacetic acid

ER- endoplasmic reticulum

FACS- fluorescent activated cell sorting

FCS- fetal calf serum

H chain- heavy chain

HRP- horseradish peroxidase

ITAM- Immunoreceptor tyrosine-based activation motif

L chain- light chain

LTR- long terminal repeat

MIIC- MHC class-II containing compartment

MHC- major histocompatibility complex

mIg- membrane immunoglobulin

PAGE- polyacrylamide gel electrophoresis

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PI3K- Phosphatidylinositol-3 phosphate kinase

PMSF-phenylmethylsulfonyl fluoride

RPMI- Roswell Park Memorial Institute

SDS- sodium dodecyl sulfate

SH2- Src homology 2 domain

TBE- tris-buffered EDTA

TBS- tris-buffered saline

TBST- TBS with 0.1% Tween 20

TLR- toll-like receptor

V region- variable region

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Linda Matsuuchi, for allowing me to work on this project and helping me through my Master's degree. You have been a wonderful teacher and very supportive of my decision to pursue a dental career. Thank you! I would also like to thank my lab mates. Firstly, thank you to May Dang-Lawson for showing me how to do almost all of my experiments, you are an incredibly patient person! Also, thank you to Teresa Corkin for putting a positive spin on negative experimental results, or for at least sharing a horror story of your own. Finally, thank you to Steve Machtaler for keeping me thoroughly entertained every day in the lab. Good luck with your PhD!

I would also like to thank my parents for their continual love and support. Completing this thesis has been a lot of work and I would not have been able to do it without your encouragement. Finally, I would like to thank Tim for putting up with all my late nights in the lab and for being so incredibly supportive of everything I do in life.

CHAPTER 1

Introduction

1.1 The innate and adaptive immune systems

Mammals are continually exposed to a barrage of foreign pathogens that can invade and infect the body, and the immune system is essential in combating this ever-present threat. In mammals, the immune system can be divided into two parts: the innate immune system and the adaptive or acquired immune system. The former is responsible for the initial recognition and destruction of foreign pathogens while the latter ensures that re-infection by the same microorganism does not occur. Without the immune system, mammals would be unable to overcome the attack of infectious microorganisms (Leonard, 2001).

The innate immune system is the non-specific branch of the immune system. It is composed of cells that identify foreign pathogens by recognizing cell surface markers specific to bacterial cells. For example, macrophages can recognize bacterial cells through their Toll-like receptors (TLRs). TLRs are cell surface proteins that bind to common, repeating, foreign components on pathogens such as glycolipids, lipoproteins, lipopolysaccaride and bacterial deoxyribonucleic acid (DNA) (Akira *et al.*, 2001, Kobayashi *et al.*, 2002). Once the macrophages recognize a foreign pathogen they destroy it by phagocytosis. Alternatively, body cells that have been infected with foreign pathogens can be destroyed by natural killer cells (Lanier, 2005). Although the innate immune system is a very effective system in preventing most microbial invasions, occasionally some pathogens evade the innate immune system and begin to multiply in the host. This is where the role of the more specific adaptive immune system comes into play.

The adaptive immune system has the ability to mount an immune response to specific antigenic targets produced by invading pathogens. The primary cells responsible for this response are B and T lymphocytes found in the blood, lymph and lymphoid organs. T lymphocytes develop in the thymus to become either cytotoxic T cells, helper T cells or T regulatory cells. The cytotoxic T cells assist in the destruction of cells that have already been infected by a foreign pathogen, whereas the helper and regulatory T cells activate other cells in the immune system (Maekawa

and Yasutomo, 2005). In mammals, B cells develop in the bone marrow and are responsible for the production of antibodies. The advantage of the adaptive immune system is that the memory cells produced prevent re-infection because they can recognize and eliminate specific pathogens that have previously infected the body at a faster rate and more efficiently than if the pathogens were novel microorganisms that were just encountered (Alt *et al.*, 1987).

The series of events that occurs during an infection involves cells from both the adaptive and innate immune systems. Initially, antigen presenting cells (APCs), which include macrophages, dendritic cells and B cells, engulf the invading pathogen, which is then proteolytically degraded into peptides. These peptides are bound to major histocompatibility (MHC) molecules within the antigen processing components of the APC and presented on the cell surface. The MHC:peptide complex is recognized by naïve T lymphocytes that subsequently proliferate and differentiate into helper T cells (Melief, 2003). The helper T cells activate B cells by releasing growth factors so the B cells will produce antibodies against the pathogen. Helper T cells also activate cytotoxic T lymphocytes to kill somatic cells that are expressing peptides from that particular pathogen (Maekawa and Yasutomo, 2005). This series of events allows the body to rid itself of the microorganism.

1.2 The BCR during B cell development

The B cell antigen receptor (BCR) is a key regulator of B lymphocyte activation, growth and differentiation. It is expressed on the surface of B lymphocyte cells and is composed of a ligand binding receptor (membrane immunoglobulin; mIg) and a signalling component (Ig-α/β) (Figure 1.1) (Hombach *et al.*, 1990). B cell development in the bone marrow requires that the BCRs signal to the cell when a functional receptor has trafficked to the cell surface and if the BCR binds to self antigen. This prevents non-functional and self-reactive B cells from escaping into the periphery. Once the B cell enters the periphery, the BCR binds foreign antigens and signals to the cell to proliferate and differentiate into an antibody producing plasma cell or a long-lived memory cell (Shapiro-Shelef and Calame, 2005). This allows the body to combat novel microorganisms and to keep a B cell repertoire that is reactive to microorganisms that have been previously encountered, allowing a faster immune response if reinfection occurs. In this sense, the functionality of the BCR is essential for the immune system to successfully eliminate foreign

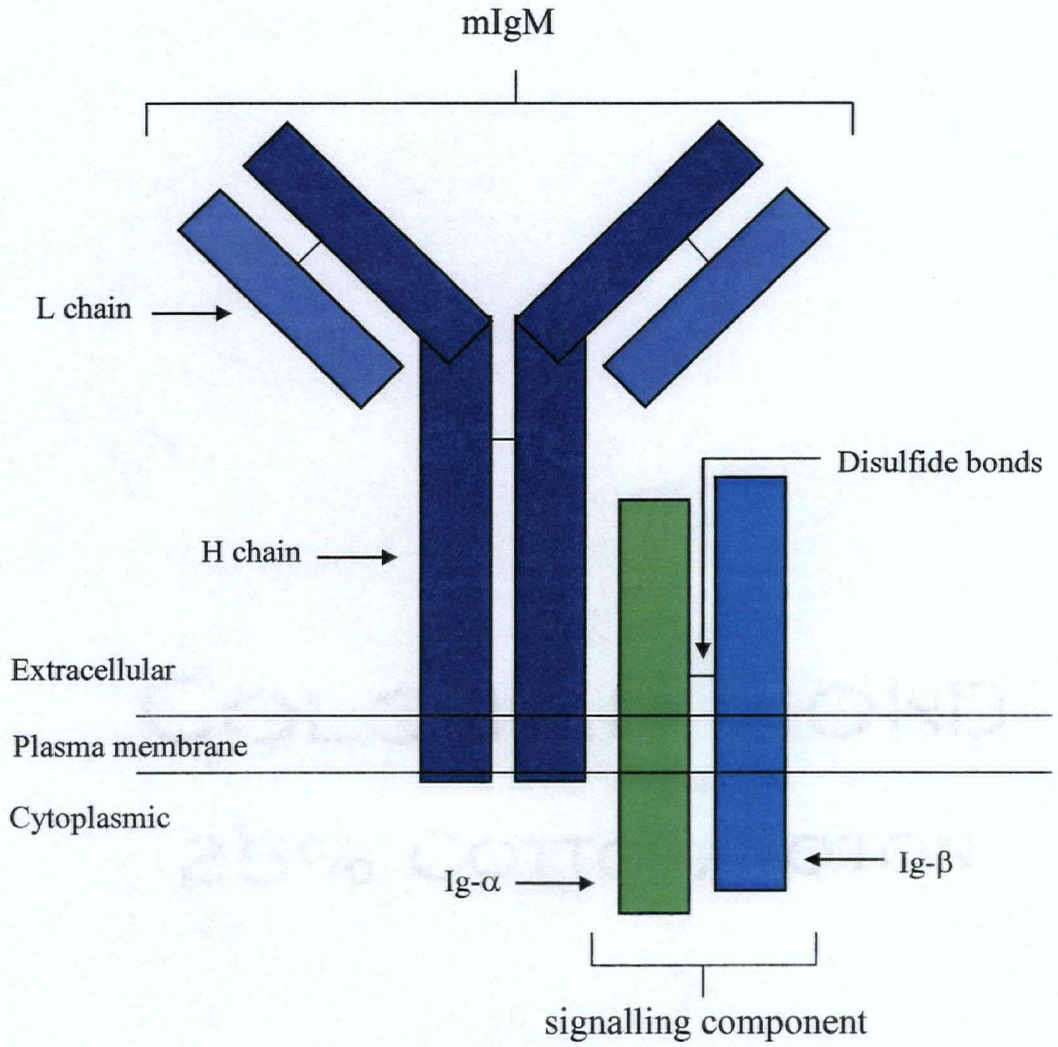


Figure 1.1 Diagram showing the proposed structure of an mIgM-containing B cell antigen receptor (BCR) on the surface of the cell. This shows the antigen binding component (mIgM) composed of the H and L chains and the signalling component composed of Ig-α and Ig-β (Hombach *et al.*, 1990).

pathogens. In addition, a non-functional BCR can lead to autoimmune diseases and B cell leukemias and lymphomas (Thompson *et al.*, 1997; Payelle-Brogard *et al.*, 1999).

The rearrangement and expression of the heavy (H) and light (L) chain genes characterize the stages of B cell development in the bone marrow. This rearrangement is what accounts for antigen specificity of individual B cells. Gene rearrangement first occurs in the stem cell derived pro-B cells that express Ig-α and Ig-β, but do not yet express membrane Ig (mIg). The Ig-α and Ig-β proteins associate with the endoplasmic reticulum (ER) chaperone protein, calnexin, and appear on the surface of the cell. Although calnexin is normally an ER resident protein, it can be found of the surface of pro-B cells where it may signal (Nagata *et al.*, 1997).

Once pro-B cells undergo heavy chain locus rearrangement and produce a membrane bound H chain they are considered pre-B cells. This is the first stage that a complete BCR is expressed on the cell surface because, although pro-B cells express Ig-α/β, they do not express any H or L chains. The pre-BCR is composed of the rearranged heavy chain, a surrogate light chain and the Ig-α and Ig-β signalling proteins. The surrogate light chain is made up of two proteins, VpreB and λ5 (Martensson and Ceredig, 2000). If the pre-BCR successfully reaches the cell surface, the cell begins light chain rearrangement.

A successful light chain gene rearrangement allows the cells to express the heavy and light chain proteins as part of the BCR. At this stage the completed BCR signals through Ig-α and Ig-β, and the cell is considered an immature B cell (Reth, 1991). These immature B cells then undergo selection in the bone marrow so that the body can eliminate any self-reactive cells. If the BCR recognizes a self-antigen then that cell will go through clonal deletion and be removed from the B cell repertoire. Alternatively, it will undergo receptor editing that will change the sequence of the mIgM variable region so that it is no longer self-reactive, or will migrate to the periphery, but will not be active. If the BCR does not react to self antigen the cell will enter the periphery and develop into a mature B cell (Gold, 2002).

The expression of another heavy chain isoform distinguishes the mature from the immature B cell. Alternative mRNA splicing determines whether or not the H chains will be μ or δ H chains that result in a BCR of either mIgM or mIgD type, respectively (Venkitaraman *et al.*, 1991).

Both mIgM and mIgD BCRs signal through the same Ig-α/β signalling proteins that are associated with them, and the BCRs have the same antigen specificity. Mature B cells primarily express the mIgD form of the BCR (Campbell *et al.*, 1991, Havran *et al.*, 1984). Mature B cells that have not yet encountered an antigen are considered naïve B cells. Upon encountering antigen they proliferate and differentiate into antibody producing plasma cells or memory cells. If the cell develops into an antibody producing plasma cell, it secretes another form of the H chain that does not have a transmembrane sequence. The secreted H chain is formed through alternate splicing of the H chain mRNA (Rogers *et al.*, 1980). The secreted antibodies then bind to foreign pathogens in the periphery and target them for destruction by phagocytosis or for lysis by complement (James, 1982; Shapiro-Shelef and Calame, 2005).

1.3 BCR structure

The B cell receptor (BCR) is found on the surface of B cells. It is composed of an antigen binding component (mIg) and a signalling component (Figure 1.1). The mIg is comprised of two membrane immunoglobulin H chains linked by a disulfide bond and two immunoglobulin L chains linked to the H chains by a disulfide bond. Alternative mRNA splicing determines whether or not the BCR contains a mIg of either mIgM or mIgD (Venkitaraman *et al.*, 1991). Mature B cells primarily express the mIgD form of the BCR (Havran *et al.*, 1984). Memory cells and plasma cells can also express BCRs on their cell surface. Surface BCRs can be of all five isotypes of mIg (mIgM, mIgD, mIgA, mIgG, mIgE) and all are normally associated with the Ig-α/β heterodimer (Venkitaraman *et al.*, 1991). Differential cleavage and polyadenylation of the mIg RNA sequence produces a secreted Ig complex that is lacking the transmembrane and cytoplasmic domains (Rogers *et al.*, 1980). Each B cell expresses BCRs with variable regions that are specific for different potential antigens.

The mIg component of the mIgM BCR is unable to signal due to its short cytoplasmic domain, therefore it must associate with two signalling proteins, Ig-α and Ig-β, to form the complete functional BCR (Hombach *et al.*, 1990). Ig-α and Ig-β are disulfide-linked, transmembrane proteins that are non-covalently associated with mIg. The current model suggests that one heterodimer of Ig-α/β associates with one mIg complex (Schamel and Reth, 2000). Ig-α and Ig-β are found on the surface of B cells during the early stages of B cell development. Antigen

binding or cross-linking of the BCR results in signalling mediated by the Ig-α/β heterodimer (Reth, 1991) (see section 1.5).

1.3.1 Membrane Immunoglobulin (mIg)

The ligand, or antigen binding portion, of the BCR is a membrane Ig. The H chain protein of mIgM has a large extracellular domain, a transmembrane domain and a small cytoplasmic domain (Figure 1.2). In mice, the extracellular domain has a variable (V), 110 amino acid VDJ_H region at the N-terminus that is followed by four 110 amino acid constant (C) regions. Each region is a barrel shaped structure and is composed of β-pleated sheets. This structure is maintained with a disulfide bond that holds the sheets together. The H chain protein of mIgM bends between the second and third barrel region (Davies and Metzger, 1983). The transmembrane portion of the protein is proposed to be an α helix consisting of 26 amino acids that span the lipid bilayer (Figure 1.3) (Rogers *et al.*, 1980). Thirteen of those amino acids are highly conserved among different immunoglobulin isotypes, and 11 of those 13 are proposed to line up on one side of the helix suggesting that they, or that side of the helix, may be important for interaction with other molecules. The transmembrane region is not composed entirely of hydrophobic amino acids, as is seen with many transmembrane proteins. There are 9 polar amino acids in the transmembrane region of mIgM. This suggests that the H chain transmembrane region could be interacting with the transmembrane regions of other proteins since one might predict that polar amino acids should be hidden or shielded in the lipid bilayer (Reth, 1992).

The cytoplasmic region of the murine μ H chain is very short, consisting of only three amino acids, lysine, valine, lysine (KVK (single letter amino acid code)). This sequence is also identical in mice, humans and sharks (Reth, 1992). The same three amino acids also make up the cytoplasmic domain of human and mouse mIgD (Reth, 1992).

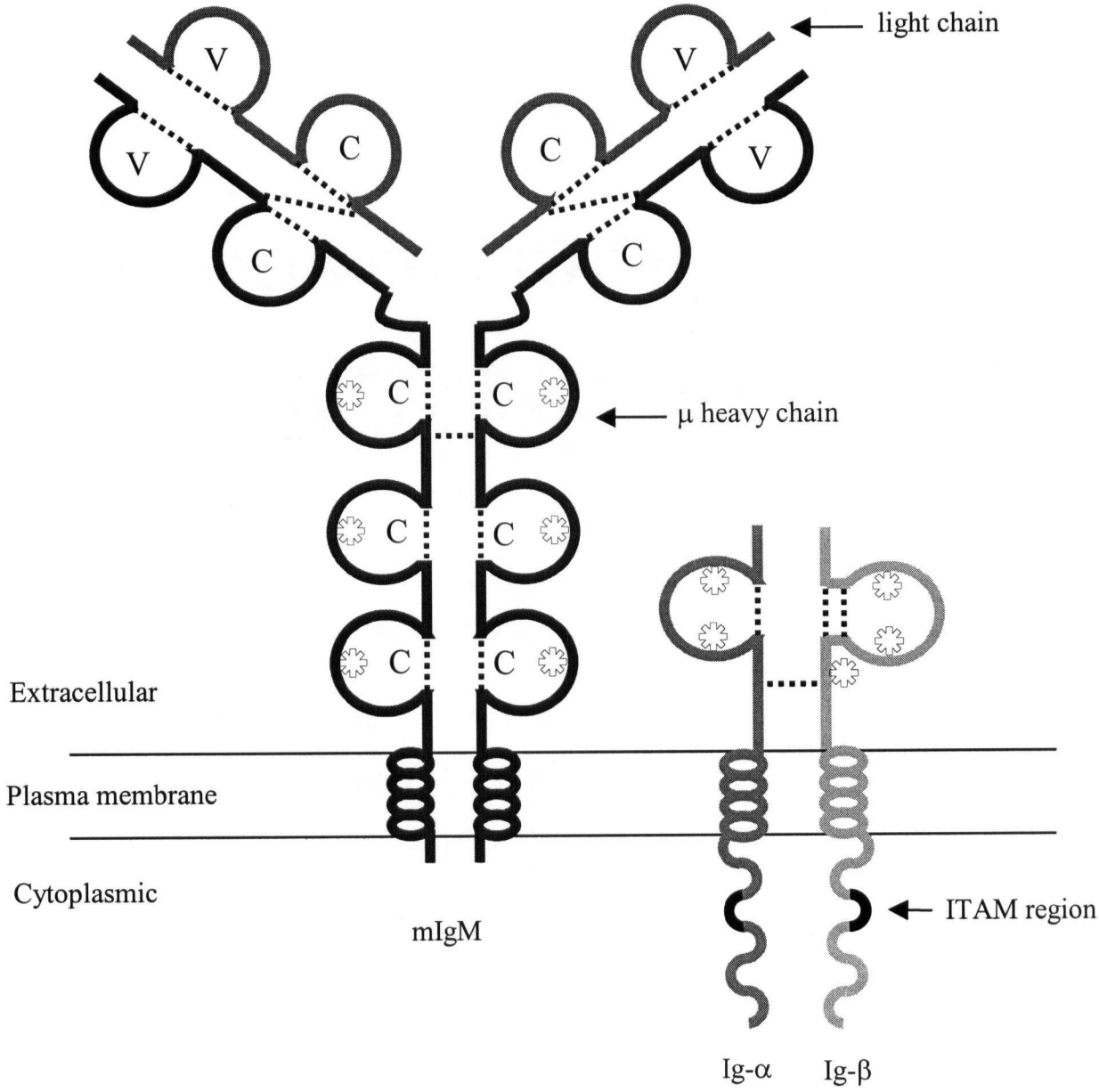


Figure 1.2 The structure of the BCR. This shows the looped barrel domains (variable (V) and constant (C) regions), the intramolecular disulfide bonds (dashed lines), the proposed extracellular glycosylation sites (), the alpha helical transmembrane region and the cytoplasmic tails with the ITAM regions (adapted from Abbas *et al.*, 2000).

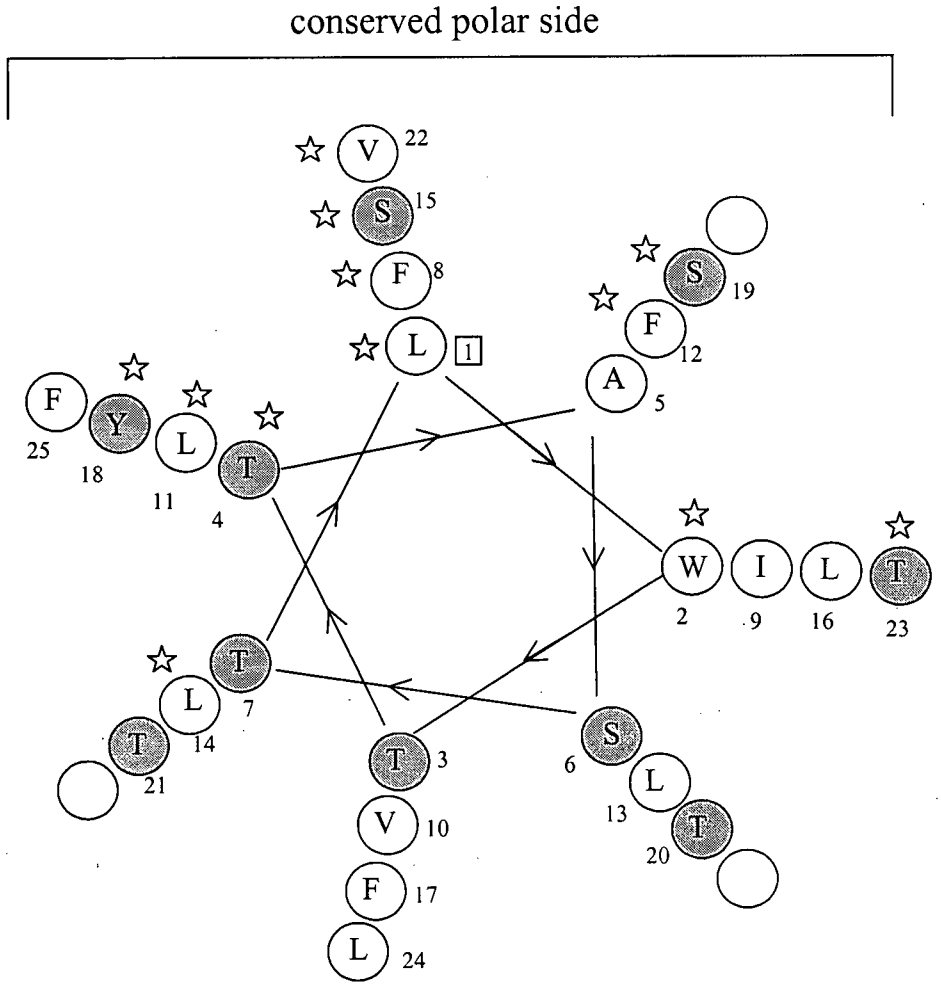


Figure 1.3 The top view of the mIgM transmembrane region showing the α helix structure (Reth, 1992). Polar amino acids are shaded. Evolutionarily conserved amino acids are represented with a star (☆). Amino acid [1] is at the extracellular side of the transmembrane region and amino acid 25 is at the cytoplasmic side. Single letter amino acid code is used.

The L chain protein is linked to the H chain protein by a disulfide bond in its C region. The L chain is a 25 kDa molecule. The variable region of the L chain is at the N-terminus of the protein and the C region is at the C-terminus. The variable region is composed of two β sheets with four and five amino acid strands composing each sheet. The C region is composed of two β sheets with three and four strands making up each sheet. These form into two barrel shapes and are stabilized with a disulfide bond (Figure 1.2) (Edelman, 1991). The barrel shape is highly conserved among immunoglobulin molecules and is known as the immunoglobulin fold (Siden *et al.*, 1981). Together the H and L chain proteins make up the antigen binding portion of the BCR.

1.3.2 Ig-α and Ig-β

The signalling portion of the BCR is composed of a heterodimer of Ig-α and Ig-β. Ig-α is a transmembrane glycoprotein with the predicted membrane topology shown in Figure 1.2. The extracellular portion of murine Ig-α is composed of 109 amino acids. The amino acid sequence indicates that the extracellular regions of Ig-α fold into a barrel domain similar to those seen in the H and L chains. There are two cysteine residues (50 and 101) that form a disulfide bond between the β sheets in the extracellular region. Another cysteine residue (113) forms a disulfide bond with Ig-β. Ig-α also two has glycosylation sites at amino acids 58 and 68 (Sakaguchi *et al.*, 1988, Hombach *et al.*, 1988). The transmembrane and cytoplasmic domains are predicted α helices consisting of 22 and 61 amino acids, respectively (Reth, 1992). The transmembrane domain of Ig-α contains two polar amino acids (Hombach *et al.*, 1999). The cytoplasmic domain has a highly conserved immunoreceptor tyrosine-based activation motif (ITAM) with the amino acid sequence $D/Ex_7D/Ex_2Yx_2L/Ix_7Yx_2L/I$ (Reth, 1989). Additionally, the cytoplasmic domain has two other tyrosine residues (176 and 204). Phosphorylation of these residues recruits the B cell linker proteins Vav and Grb2. Activated B cells lacking these residues do not present antigen to T cells, indicating that $tyrosine_{176,\ 204}$ phosphorylation is important for intracellular trafficking of the receptor after antigen crosslinking (Siemasko, 2002). The residue at position 204 may also have a role in binding the SH2 domain of the adaptor protein BLNK (Baba *et al.*, 2001, Kabak *et al.*, 2002)

The Ig-β protein is similar in structure to Ig-α. The extracellular region of Ig-β is composed of 129 amino acids that form β sheets. There are four cysteine residues that form two intramolecular disulfide bonds (43:124 and 65:120) and an additional cysteine residue (135) that forms the disulfide bond with Ig-α. The extracellular domain also has three glycosylation sites (68, 99, 130) (Hermanson *et al.*, 1988). The transmembrane region, like Ig-α, is an α helix composed of 22 amino acids. The transmembrane domain of Ig-β contains two polar amino acids (Hermanson *et al.*, 1988). The cytoplasmic tail consists of 48 amino acids and has an ITAM domain. The ITAM sequence for both Ig-α and Ig-β is conserved in different species indicating that protein function requires that the sequence remains unchanged (Reth, 1989). Tyrosine phosphorylation of the ITAM domains by Src-family tyrosine kinases leads to interaction with the SH2 domains of Syk, a protein tyrosine kinase, resulting in the activation of numerous downstream intracellular signalling cascades (Gold, M. R., 2002) . Murine Ig-β, unlike Ig-α, does not contain any additional tyrosine residues (Reth, 1989). The Ig-α/β heterodimer makes up the signalling portion of the BCR.

1.3.3 The Four Chain BCR

The four different components that make up the BCR (H chains, L chains, Ig-α and Ig-β) interact through covalent and non-covalent bonds. Disulfide bonds join the two H chains, the L and H chains and Ig-α and Ig-β to each other. A model of how these chains could interact is shown in Figure 1.1 with the transmembrane portion of mIgM interacting noncovalently with Ig-α/β.

The current model of BCR structure suggested by Schamel and Reth (2000) indicates that only one Ig-α/β heterodimer is associated with a mIg complex to make up the BCR as opposed to two heterodimers. They found that when a cell line was created that expressed both a tagged and untagged form of Ig-α, there were never any BCR complexes that contained both forms of the Ig-α protein. This indicates that there must only be one heterodimer of Ig-α/β per mIg molecule. If there were two heterodimers per molecule, as was previously hypothesized, then there should be some complexes with both forms of Ig-α. Additionally, Schamel and Reth (2000) supported their model by radiolabeling cells with ^{35}S-methionine and allowing the radiolabeled amino acid to incorporate into cellular proteins. They then quantified and compared the known number of methionine molecules contained in each component of the BCR complex to their data and

determined that the BCR complex contained only one Ig-α/β heterodimer. The difficulty with this model comes in reconciling how the single heterodimer associates with the mIg molecule. The mIg complex has two predicted polar patches in its transmembrane region (see Figure 1.3) that need to be covered over by association with Ig-α/β or perhaps by some other component. It is hypothesized that one of the proteins in the heterodimer could associate with both polar regions or each heterodimer protein could associate with a different polar region, but this remains unknown (Matsuuchi and Gold, 2001). This idea led to the controversial proposal by Schamel and Reth (2000) that the BCR is present as an oligomer on the cell surface. They suggest that the polar amino acids on one side of the transmembrane region of mIg interact with Ig-α/β and that the polar amino acids on the other side interact with other BCR complexes. This creates a cluster of BCRs in one location. This model is controversial and yet to be confirmed or disproved.

1.4 BCR assembly and surface expression

B lymphocyte chaperone proteins play a vital role in ensuring that intact BCRs are expressed on the cell surface. Because BCR signalling is required for B cell development and differentiation it is important that all the components of the BCR are properly folded and associated with one another on the cell surface. Without chaperone proteins controlling BCR assembly and lymphocyte development could be severely impaired. This could have profound effects on the adaptive immune system.

In order for the mIgM-containing BCR to be expressed on the cell surface, all four components of the receptor must pass through quality control mechanisms within the ER and be trafficked to the plasma membrane. Intermediate forms of assembled BCR chains are held in the ER by chaperone proteins until assembly is complete. If one or more components of the BCR are not expressed, then the remaining BCR proteins remain trapped in the ER (Matsuuchi *et al.*, 1992; Venkitaraman *et al.*, 1991). If a protein is incorrectly folded, mutated or improperly assembled, it will remain in the ER, bound by chaperone proteins, until it can be degraded in the cytoplasm by proteosomes (Fagioli and Sitia, 2001; Foy and Matsuuchi, 2001).

In order to ensure that unfolded proteins do not escape the ER, chaperone proteins contain amino acid sequences that identify them as ER resident proteins. As long as a chaperone is bound to an

unfolded protein, neither of the proteins will be permitted to leave the ER. Soluble ER resident chaperones, such as BiP, contain an H/KDEL amino acid sequence that binds to the membrane associated KDEL receptor. This receptor keeps the chaperone in the ER or returns it to the ER if it is found in the Golgi (Nilsson and Warren, 1994; Teasdale and Jackson, 1996). Membrane bound proteins, such as calnexin, contain a KKXX sequence on their cytoplasmic tails which ensures their retention in the ER (Teasdale and Jackson, 1996). ER localization of chaperone proteins also makes sure that target proteins can not escape until properly folded and assembled into complexes.

Chaperone proteins recognize and bind to newly synthesized proteins using common mechanisms. Generally, chaperones function by recognizing properties universal to unfolded or unassembled proteins. For example, they may bind to exposed hydrophobic patches, sulfhydryl groups or oligosaccharides, or to groups of proteins that have formed large aggregates. This method of binding allows the chaperones to recognize many different proteins rather than just those with a particular amino acid sequence (Hammond and Helenius, 1995).

Many different chaperone proteins are present in the ER at any given time, therefore it is important that the correct chaperones interact with the correct target proteins to ensure proper folding. It is believed that the position of the glycans in the glycoprotein sequence may determine the order in which the target proteins interact with certain chaperone proteins (Ellgaard and Helenius, 2001). Two chaperones, BiP(GRP78) and GRP94, interact with the BCR L chain. BiP binds to an earlier intermediate of the forming L chain, and if the internal disufide bond fails to form, BiP will target the protein for degradation (Skowronek *et al.*, 1998), but, if the protein folds correctly, GRP94 will bind to it and allow for its association with the H chain (Melnick *et al.*, 1994). The H chain is initially bound to BiP and upon formation of the disulfide bond between the H and L chains, BiP will dissociate and allow further BCR assembly (Lee *et al.*, 1999; Foy and Matsuuchi, 2001). In order for the complete BCR to form, Ig-α and Ig-β must associate with one another and then with mIgM. Ig-α is initially associated with GRP94 and dissociates upon bond formation between Ig-α and Ig-β (Foy and Matsuuchi, 2001).

In addition to being folded in the ER the BCR components are glycosylated. Folded proteins are glycosylated with both N-linked and O-linked oligosaccharides. Calnexin, a membrane bound chaperone, has been shown to interact with unfolded proteins in the ER and dissociate from them

when they are glycosylated. This could be a quality control mechanism that ensures that unfolded proteins cannot leave the ER until they are properly glycosylated. After ER glycosylation the glycoproteins traffic to the Golgi where the oligosaccharides are processed and the proteins are sorted to the cell surface (Wu *et al.*, 1997).

If the mIgM-containing BCR is missing a component, then the other proteins in the mIgM-containing BCR remain trapped in the ER and are eventually degraded. Pulse-chase experiments have shown that the μ H chain is degraded much more quickly when not associated with other components of the BCR. Interestingly, Ig-α and the L chain are not more rapidly degraded when lacking associations with other BCR components. This may be because the μ H chain is bound to BiP when not bound to L chain, and BiP enhances the rate of degradation (Foy and Matsuuchi, 2001). As Ig-α synthesis is the rate-limiting step in human BCR complex formation and Ig-β, H and L chain are produced in excess, Ig-α may be used to assemble complete BCRs immediately, leaving little time for it to be sequestered in the ER by chaperones (Brouns *et al.*, 1995).

1.5 BCR signalling and antigen processing

It has been proposed that once the BCR has reached the cell surface, the receptors form oligomeric complexes. Schamel and Reth (2000) found that when the membranes of BCR expressing cells are solubilized, the extracted BCR complexes appear to form oligomers of their particular isotype (i.e. mIgM interacts with mIgM and not mIgD, and vice versa). They propose that the isotype specific complexes are a result of interactions between the transmembrane regions of the mIg proteins suggesting that the polar amino acids on one side of the transmembrane helix (Figure 1.3) interact with Ig-α/β and those on the other side interact with isotype-specific mIg molecules.

Upon BCR activation and cross-linking the receptors cluster further and migrate into lipid rafts (Pierce, 2002). Lipid rafts are regions of cell membranes that are enriched in cholesterol and sphingolipids, whereas other regions of the membrane contain unsaturated phosphatidylcholine and cholesterol. Lipid rafts also contain GPI-anchored proteins, transmembrane proteins and tyrosine kinases of the Src family. The GPI-anchored proteins are incorporated into plasma membrane rafts immediately after transport from the Golgi and remain there indefinitely. It is believed that proteins that are doubly acylated by saturated chains, such as the Src-family kinases

(in particular, Lyn), are partitioned into rafts (Pierce, 2002) whereas transmembrane proteins may have an affinity to rafts, but they may travel in and out of them (Simons and Ikonen, 1997).

The current lipid raft model is that the resting BCR has a low affinity for lipid rafts, but upon crosslinking, the BCR's affinity for the lipid raft increases and it moves into the rafts. The rafts contain signalling proteins such as Lyn, which phosphorylate Ig-α/β and initiate intracellular signalling cascades. The rafts also contain BCR associated proteins such as CD19 (Carroll, 1998). It is suggested that lipid rafts help to bring the signalling proteins and the BCR in close proximity to aid in signal transduction (Pierce, 2002).

BCR signalling begins with phosphorylation of the ITAM domains of Ig-α/β by the Src family protein tyrosine kinases (Fyn, Blk and Lyn) and the protein tyrosine kinase, Syk. These kinases are believed to associate weakly with some inactivated BCRs, but upon BCR activation and clustering they are believed to activate each other and promote ITAM phosphorylation. ITAM phosphorylation leads to the activation of three signalling pathways controlled by PI3K, PLCγ2 and Ras (Gold, 2002). PI3K activation causes the B cell to proliferate by activating the enzyme AKT, which inhibits pro-apoptotic factors. In the second pathway, PLCγ2 is recruited to the membrane by BLNK, and this produces inositol-1,4,5-triphosphate and DAG. This results in the expression of genes that determine B cell fate and the transcription of anti-apoptotic genes (Niiro and Clark, 2002). The third pathway activated by BCR antigen binding is the Ras pathway. Ras is a GTPase that controls Raf-1 activation, leading to activation of the Erk cascade. This pathway is important for B cell development and proliferation of mature B cells (Gold, 2002).

In addition to signalling from an antigen-bound BCR, the receptor can be internalized and the antigen processed for presentation on MHC class II molecules. The MHC class II molecule presents a peptide fragment from the antigen to helper T cells. The T cells then activate the B cells and stimulate them to produce antibodies (McHeyzer-Williams, 2003).

In order for receptor internalization to occur there must be signalling from the crosslinked BCR to the rest of the cell. In mIgG BCRs this can occur through a tyrosine residue on the H chain cytoplasmic tail. In other BCR isotypes this occurs through Ig-α signalling. The signal depends on the first tyrosine residue in the ITAM domain of Ig-α (Cassard *et al.*, 1998). Stoddart *et al.*

(2002) hypothesize that BCR signalling is supported through BCR localization in lipid rafts because lipid rafts have been shown to contain the signalling molecules necessary for internalization. In their model, BCR signalling activates Src-family kinases such a Lyn, which phosphorylate clathrin. The clathrin forms a clathrin-coated pit either beside or within the lipid raft, and this allows BCR internalization by invagination of the pit and pinching-off of the membrane to form a cytoplasmic vesicle.

Once internalized, the contents of the vesicle are degraded in endosomes and targeted to the MHC class-II containing compartment (MIIC) where MHC class II molecules are assembled. The peptides in the vesicles are then loaded onto MHC class II molecules. The intracellular trafficking of the vesicle appears to be mediated by the cytoplasmic tails of Ig-α/β. If chimeric proteins with only Ig-α or only Ig-β cytoplasmic tails are expressed in cells, they are not targeted to the MIIC compartment, but if both Ig-α and Ig-β tails are expressed, then there is targeting to the MIIC compartment resulting in MHC presentation. Additionally, the cytoplasmic tail of Ig-α slows down vesicle trafficking, whereas the cytoplasmic tail of Ig-β speeds it up. This also corresponds to how quickly they are degraded into peptides for MHC class II binding (Li *et al.*, 2002). Therefore, Ig-α and Ig-β appear to play distinct roles in the trafficking of the BCR-antigen-containing vesicle to the MIIC compartment.

In the adaptive immune system the B cell is both an antibody producing cell and an antigen-presenting cell. The BCR is an integral component in both of these processes. In order to reach the antibody producing stage the BCR must signal through many developmental stages, eventually promoting differentiation into an antibody producing plasma cell. In addition to signalling, a second function of the BCR is antigen internalization for processing and presentation to T lymphocytes (Niiro and Clark, 2002).

1.6 BCR mutations

1.6.1 BCR mutations affect B cell development

B cell development can be divided into two parts, that which occurs in the bone marrow and that which occurs in the periphery (see section 1.2). Both of these stages are BCR dependent, and

mutations in the BCR can lead to a block in B cell development in either the bone marrow or the periphery. In order to understand the functions of different components of the BCR during B cell development and differentiation, many researchers have created strains of mice with deletions or modifications in one or more of their BCR proteins. Kitamura *et al.* (1991) found that disruption of the transmembrane region of mIgM in mice results in blockage of B cell development at the pro-B cell stage, potentially due to the inability of Ig-α/β to associate with mIg and get expressed on the cell surface with the pre-BCR. This would prevent signals from the pre-BCR from being generated, and B cell development would be impaired. Additionally, Pelanda *et al.* (2002) generated Ig-α and Ig-β deficient mice. They found that B cell development in these mice does not proceed past the pro-B cells stage, but that H chain recombination can occur. This is expected because after the pro-B cell stage the BCR is expressed on the cell surface and requires Ig-α/β to signal that it has reached the cell surface. If a pro-B cell is lacking Ig-α/β, then it will not be able to signal and B cell development will be blocked at the pro-B cell stage. This also indicates that B cell development does not require Ig-α/β signalling before the pre-B cell stage.

In a similar study, Reichlin *et al.* (2001) examined the effects of mutating Ig-α or Ig-β on B cell development. They found that mice that have a deletion of the cytoplasmic domain of Ig-β proceed normally through the initial stages of B cell development and produce immature B cells, but that development is arrested at the immature B cell stage. In contrast, when they examined mice that contained a deletion of the cytoplasmic domain of Ig-α, they found that very few of these cells even make it to the immature B cell stage and that most begin to apoptose at the pre-B cell stage. This indicates that the cytoplasmic tails of Ig-α and Ig-β have different functions during B cell development and that the Ig-α cytoplasmic tail allows development to a later stage than the Ig-β cytoplasmic tail.

Wang *et al.* (2004) further examined the specific roles of Ig-α and Ig-β in B cell development. They created two Ig-β constructs, one that was cytoplasmically truncated (ΔCY (β/β/0)) and one that had an Ig-α cytoplasmic domain (Cα (β/β/α)). They found that the ΔCY construct rescued B cell development in the bone marrow, but not the periphery (similar to the results seen by Reichlin *et al.* (2001)) and that the Cα construct rescued development in the bone marrow and

variably in the periphery. This indicates that signalling from two Ig-α cytoplasmic tails enhances the B cell's ability to develop and differentiate.

1.6.2 BCR mutations affect BCR cell surface expression

In order for the mIgM-containing BCR to be expressed on the cell surface, all four components of the BCR must be expressed in the cells. If one or more of the components are lacking, the rest of the BCR remains trapped in the ER (Matsuuchi *et al.*, 1992; Venkitaraman *et al.*, 1991). In addition, certain mIgM or Ig-α/β mutations can prevent cell surface expression by disrupting the association of the various BCR components (described further below).

Shaw *et al.* (1990) found that if certain polar amino acids in the transmembrane region of mIgM are changed to hydrophobic residues, mIgM does not interact with Ig-α/β. They found that mutating the tyrosine-serine dipeptide, the 19^{th} and 20^{th} amino acids in the transmembrane region, to valines (YS/VV) affected both signal transduction and antigen presentation, presumably by interfering with proper Ig-α/β association with mIgM, which is essential for these events. Blum *et al.* (1993) found that switching 41 amino acids in the C-terminal (transmembrane) region of the H chain with amino acids from hCD8α permitted surface BCR expression, but prevented any interaction with Ig-α/β and, therefore, prevented signalling from occurring. Blum *et al.* (1993) also individually mutated eight of the polar amino acids in the transmembrane region of mIgM and found that none of the mutations disrupted signalling completely. From this they were able to conclude that Ig-α/β must interact with more than one residue in the transmembrane region and that the transmembrane region is important for the association of the various BCR components.

It also appears important that the extracellular domains of Ig-α and Igβ remain intact in order for BCR cell surface expression to occur. Some mutations in the extracellular domains of Ig-α or Ig-β will prevent BCR cell surface expression or allow an incomplete BCR to be trafficked to the cell surface without all of its components. For example, if a particular proline residue (amino acid 126) in the extracellular domain of Ig-α is mutated, the protein is glycosylated differently and is unable to associate with mIgM, but it does traffic to the cell surface with Ig-β. Therefore, the extracellular domain of Ig-α appears to be important for interacting with quality control

mechanisms within the cell that would normally sequester the incomplete BCR in the ER (Condon *et al.*, 2000).

Additionally, a truncation of the extracellular domain of Ig-β prevents heterodimerization with Ig-α and will not allow trafficking of the BCR to the cell surface. Or, if a chimeric Ig-β protein is created that has an extracellular Ig-α domain and Ig-β transmembrane and cytoplasmic domains, it will not associate with Ig-α and will not allow BCR cell surface trafficking (Wang *et al.*, 2004). This demonstrates that quality control mechanisms interact with the extracellular or transmembrane domains of Ig-α/β, but not the cytoplasmic domains, and therefore, BCR cell surface trafficking is dependent on both the extracellular and transmembrane domains.

If the above mutations were to occur in an immature B cell, the cell should be eliminated from the body in the bone marrow due to its inability to signal through the BCR. But, somatic hypermutation can introduce mutations in mature B cells that are already circulating in the periphery. Somatic hypermutation is a process that occurs after the B cell encounters antigen in the periphery and has begun to proliferate. It involves introducing point mutations into the H and L chain genes so that they will bind to the foreign antigen more effectively (Kruppers *et al.*, 1999). In some cases errors occur during somatic hypermutation and the point mutations are inserted in the genes encoding Ig-α and Ig-β instead. This is believed to be due to sequence similarity in the transcriptional control regions between Ig-α/β and the target genes (Gordon *et al.*, 2003). In a study of the B cell mutations in patients with B cell chronic lymphocytic leukemia (BCLL), a leukemia resulting from an accumulation of non-functional, mature B cells in the body, Gordon *et al.* (2003) found that the genes encoding Ig-α and Ig-β were often mutated, primarily in the extracellular domain or in the cytoplasmic ITAM region. This indicates that mutations in Ig-α and Ig-β can cause improper BCR assembly or signalling leading to an accumulation of ineffective B cells resulting in BCLL. In fact, mature B cells with a BCR that contains two Ig-α cytoplasmic tails have lower levels of BCR surface expression, are anergic and appear to live longer than wild-type B cells. These are all characteristics of BCLLs and indicate that the Ig-α/β heterodimer may help control B cell longevity (Reichlin *et al.*, 2004). Further understanding of the role of Ig-α/β in BCR assembly, trafficking and signalling is vital to further understanding this disease.

1.7 Purpose of thesis study

The interest in studying how BCR mutations affect BCR assembly, cell surface expression and, ultimately, B cell development is in an effort to further understand the generation of B cell leukemias and lymphomas. Initial studies on BCR cell surface expression showed that correct assembly and cell surface trafficking of the mIgM BCR requires that all four chains of the BCR be expressed in the cells (Matsuuchi *et al.*, 1992; Venkitaraman *et al.*, 1991). Deletion of Ig-α or Ig-β prevents B cell development past the pro-B cell stage (Pelanda *et al.*, 2002). However, if only the cytoplasmic tails of Ig-α or Ig-β are truncated or mutated, the mIgM BCR can still traffic to the cell surface, but B cell signalling and development are disrupted (Reichlin *et al.*, 2001, Wang *et al.*, 2004). If the B cell is allowed to mature prior to a BCR mutation that creates a BCR with two Ig-α cytoplasmic tails, the cell become anergic and long-lived, likely due to lower BCR cell surface expression (Reichlin *et al.*, 2004). This could be a analogous to a B cell leukemia in which somatic hypermutation disrupts BCR cell surface trafficking so that only low levels of signalling can occur, resulting in B cells that are nonfunctional and long-lived (Gordon *et al.*, 2003). In order to further understand BCLLs, it is important to determine which portions of the BCR are important for receptor assembly and cell surface trafficking.

The purpose of this study is to test the hypothesis that specific protein interactions between the transmembrane and extracellular domains of Ig-α and Ig-β are necessary for BCR assembly and cell surface trafficking. The approach I have used is to create mutant Ig-α and Ig-β genes and express them in cell lines containing the other chains of the BCR. The association of the various BCR components was examined by immunoprecipitation experiments. Cell surface expression of the BCR was studied by cell surface fluorescence or fluorescent activated cell sorting (FACS).

Two different expression systems were used for this study. The first being a series of non-lymphoid cultured cell lines (AtT20 endocrine cells) that have been previously transfected with various combinations of the BCR components (Matsuuchi and Kelly, 1991, Matsuuchi *et al.*, 1992). The advantages of using this non-lymphoid cell line are that the cell lines created are stable and can express high levels of the BCR components so that BCR assembly and membrane trafficking can be examined. Also, the number of cells produced is unlimited, so recovering enough material for biochemical analyses is not the problem that it would be in other transient expression systems. The disadvantage of the AtT20 system is that it is a non-lymphoid system

so the cells do not contain all the components of a lymphoid cell and, therefore, may not behave in the same manner. This drawback, however, appears to be more important when studying downstream signalling events than for receptor assembly and secretion or trafficking studies (Matsuuchi et al., 1992; Foy and Matsuuchi, 2001).

The second expression system is based on a lymphoid tissue cultured cell line derived from the J558 plasmacytoma. Normally these cells do not express the BCR on their surface since they are antibody-secreting plasma cells. We have obtained four J558 cell lines that have been transfected with, and express, various components of the BCR. The advantage of this system is that the expression studies can be done transiently as well as stably. The transient expression studies allow for much quicker analysis because screening of clones and their recovery as isolated clonal cultures is not required. This allows for more efficient experimentation and analysis.

In order to test the importance of specific protein interactions between the transmembrane and extracellular domains of Ig-α/β in BCR assembly and cell surface trafficking, I created seven mutant Ig-α/β constructs (Xα2, Cβ, MPβ, ΔXβ, ΔXα, MPα and Xβ) (Table 1.1) using either polymerase chain reaction (PCR) or by ligating digested Ig-α DNA fragments into a plasmid already containing Ig-β DNA. Additionally, I altered one construct to make it expressible in our cell lines (ΔαKVK), and I used one construct that was used in a previous study (Cα) (Wang *et al.*, 2004) (Table 1.1). Throughout this thesis the constructs will be named and then followed by a description in parentheses using the format: (extracellular domain/ transmembrane domain/ cytoplasmic domain). For example, the Xα2 construct has an Ig-α extracellular domain and Ig-β transmembrane and cytoplamic domains and will be described as: Xα (α/β/β).

To verify that a heterodimer in the cytoplasmic domain is not sufficient to allow BCR cell surface expression I used the Cα (β/β/α) construct (Figure 1.4). Cα is extracellularly and transmembrane Ig-β and cytoplasmically Ig-α and, when associated with Ig-β (β/β/β), there will only be a heterodimer in the cytoplasmic domain.

To determine whether having an Ig-α/β heterodimer in the extracellular domain was important I created the Ig-α mutant Xα2 (α/β/β) (Figure 1.4). Xα2 is extracellularly Ig-α and

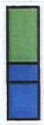
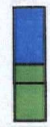
transmembrane and cytoplasmically Ig-β. When associated with Ig-β (β/β/β), an Ig-α/β heterodimer will only occur in the extracellular domain.

To examine the significance of having an Ig-α/β heterodimer in the transmembrane and extracellular domains I created the Cβ (α/α/β) and ΔαKVK (α/α/0) constructs (Figure 1.4). Cβ is extracellularly and transmembrane Ig-α and cytoplasmically Ig-β. ΔαKVK is an Ig-α mutant that is truncated cytoplasmically. When associated with Ig-β (β/β/β), both Cβ and ΔαKVK will form a heterodimer in the extracellular and transmembrane domains.

To test the importance of having an Ig-α/β heterodimer in the transmembrane region I created an Ig-α mutant construct, ΔXα (o/α/α), that is truncated in the extracellular domain (Figure 1.4). When Ig-β (β/β/β) is associated with ΔXα there will be an Ig-α/β heterodimer in the transmembrane and cytoplasmic domains.

My data showed that the Cβ (α/α/β) construct is able to associate with both the μ H chain and with Ig-β (β/β/β) and is able to traffic to the cell surface with the rest of the BCR. The ΔαKVK (α/α/0) construct is able to associate with the μ H chain, but not Ig-β and, therefore, does not traffic to the cell surface. Similarly, the ΔXα (0/α/α) construct is able to associate with the μ H chain, but not with Ig-β and, also, does not traffic to the cell surface. The rest of the constructs do not associate with any BCR components so BCR cell surface trafficking does not occur. A summary of these findings is shown in Table 1.2. This data indicates that a heterodimer in the extracellular and transmembrane domains and, potentially, dimerization in the cytoplasmic domain, is required for BCR assembly and cell surface expression.

Table 1.1: Summary of mutant constructs. The green colouration represents Ig-α and the blue colouration represents Ig-β. EX: the extracellular domain, TM: the transmembrane domain, CY: the cytoplasmic domain, MP: membrane proximal region of the extracellular domain.

Construct name	Construct diagram*	Construct description*	Approximate molecular weight
Ig-α (α/α/α)		WT Ig-α	34 kDa
Ig-β (β/β/β)		WT Ig-β	40 kDa
Xα2 (α/β/β)		EX: Ig-α TM: Ig-β CY: Ig-β	27 kDa
Cβ (α/α/β)		EX: Ig-α TM: Ig-α CY: Ig-β	27 kDa
MPβ (MPβ/β/β)		EX: Ig-β MP region TM: Ig-β CY: Ig-β	14 kDa
ΔXβ (0/β/β)		EX: none TM: Ig-β CY: Ig-β	13 kDa
MPα (MPα/α/α)		EX: Ig-α MP region TM: Ig-α CY: Ig-α	16 kDa
ΔXα (0/α/α)		EX: none TM: Ig-α CY: Ig-α	13 kDa
Xβ (β/α/α)		EX: Ig-β TM: Ig-α CY: Ig-α	39 kDa
ΔαKVK (α/α/0)		EX: Ig-α TM: Ig-α CY: none	20 kDa
Cα (β/β/α)		EX: Ig-β TM: Ig-β CY: Ig-α	39 kDa

* structure of proposed protein following removal of the signal peptide

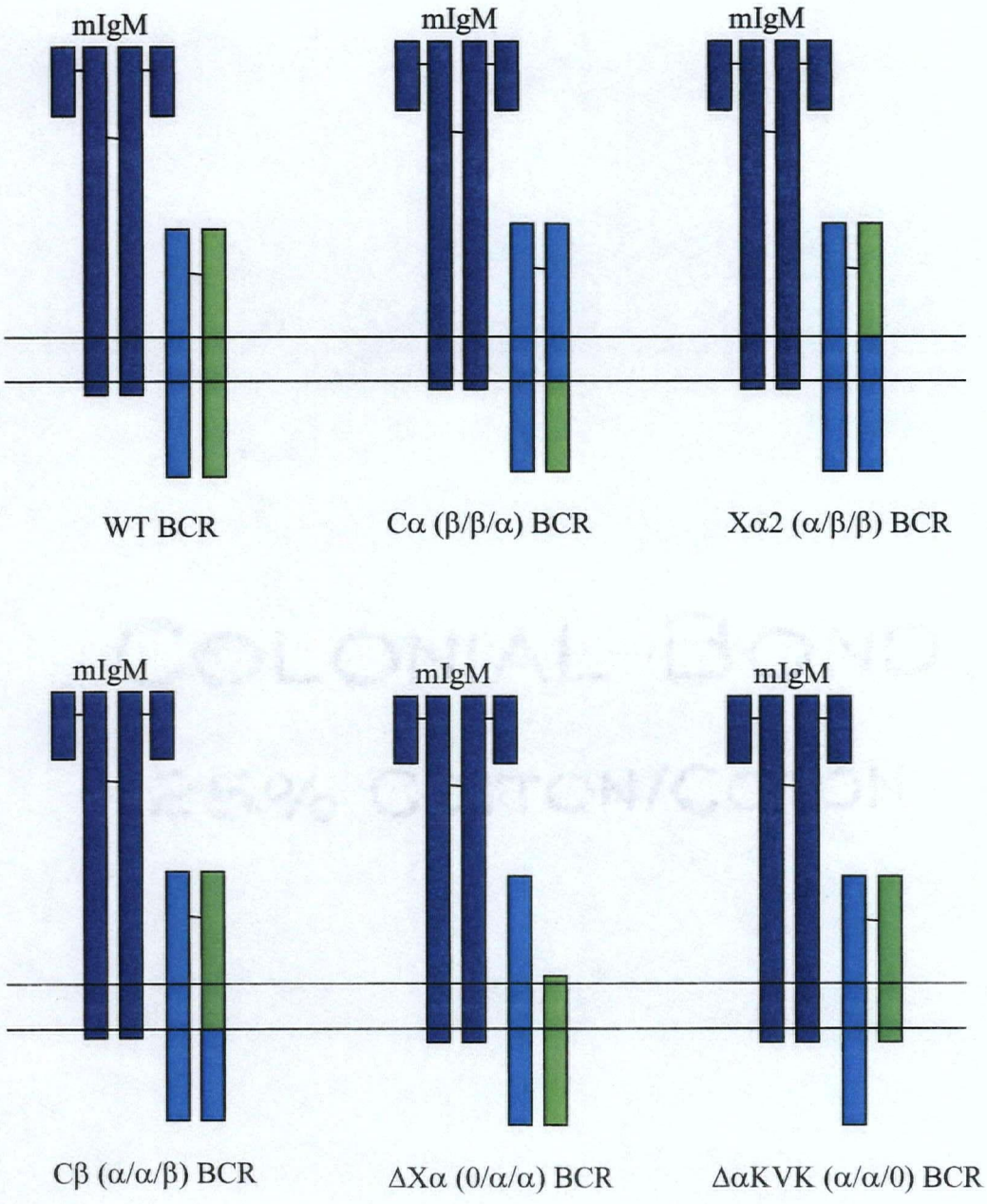


Figure 1.4: Depiction of mutant BCRs on the cell surface to display the interactions that *could* occur between mutant Ig-α constructs and the rest of the BCR. These interactions were tested by co-immunoprecipitation studies (green colouration represents Ig-α, light blue colouration represents Ig-β, dark blue colouration represents mIgM).

Table 1.2: Summary of results from Ig-α mutant constructs*. The green colouration represents Ig-α and the blue colouration represents Ig-β. EX: the extracellular domain, TM: the transmembrane domain, CY: the cytoplasmic domain, MP: membrane proximal region of the extracellular domain.

Construct name	Construct diagram	Construct description	Association with mIgM	Association with Ig-β	BCR cell surface expression
Ig-α (α/α/α)		WT Ig-α	yes	yes	yes
Cα (β/β/α)		EX: Ig-β TM: Ig-β CY: Ig-α	no	no	no
Xα2 (α/β/β)		EX: Ig-α TM: Ig-β CY: Ig-β	no	no	no
Cβ (α/α/β)		EX: Ig-α TM: Ig-α CY: Ig-β	yes	yes	yes
ΔαKVK (α/α/0)		EX: Ig-α TM: Ig-α CY: none	yes	no	no
ΔXα (0/α/α)		EX: none TM: Ig-α CY: Ig-α	yes	no	no

* the constructs not included in this table (MPα, MPβ, ΔXβ and Xβ) will be used to study BCR formation and cell surface expression in the future.

CHAPTER 2

Materials and Methods

2.1 Reagents

2.1.1 Antibodies

The rabbit anti-mouse IgM (μ chain specific) antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pennsylvania). The rabbit polyclonal anti-mouse λ light chain antibody was from Bethyl Laboratories (Montgomery, Texas). The polyclonal rabbit anti-mouse Ig-α cytoplasmic antibody, produced against a 34 amino acid peptide from the Ig-α cytoplasmic tail (amino acids 187-220), was previously described (Gold, *et al.*, 1991). The polyclonal rabbit anti-mouse Ig-α extracellular antibodies, produced with a 30 amino acid peptide to the extracellular domain of Ig-α (amino acids 29-58) and the polyclonal rabbit anti-mouse Ig-β extracellular antibody, produced with a 30 amino acid peptide to the extracellular domain of Ig-β (amino acids 71-100) were a gift from Abeome (Athens, Georgia) and Dr. Richard Meagher (University of Georgia, Athens, Georgia). The rabbit anti-mouse Ig-β cytoplasmic antibody that recognizes the cytoplasmic tail of Ig-β was a gift from Dr. Marcus Clark (University of Chicago, Chicago, Illinois).

The horseradish peroxidase (HRP)-conjugated protein A secondary reagent used for Western immunoblotting was from Amersham Biosciences (Baie d'Urfe, Quebec). The goat anti-rabbit IgG-HRP was from Jackson Immunoresearch Laboratories, Inc. (West Grove, Pennsylvania).

The fluorescein (FITC)-conjugated goat anti-mouse IgM (μ chain specific) antibody used for fluorescence activated cell sorting (FACS) was purchased from Jackson ImmunoResearch Laboratories, Inc. The rhodamine labeled goat anti-mouse IgM (μ chain specific) used for cell surface fluorescence was from BD Biosciences (Palo Alto, California).

2.1.2 Plasmids

The *pMX-puro* retroviral expression vector was a gift from Dr. Alice Mui (Jack Bell Research Centre, Vancouver, BC). The *pWZL-Blast1* and *pWZL-Blast3* retroviral expression vectors were a gift from Dr. Stephen Robbins (University of Calgary, Calgary, Alberta). The *pMSCV-puro* retroviral expression vector was purchased from BD Biosciences. The *pMIGR1-Xα*, *pMIGR1-Igβ*, *pMIGR1-ΔCY* and *MIGR1p-Cα* expression vectors were gifts from Dr. Marcus Clark (University of Chicago, Chicago, Illinois) (Wang *et al.*, 2004).

2.1.3 Plasmids created

***pMX-puro*-ΔαKVK (α/α/0)**

This plasmid encodes an Ig-α protein that is truncated cytoplasmically (Table 1.1). The initial construction of this plasmid was done by May Dang-Lawson, the laboratory technician and lab manager. The DNA for the extracellular and transmembrane portions of Ig-α was amplified using polymerase chain reaction (PCR), and the fragment was inserted into the *pMX-puro* vector between the upstream Apa I and downstream Not I restriction enzyme sites, creating *pMX-puro-Δα*. To create *pMX-puro*-ΔαKVK the three amino acids in the cytoplasmic tail of Δα were then changed from Arg Lys Arg to Lys Val Lys (KVK, single digit amino acid code) using site-directed mutagenesis (Figure 2.1). The Lys Val Lys amino acids are the same three amino acids found in the cytoplasmic tail of the heavy chain of mIgM. They were used to ensure that the construct was anchored in the plasma membrane. The primers used for site-directed mutagenesis were mb1-1 and mb1-2 (the sequences of which can be found in Table 2.1). Details of the molecular biology techniques will be discussed in section 2.2.

***pMSCV-puro*-Cβ and *pWZL-Blast3*-Cβ (α/α/β)**

This plasmid encodes an Ig-α protein that is extracellularly and transmembrane Ig-α and cytoplasmically Ig-β (Table 1.1). Site-directed mutagenesis was used to insert a Bam HI restriction enzyme site into *pMX-puro*-Δα between the transmembrane and cytoplasmic domains. The primers used for site-directed mutagenesis were JDXA1 and JDXA2 (the sequences of which can be found in Table 2.1). The Ig-α fragment from *pMX-puro*-Δα was then excised using Xho I and Bam HI restriction enzymes and inserted into *pMIGRI*-Xα to create *pMIGRI-*

Cβ. The DNA encoding Cβ was then excised from pMIGR1 using Xho I and Eco RI restriction enzymes and ligated into *pWZL-Blast3* and *pMSCV-puro* to create *pWZL-Blast3*-Cβ and *pMSCV-puro*-Cβ (Figure 2.2).

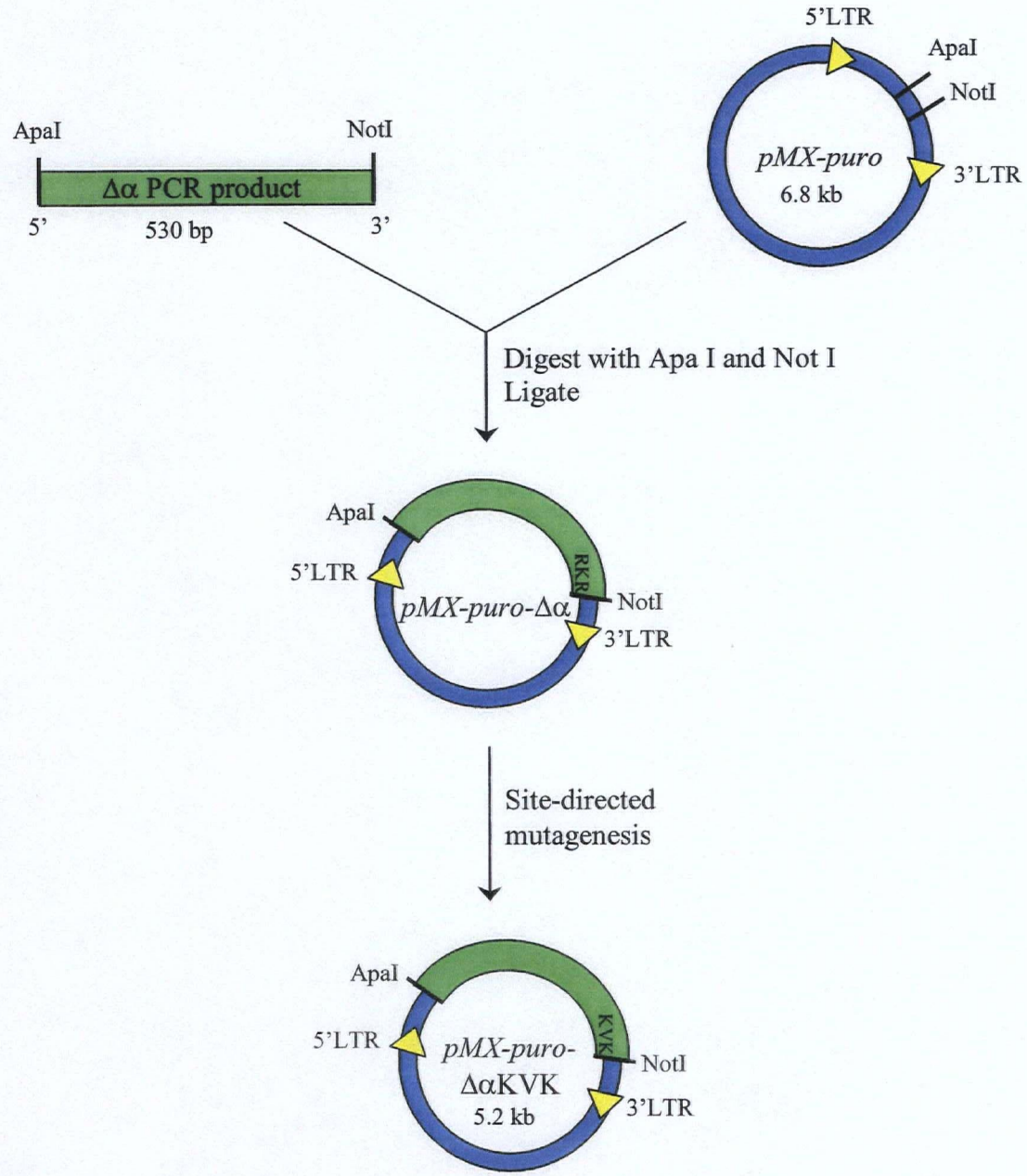


Figure 2.1 Creation of the pMX-puro-ΔαKVK expression vector. This expression vector encodes an Ig-α protein lacking its cytoplasmic tail. The LTR sequences and the direction of transcription are represented by the yellow arrows. See section 2.1.3 for more details.

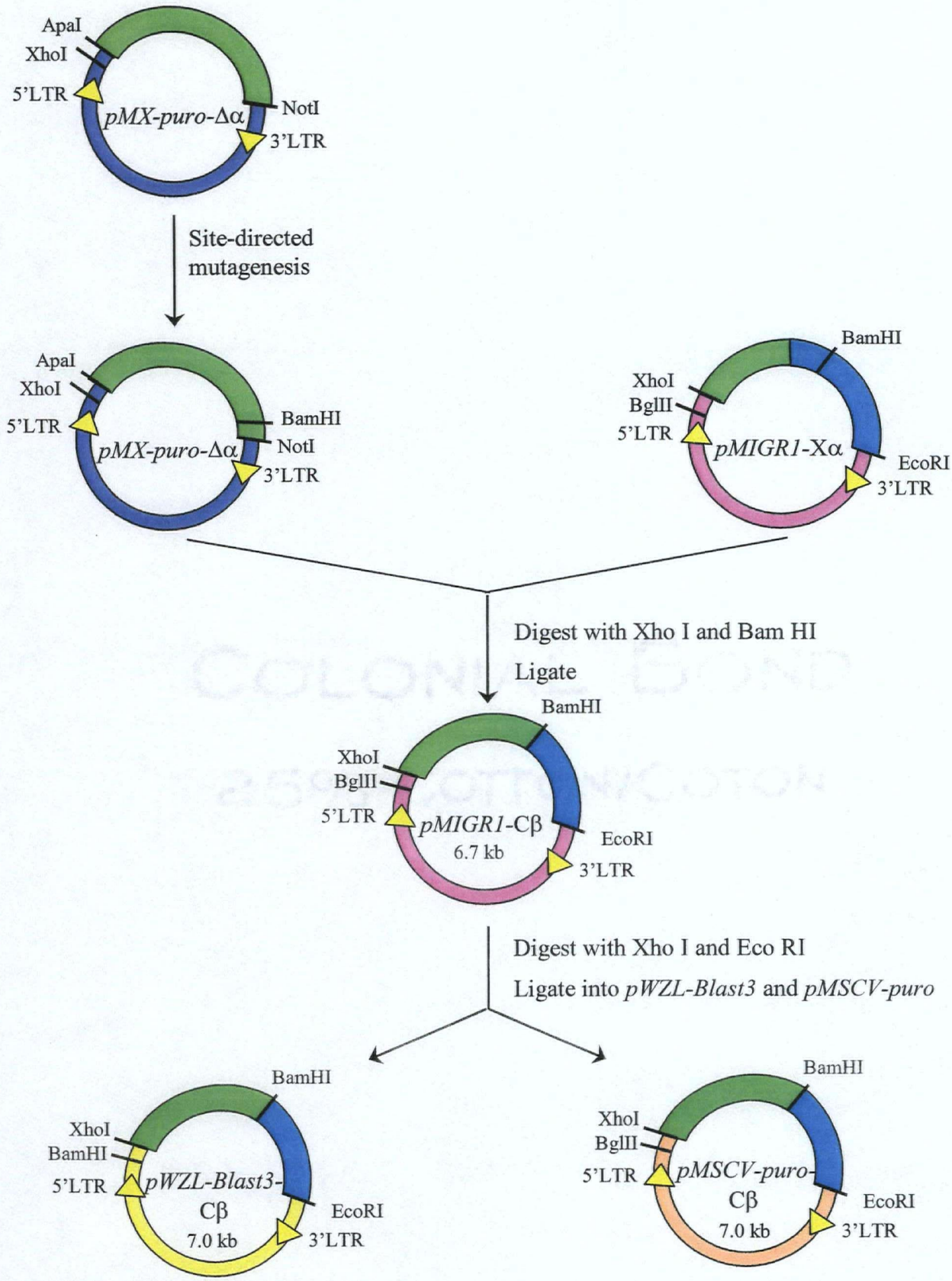


Figure 2.2 Creation of the *pMSCV-puro*-Cβ and *pWZL-Blast3*-Cβ expression vectors. These vectors encode a protein that is extracellularly and transmembrane Ig-α and cytoplasmically Ig-β. The green colour represents Ig-α DNA and the blue colour represent Ig-β DNA. The LTR sequences and the direction of transcription are represented by the yellow arrows. See section 2.1.3 for more details.

Table 2.1: **Oligonucleotide primers used for site-directed mutagenesis reactions.** The highlighted nucleotides are the ones that were changed from the wild-type sequences. Underlined nucleotides indicate restriction enzyme cut sites.

Primer Name	Primer Sequence (5'-3')	Primer binding site
mb1-1	GGG ACG CTG CTG CTA TTC AAG GTA AAG TAA TTG CGG CCG CC	The noncoding strand of Ig-α between the transmembrane and cytoplasmic domains
mb1-2	GG CGG CCG CAA TAA TTA CTT TAC CTT GAA TAG CAG CAG CGT CCC	The coding strand of Ig-α between the transmembrane and cytoplasmic domains
JDXA1	G CTG CTA TTC AGG ATC CGG TAA TTG CGG CCG	The noncoding strand of Ig-α between the transmembrane and cytoplasmic domains, creating a Bam HI restriction site
JDXA2	CGG CCG CAA TAA CCG GAT CCT GAA TAG CAG C	The coding strand of Ig-α between the transmembrane and cytoplasmic domains, creating a Bam HI restriction site
SacB1	CGG CGG AAC ACA CTG AAC CGC GGC ATT ATC TTG ATC CAG ACC	The noncoding strand of Ig-β between the extracellular and transmembrane domains, creating a Sac II restriction site
SacB2	GGT CTG GAT CAA GAT AAT GCC GCG GTT CAG TGT GTT CCG CCG	The coding strand of Ig-β between the extracellular and transmembrane domains, creating a Sac II restriction site
SacA1	GGG GAA GGT ACC AAG AAC CGC GGC ATC ACA GCA GAA GGG	The noncoding strand of Ig-α between the extracellular and transmembrane domains, creating a Sac II restriction site
SacA2	CCC TTC TGC TGT GAT GCC GCG GTT CTT GGT ACC TTC CCC	The coding strand of Ig-α between the extracellular and transmembrane domains, creating a Sac II restriction site
fixXB1	CGG CGG AAC ACA CTG AAA GAT ATC ATC ACA GCA GAA GGG	The noncoding strand of Xβ between the extracellular and transmembrane domains, erasing a Sac II restriction site.
fixXB2	CCC TTC TGC TCT GAT GAT ATC TTT CAG TGT GTT CCG CCG	The coding strand of Xβ between the extracellular and transmembrane domains, erasing a Sac II restriction site.

***pMSCV-puro*-Xα2 and *pWZL-Blast3*-Xα2 (α/β/β)**

These plasmids encode a protein that is extracellularly Ig-α and transmembrane and cytoplasmically Ig-β (Table 1.1). Site-directed mutagenesis was used to insert a Sac II restriction enzyme site into *pMIGR1*-Igβ and *pMX-puro*-Δα between the extracellular and transmembrane domains. The primers used for *pMIGR1*-Igβ were SacB1 and SacB2, and the primers used for *pMX-puro*-Δα were SacA1 and SacA2 (the sequence of these primers can be found in Table 2.1). The Ig-α extracellular domain DNA fragment was then excised from *pMX-puro-Δα* using the Xho I and Sac II restriction enzyme sites and inserted into *pMIGR1*-Igβ, replacing the Ig-β extracellular domain and creating *pMIGR1*-Xα2. The Xα2 DNA fragment was then excised from *pMIGR1*-Xα2 using Xho I and Eco RI restriction enzymes and ligated into *pWZL-Blast3* and *pMSCV-puro* to create *pWZL-Blast3*-Xα2 and *pMSCV-puro*-Xα2 (Figure 2.3).

***pWZL-Blast1*-Cα (β/β/α)**

This plasmid encodes a protein that is extracellularly and transmembrane Ig-β and cytoplasmically Ig-α (Table 1.1). The DNA encoding Cα was excised from *MIGR1p*-Cα with Bgl II and Xho I restriction enzymes. It was then ligated into *pWZL-Blast1* that had been digested with Bgl II and Sal I (Xho I and Sal I have compatible cohesive ends). This created *pWZL-Blast1*-Cα (Figure 2.4).

***pMIGR1*-Xβ (β/α/α)**

This plasmid encodes a protein that is extracellularly Ig-β and transmembrane and cytoplasmically Ig-α (Table 1.1). Site-directed mutagenesis was used to insert a Sac II restriction enzyme site into *pMIGR1*-Igβ and *pMX-puro*-Δα between the extracellular and transmembrane domains. The primers used for *pMIGR1*-Igβ were SacB1 and SacB2, and the primers used for *pMX-puro*-Δα were SacA1 and SacA2 (the sequence of these primers can be found in Table 2.1). Polymerase chain reaction (PCR) was used to amplify a segment of DNA encoding the Ig-α transmembrane and cytoplasmic domains. The primers used for PCR were SacA1 and α3Eco. The plasmid and the PCR product were then digested with Eco RI and Sac II restriction enzymes and ligated together to form *pMIGR1*-Xβ (Figure 2.5).

***pMSCV-puro*-MPα and *pWZL-Blast3*-MPα (MPα/α/α)**

These plasmids encode an Ig-α protein that is truncated in the NT and IG regions of the extracellular domain (Table 1.1). PCR was used to amplify the Ig-α DNA fragment encoding the membrane proximal (MP) region (amino acids 121 to 137), the transmembrane domain and the cytoplasmic domain. The DNA fragment had a Xho I restriction enzyme site upstream and an Eco RI restriction enzyme site downstream. The primers used were MPAXho and α3Eco (the sequence of these primers can be found in Table 2.2). This fragment was digested and ligated into *pWZL-Blast3* using the Xho I and Eco RI restriction enzyme sites. PCR was then used to amplify the DNA encoding the signal peptide of Ig-α (amino acids 1 to 22) with a Bam HI restriction enzyme sequence upstream and a Xho I restriction enzyme sequence downstream. The primers used for PCR were SRSα1 and SRSα2 (the sequence of these primers can be found in Table 2.2). The DNA fragment was digested with Bam HI and Xho I restriction enzymes and inserted into *pWZL-Blast3* that already contained the MPα insertion described above; this created *pWZL-Blast3*-MPα. To create *pMSCV-puro*-MPα the DNA encoding MPα was excised with Bam HI and Eco RI restriction enzymes and ligated into *pMSCV-puro* that had been cleaved at the Bgl II and Eco RI restriction enzyme sites (Bam HI and Bgl II have compatible cohesive ends) (Figure 2.6).

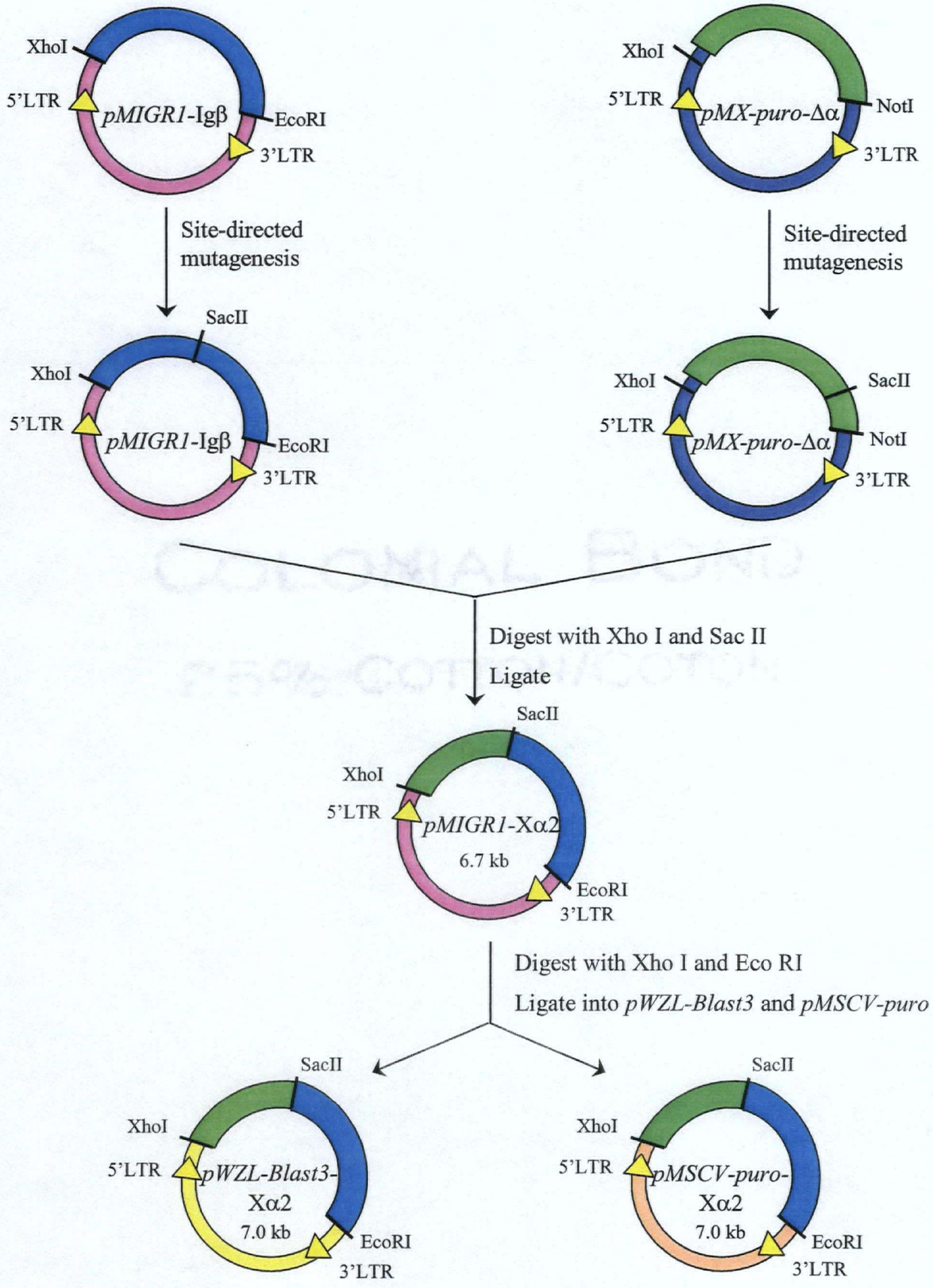


Figure 2.3 Creation of the *pMSCV-puro*-Xα2 and *pWZL-Blast3*-Xα2 expression vectors. These plasmids encode a protein that is extracellularly Ig-α and transmembrane and cytoplasmically Ig-β. The green colour represents Ig-α DNA and the blue colour represents Ig-β DNA. The LTR sequences and the direction of transcription are represented by the yellow arrows. See section 2.1.3 for more details.

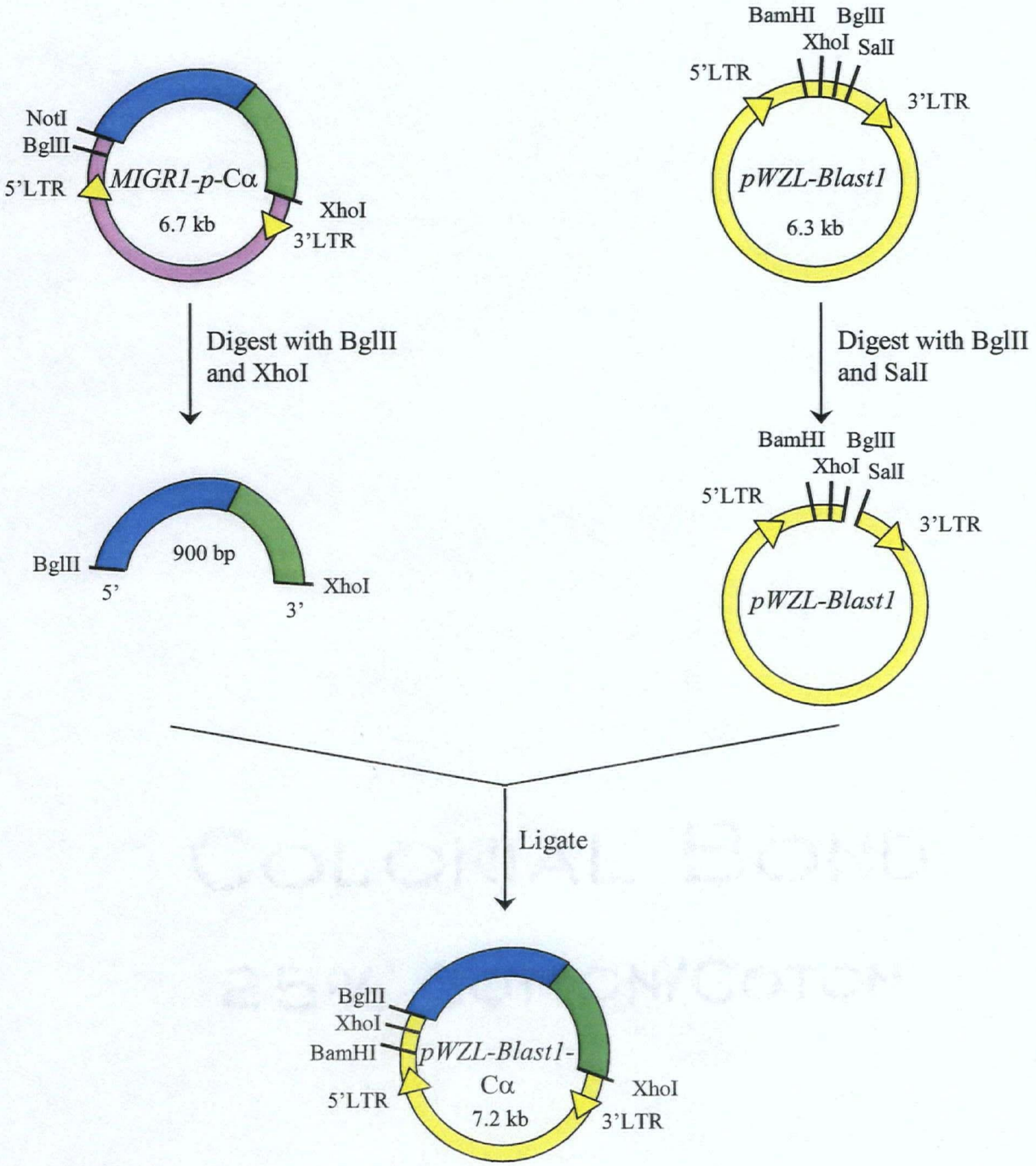


Figure 2.4 Creation of the *pWZL-Blast1*-Cα expression vector. This vector encodes a protein that is extracellularly and transmembrane Ig-β and cytoplasmically Ig-α. The green colour represents Ig-α DNA and the blue colour represents Ig-β DNA. The LTR sequences and the direction of transcription are represented by the yellow arrows. See section 2.1.3 for more details.

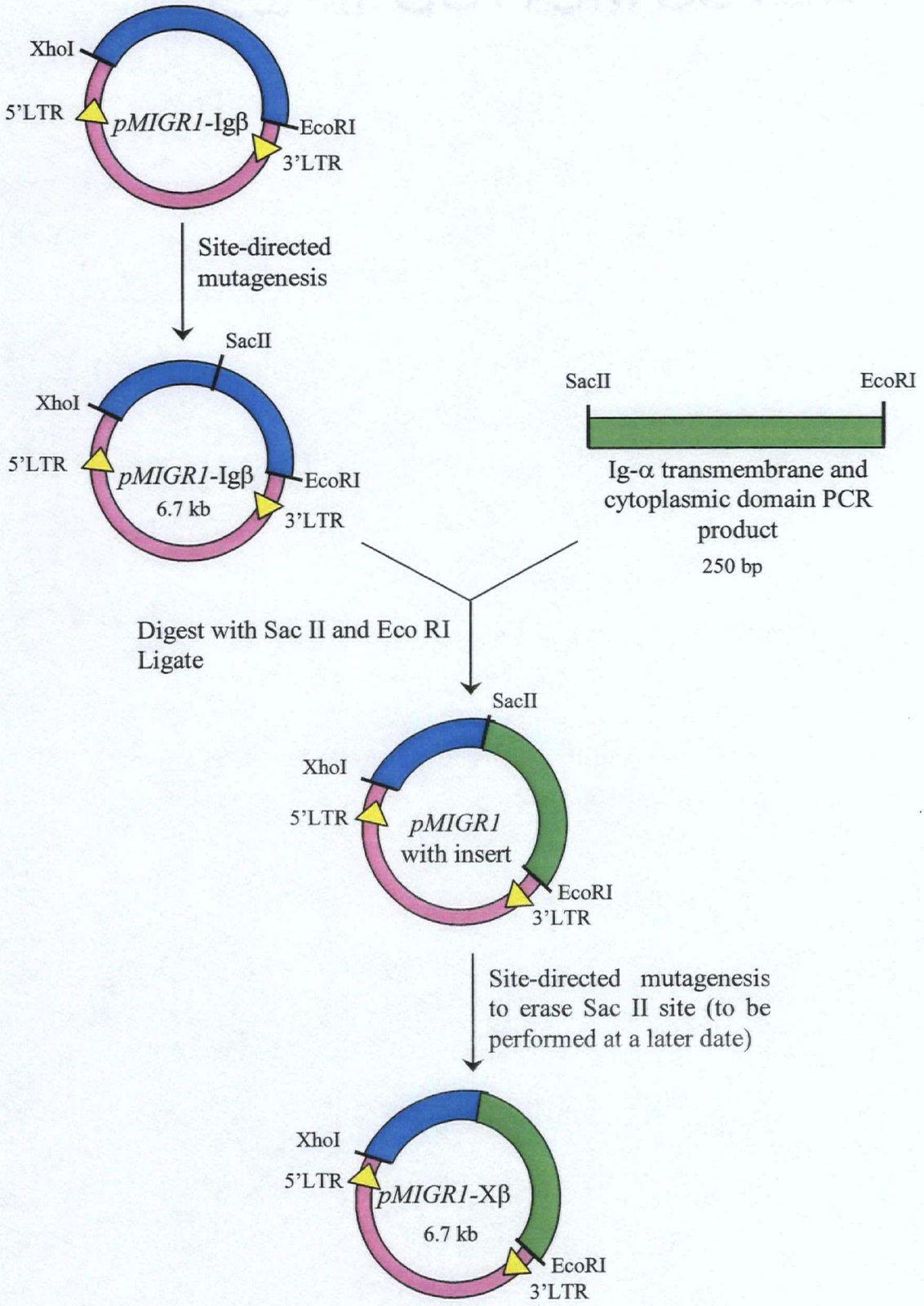


Figure 2.5 Creation of the *pMIGR1*-Xβ expression vector. This plasmid encodes a protein that is extracellularly Ig-β and transmembrane and cytoplasmically Ig-α. The green colour represents Ig-α DNA and the blue colour represents Ig-β DNA. The LTR sequences and the direction of transcription are represented by the yellow arrows. See section 2.1.3 for more details.

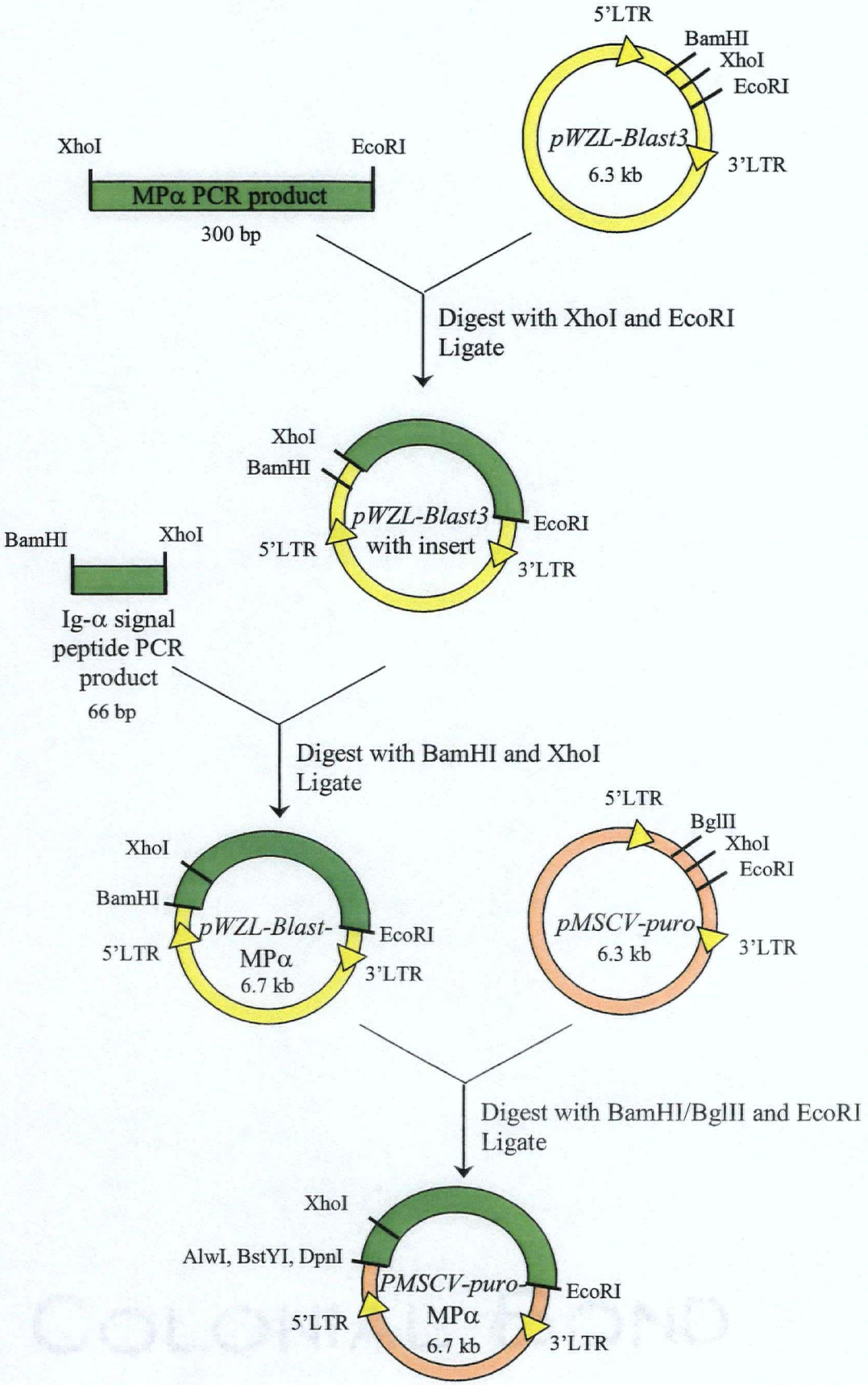


Figure 2.6 Creation of the *pMSCV-puro*-MPα and *pWZL-Blast3*-MPα expression vectors. These vectors encode an Ig-α protein that is truncated in the NT and IG regions of the extracellular domain. The LTR sequences and the direction of transcription are represented by the yellow arrows. See section 2.1.3 for more details.

***pMSCV-puro*-MPβ and *pWZL-Blast3*-MPβ (MPβ/β/β)**

These plasmids encode an Ig-β protein that is truncated in the NT and IG regions of the extracellular domain (Table 1.1). PCR was used to amplify the Ig-β DNA fragment encoding the membrane proximal (MP) region (amino acids 143 to 180), the transmembrane domain and the cytoplasmic domain. The DNA fragment had a Xho I restriction enzyme site upstream and an Eco RI restriction enzyme site downstream. The primers used were MPBXho and β3Eco (the sequence of these primers can be found in Table 2.2). This fragment was digested and ligated into *pWZL-Blast3* using the Xho I and Eco RI restriction enzyme sites. PCR was then used to amplify the DNA encoding the signal peptide of Ig-β (amino acids 1 to 25) with a Bam HI restriction enzyme sequence upstream and a Xho I restriction enzyme sequence downstream. The primers used for PCR were SRSβ1 and SRSβ2 (the sequence of these primers can be found in Table 2.2). The DNA fragment was digested with Bam HI and Xho I restriction enzymes and inserted into *pWZL-Blast3* that already contained the MPβ insertion described above; this created *pWZL-Blast3*-MPβ. To create *pMSCV-puro*-MPβ the DNA encoding MPβ was excised with Bam HI and Eco RI restriction enzymes and ligated into *pMSCV-puro* that had been cleaved at the Bgl II and Eco RI restriction enzyme sites (Bam HI and Bgl II have compatible cohesive ends) (Figure 2.7).

***pMSCV-puro*-ΔXα and *pWZL-Blast3*-ΔXα (0/α/α)**

These plasmids encode an Ig-α protein that is truncated in the extracellular domain (Table 1.1). PCR was used to amplify the Ig-α DNA fragment encoding the transmembrane domain and cytoplasmic domains. The DNA fragment had a Xho I restriction enzyme site upstream followed by nucleotides encoding methionine and lysine (as an extracellular domain to anchor the protein in the membrane (Johansson *et al.*, 1999)) and an Eco RI restriction enzyme site downstream. The primers used were ΔXαXho and α3Eco (the sequence of these primers can be found in Table 2.2). This fragment was digested and ligated into *pWZL-Blast3* using the Xho I and Eco RI restriction enzyme sites. PCR was then used to amplify the DNA encoding the signal peptide of Ig-α (amino acids 1 to 22) with a Bam HI restriction enzyme sequence upstream and a Xho I restriction enzyme sequence downstream. The primers used for PCR were SRSα1 and SRSα2 (the sequence of these primers can be found in Table 2.2). The DNA fragment was digested with Bam HI and Xho I restriction enzymes and inserted into *pWZL-Blast3* that already contained the ΔXα insertion described above; this created *pWZL-Blast3*-ΔXα. To create

pMSCV-puro-ΔXα the DNA encoding ΔXα was excised with Bam HI and Eco RI restriction enzymes and ligated into *pMSCV-puro* that had been cleaved at the Bgl II and Eco RI restriction enzyme sites (Bam HI and Bgl II have compatible cohesive ends) (Figure 2.8).

***pMSCV-puro*-ΔXβ and *pWZL-Blast3*-ΔXβ (0/β/β)**

These plasmids encode an Ig-β protein that is truncated in the extracellular domain (Table 1.1). PCR was used to amplify the Ig-β DNA fragment encoding the transmembrane and cytoplasmic domains. The DNA fragment had a Xho I restriction enzyme site upstream followed by nucleotides encoding methionine and lysine (as an extracellular domain to anchor the protein in the membrane (Johansson *et al.*, 1999)) and an Eco RI restriction enzyme site downstream. The primers used were ΔXβXho and β3Eco (the sequence of these primers can be found in Table 2.2). This fragment was digested and ligated into *pWZL-Blast3* using the Xho I and Eco RI restriction enzyme sites. PCR was then used to amplify the DNA encoding the signal peptide of Ig-β (amino acids 1 to 25) with a Bam HI restriction enzyme sequence upstream and a Xho I restriction enzyme sequence downstream. The primers used for PCR were SRSβ1 and SRSβ2 (the sequence of these primers can be found in Table 2.2). The DNA fragment was digested with Bam HI and Xho I restriction enzymes and inserted into *pWZL-Blast3* that already contained the ΔXβ insertion described above; this created *pWZL-Blast3*-ΔXβ. To create *pMSCV-puro*-ΔXβ the DNA encoding ΔXβ was excised with Bam HI and Eco RI restriction enzymes and ligated into *pMSCV-puro* that had been cleaved at the Bgl II and Eco RI restriction enzyme sites (Bam HI and Bgl II have compatible cohesive ends) (Figure 2.9).

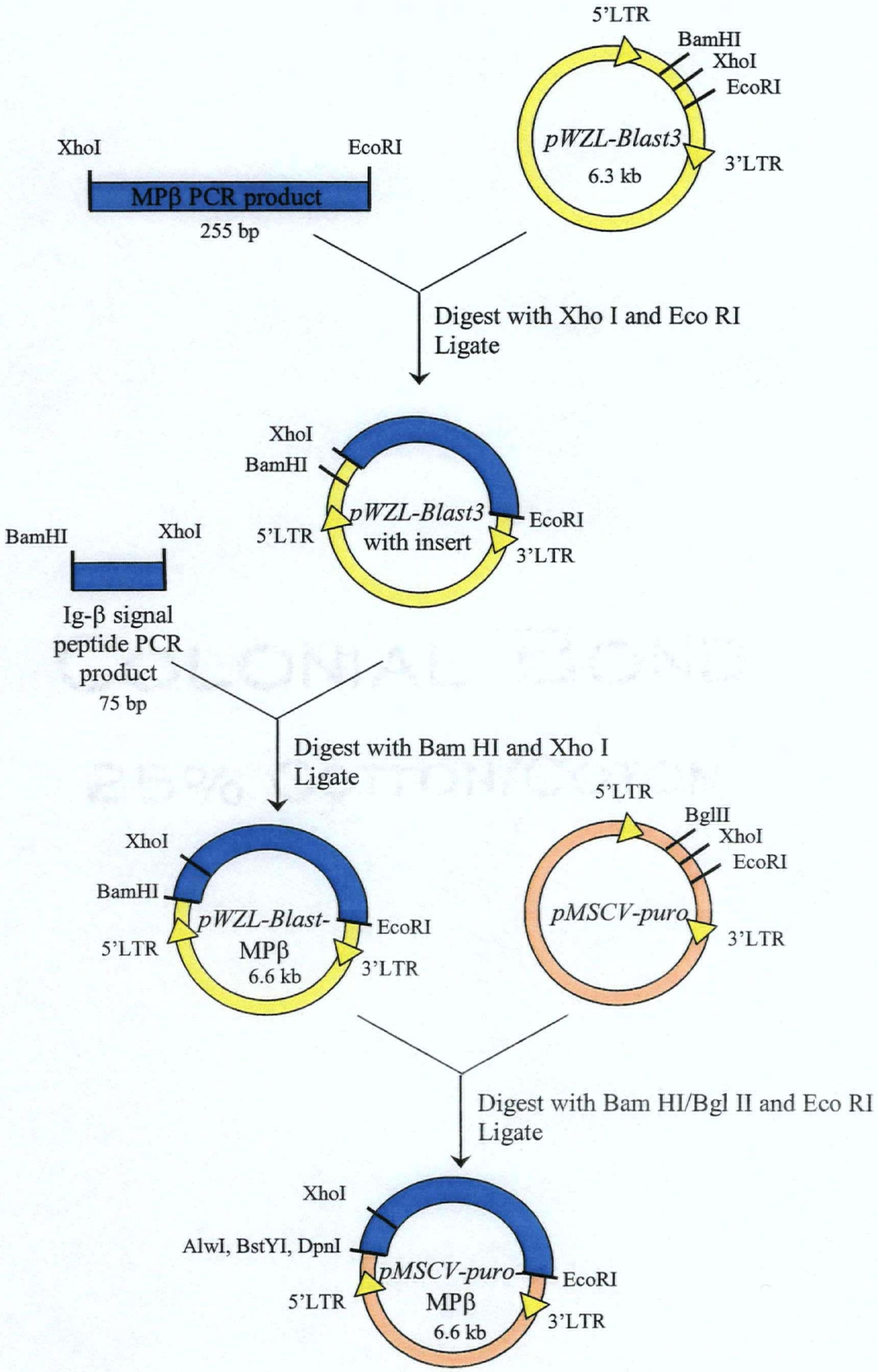


Figure 2.7 Creation of the *pMSCV-puro*-MPβ and *pWZL-Blast3*-MPβ expression vectors. These vectors encode an Ig-β protein that is truncated in the NT and IG regions of the extracellular domain. The LTR sequences and the direction of transcription are represented by the yellow arrows. See section 2.1.3 for more details.

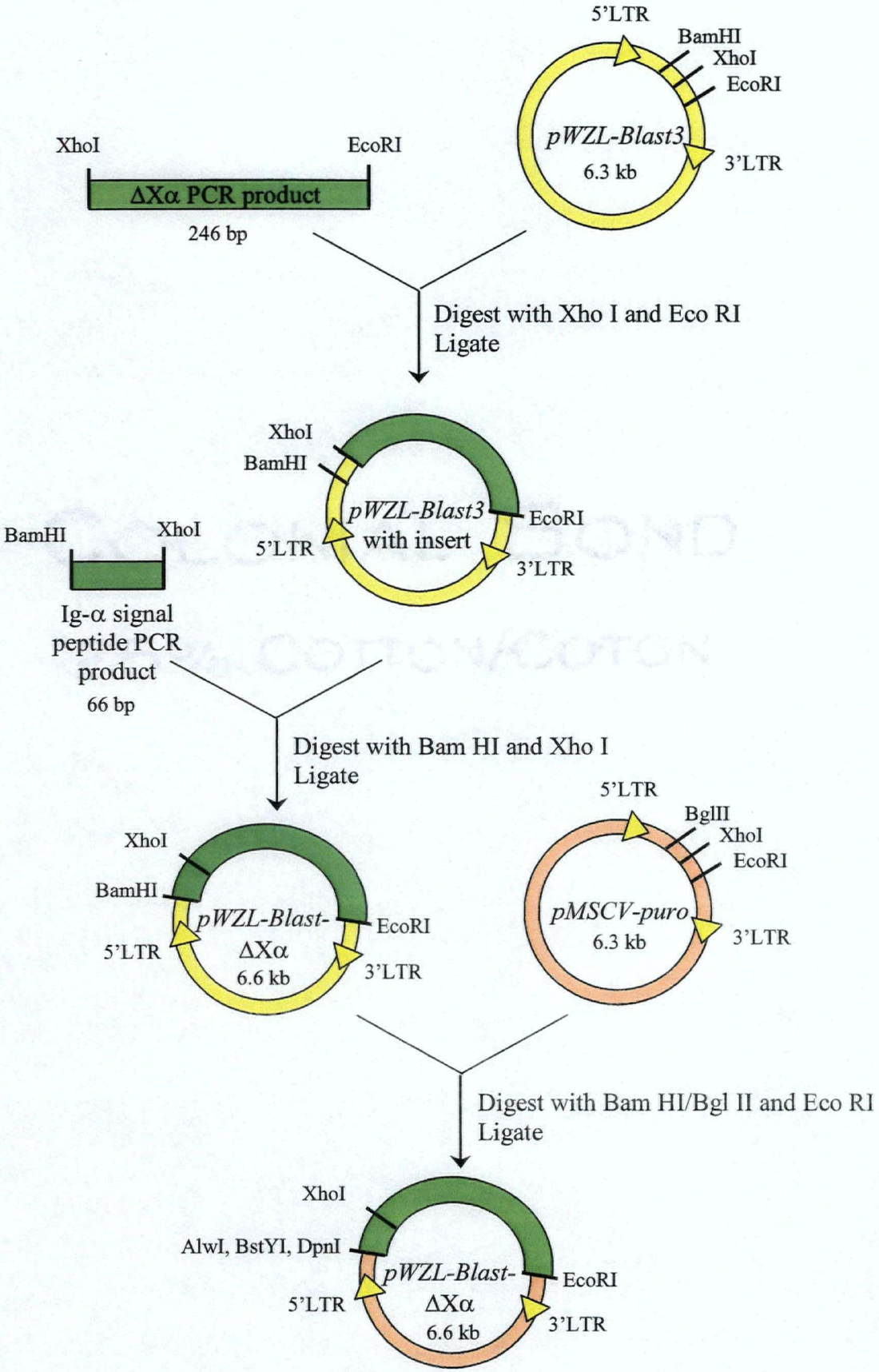


Figure 2.8 Creation of the *pMSCV-puro*-ΔXα and *pWZL-Blast3*-ΔXα expression vectors. These vectors encode an Ig-α protein that is truncated in the extracellular domain. The LTR sequences and the direction of transcription are represented by the yellow arrows. See section 2.1.3 for more details.

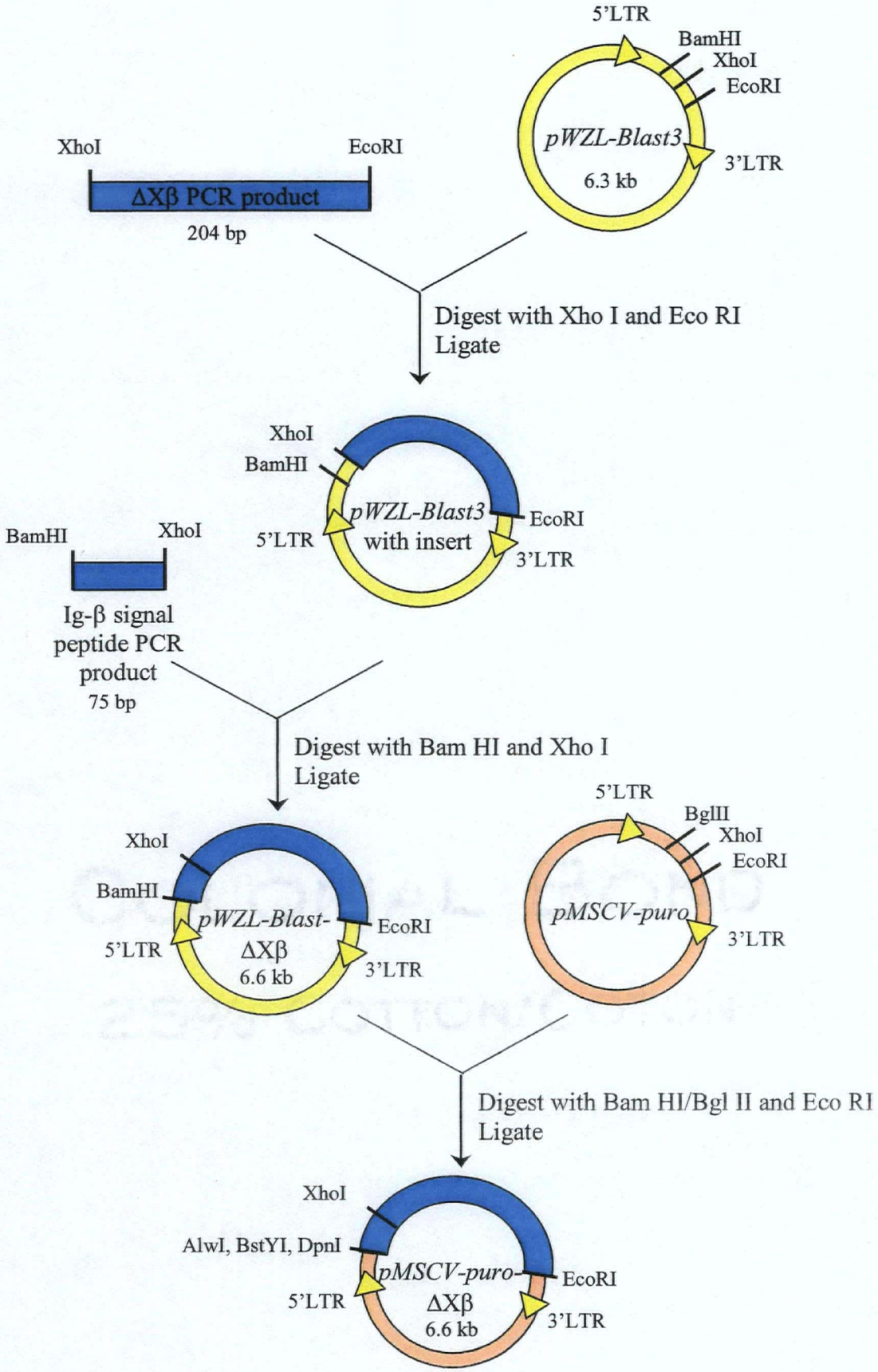


Figure 2.9 Creation of the *pMSCV-puro*-ΔXβ and *pWZL-Blast3*-ΔXβ expression vectors. These vectors encode an Ig-β protein that is truncated in the extracellular domain. The LTR sequences and the direction of transcription are represented by the yellow arrows. See section 2.1.3 for more details.

Table 2.2: **Oligonucleotide primers used for polymerase chain reaction (PCR).** Underlined nucleotides indicate restriction enzyme recognition sites.

Primer Name	Primer Sequence (5'-3')	Primer binding site
MPAXho	CCG CTC GAG CGG ATG AAT CCA GTC CCT AGG CCC TTC	The noncoding strand of Ig-α, amino acid 121, with a Xho I restriction site added
α3Eco	CCG GAA TTC CGG TCA TGG CTT TTC CAG CTG GGC	The coding strand of Ig-α at the stop site with an Eco RI restriction site added
SRSα1	CGC GGA TCC GCG ATG CCA GGG GGT CTA G	The noncoding strand of Ig-α at start site with Bam HI restriction site added
SRSα2	CCG CTC GAG CAA ACA GGC GTA TGA CAA G	The coding strand of Ig-α, amino acid 22, with a Xho I restriction site added
MPBXho	CCG CTC GAG CGG ATG GGA TTC AGC ACG TTG GAC CAA	The noncoding strand of Ig-β, amino acid 143, with a Xho I restriction site added
β3Eco	CCG GAA TTC CGG TCA TTC CTG CCG TGG ATG	The coding strand of Ig-β at the stop site with an Eco RI restriction site added
SRSβ1	CGC GGA TCC GCG ATG GCC ACA CTG GTG CTG	The noncoding strand of Ig-β at start site with Bam HI restriction site added
SRSβ2	CCG CTC GAG CGG CTC ACC TGA GAA GAG	The coding strand of Ig-β, amino acid 25, with a Xho I restriction site added
ΔXαXho	CCG CTC GAG ATG AAG ATC ATC ACA GCA GAA GGG	The noncoding strand of Ig-α, between the extracellular and transmembrane domains, with a Xho I restriction site added
ΔXβXho	CCG CTC GAG ATG AAG GGC ATT ATC TTG ATC CAG	The noncoding strand of Ig-β, between the extracellular and transmembrane domains, with a Xho I restriction site added

2.2 Molecular Biology Techniques

2.2.1 Restriction endonuclease digestion

Restriction enzymes (Invitrogen Life Technologies (Burlington, Ontario) or New England Biolabs (Pickering, Ontario)) were added to the DNA according to manufacturer's instructions and digested at 37°C for 2 hours. If a double digest was to be performed, a mutually compatible buffer for both enzymes was used and the enzymes were added to the DNA to a total volume of no more than 10%. The reactions were then run on an agarose gel to visualize or extract the digested fragments.

2.2.2 Agarose gel electrophoresis

Agarose gels between 0.8 and 1.0% agarose (Fisher Scientific, Ottawa, Ontario) in Tris-buffered EDTA (TBE) (90 mM Tris-HCl pH 8.2, 90 mM boric acid (Fisher Scientific), 2 mM EDTA) with 300 ng/mL of ethidium bromide (Invitrogen Life Technologies) were used to separate DNA fragments. The samples were prepared with DNA sample buffer (final concentration 0.04% bromophenol blue, 0.04% xylene cyanol FF ad 10% sucrose) and loaded onto the gel. A 1 kB or a 100 bp molecular weight marker (Invitrogen Life Technologies) was also loaded onto the gel. The gels were run at 100 V for approximately 1 hour in TBE. Ultraviolet light was used to visualize the DNA fragments.

2.2.3 Gel purification of DNA fragments

Digested DNA fragments run in an agarose gel were removed from the gel with a razor blade and purified using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, Ontario) following manufacturer's instructions.

2.2.4 Ligation of DNA fragments

DNA ligations were performed by combining approximately 1.0 μg of digested vector DNA with varying amounts of digested insert DNA (between 3 and 20 μg). T4 DNA ligase and 5X T4

DNA ligase buffer (both from Invitrogen Life Technologies) were added to each tube to make up 10% and 20% of the total volume, respectively. The mixture was incubated at room temperature for 2 hours before being used to transform competent bacteria.

2.2.5 Transformation of competent *E. coli*

Competent DH5α (Invitrogen Life Technologies) or XL1-Blue (Stratagene, La Jolla, California) *E. coli* strains (prepared by May Dang-Lawson) were transformed with 100 ng of plasmid DNA or with the ligation mixture described previously. Typically, 100 μL of bacteria was mixed with the DNA by vortexing for 10 seconds and then the mixture was incubated on ice for 10 minutes. If very few colonies were anticipated, the mixture was heat-shocked for 2 minutes at 42°C and placed back on ice for 5 minutes. It was then plated onto LB agar plates (5 g yeast extract, 10 g tryptone, 15 g agar (all from Difco Laboratories, Sparks, Maryland) and 5 g NaCl per litre) containing 100 μg/mL of ampicillin (Sigma) and incubated at 37°C overnight.

2.2.6 Small scale DNA preparation

Small volumes of plasmid DNA were purified using the GenElute Plasmid Miniprep Kit (Sigma Aldrich Canada, Oakville, Ontario). An individual bacterial colony was used to inoculate 3 mL of Luria-Bertani (LB) broth (5 g yeast extract, 10 g tryptone (Difco Laboratories) and 5 g NaCl per litre) containing 100 μg/mL of ampicillin and was incubated for 16 hours at 37°C with shaking. One and a half milliliters of the overnight culture was centrifuged and the plasmid was isolated and purified following manufacturer's instructions.

2.2.7 Large scale DNA preparation

Large volumes of plasmid DNA were isolated and purified using the GenElute HP plasmid Maxiprep Kit (Sigma Aldrich Canada). An individual bacterial colony was used to inoculate 150 mL of LB broth containing 100 μg/mL of ampicillin. The bacteria were grown for 16 hours at 37°C while shaking. The culture was then used to isolate and purify the plasmid DNA according to manufacturer's instructions.

2.2.8 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed using puReTaq Ready-To-Go PCR beads (Amersham Biosciences Canada) according to manufacturer's instructions. Twenty-five picomole of each of the two DNA primers (Nucleic Acid Protein Service Unit, UBC) were added to each tube, along with 5.0 ng of template DNA. The reactions were overlaid with 10 μL of sterilized mineral oil before being placed in a DNA Thermal Cycler (Perkin Elmer Cetus, Woodbridge, Ontario). The reactions were run at 95°C for 45 seconds, 55°C for 2 minutes and 72°C for 2 minutes for a total of 35 cycles.

2.2.9 Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All oligonucleotide primers were purchased from the Nucleic Acid Protein Service Unit (UBC) and the reactions were run in a DNA Thermal Cycler (Perkin Elmer Cetus, Woodbridge, Ontario) according to manufacturer's instructions. After transformation of the XL1-Blue Supercompetent bacteria, a single colony was used to inoculate LB broth for small scale DNA preparation. The resulting DNA was tested for the presence of the intended mutation by restriction enzyme digestion and DNA sequencing (Nucleic Acid Protein Service Unit).

2.3 Tissue Culture

2.3.1 Tissue culture cell lines

The AtT20 murine pituitary tumor cell line was from Dr. Regis Kelly (University of California, San Francisco, California) and has been previously described (Matsuuchi and Kelly, 1991). The ASS cell line is an AtT20 cell line that does not contain any components of the BCR. The R142 cell line in an AtT20 cell line that expresses mIgM and Ig-α (Matsuuchi *et al.*, 1992). The WT5 cell line is an AtT20 cell line that expresses mIgM and Ig-β (Lopes and Matsuuchi, unpublished). The Syk 13 cell line is an AtT20 cell line that expresses mIgM, Ig-α and Ig-β (Richards *et al.*, 1996). The J558 μμ3 and J558 15-25 murine plasmacytoma cell lines were

from Dr. Louis Justement (University of Alabama, Birmingham, Alabama) and the J558 Lμm and J558 L murine plasmacytoma cell lines were from Dr. Marcus Clark (University of Chicago, Chicago, Illinois). The BOSC 23 human fibroblast cell line was a gift from Dr. Warren Pear (University of Pennsylvania, Philadelphia, Pennsylvania).

2.3.2 Culture of cell lines

The AtT20 and BOSC 23 cell lines were grown on 10 cm polystyrene tissue culture dishes (Falcon, Franklin Lakes, New Jersey) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies) containing 4.5 g/L glucose, 2 mM L-glutamine and 110 mg/L sodium pyruvate. Ten percent heat inactivated fetal calf serum (FCS) (Invitrogen Life Technologies), 50 units/mL penicillin and 50 μg/mL streptomycin sulfate (Invitrogen Life Technologies) were also added to the media. The cell lines were incubated in an air-jacketed incubator (ThermoForma, Marietta, Ohio) at 37°C in a 10% CO2 environment. The cells were grown to 90% confluency before being split into new tissue culture dishes. For long-term storage the cell lines are frozen in liquid nitrogen. The freezing media used for AtT20 and BOSC 23 cells is complete DMEM with 10% dimethyl sulphoxide (DMSO) (Sigma Aldrich).

The adherent cells were removed from the tissue culture dish by aspirating off the media and replacing it with 2 mL of Trypsin-EDTA (0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA)) (Invitrogen Life Technologies). Once the cells were no longer adhered to the plate they were resuspended in 10 mL of complete DMEM by pipeting up and down with a 10 mL sterile plastic pipette (Corning, Corning, New York). Approximately 1 mL of the cell suspension was then placed in a new 10 cm tissue culture dish with 10 mL of complete DMEM and grown as described above.

The J558 plasmacytoma cell lines were grown in 10 cm polystyrene tissue culture dishes (Falcon) in Roswell Park Memorial Institute (RPMI)-1640 media (Invitrogen Life Technologies) containing 10% heat inactivated FCS (Invitrogen Life Technologies), 1mM sodium pyruvate (Invitrogen Life Technologies), 50 μM β-mercaptoethanol (Sigma Aldrich), 50 units/mL penicillin and 50 μg/mL streptomycin sulfate (Invitrogen Life Technologies). The cells were incubated in a water-jacketed incubator (Forma Scientific, Marietta, Ohio) at 37°C in a 5% CO_2

environment. The cells were split 1:10 in fresh media into new tissue culture dishes every 4-5 days. For long-term storage the cell lines are frozen in liquid nitrogen. The freezing media used for J558 plasmacytoma cells is heat inactivated FCS with 10% dimethyl sulphoxide (DMSO) (Sigma Aldrich).

2.3.3 Cell lysis

AtT20 cells were grown to confluency on a 10 cm tissue culture dish, the media was removed and the cells were washed twice with phosphate buffer saline (PBS) (1.5 mM NaCl, 1.9 mM $NaH_2PO_4 \cdot H_2O$, 8.4 mM Na_2HPO_4, pH 7.2 with 1 mM Na_3VO_4 (Sigma Aldrich)). The cells were then lysed with 0.5-1.0 mL of cold Triton X-100 lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% Triton X-100, 2 mM EDTA (Fisher Scientific Canada, Ottawa, Ontario), 10% glycerol, 10 μg/mL leupeptin (Roche Diagnostics, Indianapolis, Indiana), 1 μg/mL aproprotin (Roche Diagnostics), 1 mM pepstatin A (Sigma Aldrich), 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Roche Diagnostics)) or cold digitonin lysis buffer (1% digitonin (Sigma Aldrich), 10 mM triethanolamine pH 7.8, 150 mM NaCl, 1 mM EDTA, 10 μg/mL leupeptin, 1 μg/mL aproprotin, 1 mM pepstatin A, 1 mM PMSF). The cells were incubated on ice for 20 minutes (for Triton X-100 buffer) or 45 minutes (for digitonin buffer) and then transferred to a 1.5 mL tube (Axygen Scientific, Union City, California) to be centrifuged at 14000 rpm for 15 minutes at 4 °C. The soluble lysates were then transferred to a new tube and the protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, Illinois). The samples were frozen at -20°C for long-term storage.

J558 plasmactoma cells were centrifuged at 1500 rpm for 5 minutes at 12°C in an IEC Centra-8R tabletop centrifuge (International Equipment Company, Needham Heights, Massachusetts). The media was removed and the cells were washed twice with PBS and then lysed as described above.

2.4 Transfection of BOSC 23 Cells

The BOSC cells were split into a 6 well tissue culture dish (Falcon) or a 10 cm tissue culture dish, depending on the amount of retroviral particles or cell lysate required, so that they were

approximately 40% confluent. They were grown to 80% confluency as described in section 2.2.2. To transfect one well of a 6 well dish, 2.0 μg of DNA was added to 200 μL of $CaCl_2$ in a clear polystyrene tube (Falcon 2054). The DNA mixture was vortexed for 10 seconds while 200 μL of 2X HEPES-buffered saline (50 mM HEPES pH 7.2, 10 mM KCl, 12 mM NaCl, 1.5 mM Na_2HPO_4) was added to the tube dropwise. The media was then removed from the BOSC cells and replaced with 1 mL of complete DMEM containing 25 μM chloroquinone (Sigma Aldrich). The DNA mixture was then added to the cells and the cells were incubated at 37°C in a 5% CO_2 environment for 7-8 hours. The media was then replaced with 2 mL of complete DMEM and the cells were incubated at 37°C in a 5% CO_2 environment for 48 hours from the time of DNA addition. The cells were then lysed if they were to be used to determine if a particular protein construct was expressible. To recover the retroviral particle the media was removed and used immediately for retroviral infection or frozen at –80°C for long-term storage.

To transfect BOSC cells in a 10 cm tissue culture dish, 8 μg of DNA was added to 800 μL of 250 mM $CaCl_2$ and 800 μL of 2X HEPES-buffered saline was added dropwise while vortexing for 10 seconds. The media was then removed from the BOSC cells and replaced with 5 mL of complete DMEM containing 25 μM chloroquinone and the DNA mixture was added to the cells. After incubating the cells for 7-8 hours at 37°C in a 5% CO_2 environment the media was replaced with 6 mL of complete DMEM. The cells were incubated for 48 hours as described above and then either lysed or the virus particle was collected.

2.5 Retroviral Infection of Cells

2.5.1 Retroviral infection of AtT20 cells

AtT20 cells were grown to 80% confluency on a 10 cm tissue culture dish, the media was aspirated off and replaced with 5-6 mL of sterile filtered media containing virus particle from the transfected BOSC cells (syringe filtered with a 0.22 μm filter (Millipore, Billerica, Massachussetts)). Ten μg/mL of polybrene (hexadimethrine bromide, Sigma Aldrich) was added to the cells, and they were incubated for 6-7 hours at 37°C in a 10% CO_2 environment. The media was then aspirated off the cells and replaced with complete DMEM, and the cells were grown for 48 hours at 37°C in a 10% CO_2 environment before being placed in drug selection.

2.5.2 Retroviral infection of J558 plasmacytoma cells

Half a million J558 plasmacytoma cells were plated into one well of a 6 well tissue culture dish (Falcon). Two milliliters of sterile filtered media containing virus particles from transfected BOSC cells (syringe filtered with a 0.22 μm filter (Millipore)) was added to the cells along with 2.5 mL of polybrene (hexadimethrine bromide) (Sigma Aldrich). The cells were incubated for 6-20 hours at 37°C in a 5% CO_2 environment. They were then centrifuged at 1500 rpm for 5 minutes in an IEC Centra-8R tabletop centrifuge (International Equipment Company), and the media was replaced with complete RPMI media. The cells were grown for 48 hours at 37°C in a 5% CO_2 environment before being placed in drug selection.

2.5.3 Drug selection and clone isolation of infected cells

Forty-eight hours after infection of the AtT20 cells with a retroviral plasmid containing a puromycin resistance gene, the media was aspirated from the cells and they were removed from the tissue culture dish with 2 mL of Trypsin-EDTA (0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA)) (Invitrogen Life Technologies). The cells were resuspended in 20 mL of complete DMEM with 0.4 μg/mL puromycin (Calbiochem, La Jolla, California) and pipetted up and down to break up any cell clumps. One drop to 1 mL of cell suspension was added to a 10 cm tissue culture dish containing 20 mL of complete DMEM with puromycin. The cell suspension from these plates was pipetted into 96 well tissue culture dishes (Falcon), putting 150 μL into each well. The cells were then allowed to grow at 37°C in a 10% CO_2 environment until an individual clone could be seen in a single well. The media from wells containing one clone was aspirated and 100 μL of Trypsin-EDTA was added to each well. The cells were resuspended in 100 μL of complete DMEM with puromycin and added to 24 well tissue culture dishes (Falcon) contained complete DMEM with puromycin. The cells were then grown to confluency and transferred to 6 well tissue culture plates (Falcon) so that they could be screened for protein construct expression by Western immunoblotting.

To select for a population of J558 cells that transiently express a protein of interest, the cells were placed into complete RPMI media containing 4 μg/mL of blasticidin S (Invitrogen Life Technologies) 48 hours after infection. The cells that did not contain the blasticidin resistance

gene in the retroviral plasmid were allowed to die over 5 days and the remaining cells were considered a transient population of protein expressing cells.

To select for J558 cells that stably expressed a protein of interest, a population of identical clones from a single cell were isolated. Forty-eight hours after infection, the cells were diluted to varying concentrations (10^4, 10^3 and 10^2 cells/ mL) in selection media (RPMI media containing 4 μg/mL of blasticidin S (Invitrogen Life Technologies)). The cells from these plates were pipetted into 96 well tissue culture dishes (Falcon), putting 150 μL into each well. They grew at 37°C in a 5% CO_2 environment until cells could be seen growing in the individual wells (usually 10-14 days). If fewer than 15 wells on the plate had cells growing in them, the cells from those wells were removed and plated into a 24 well plate (Falcon) in selection media. They were then screened for the expression of the protein of interest by Western immunoblotting.

2.6 Immunoprecipitations

All cells to be used for immunoprecipitations were lysed in digitonin lysis buffer as described previously. One millilitre of cell lysate was added to 20 μL of washed Protein A-Sepharose 4B beads (Sigma Aldrich) in 1.5 mL tubes (Axygen Scientific). Five microliters of antibody was added to each tube, and the mixture was rocked for 1 hour at 4°C. The samples were centrifuged after incubation, and the lysate was aspirated off. The beads were then washed twice with lysis buffer, and the bound proteins were removed from the beads by adding SDS-PAGE (sodium dodecyl sulphate polyacrylamide electrophoresis) reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 4% glycerol, 2.5% SDS, 0.02% bromophenol blue, 100 mM dithiothreitol (DTT) (Sigma Aldrich) and boiling for 5 minutes in a water bath. The samples were then loaded onto an SDS-PAGE mini-gel and analyzed by Western immunoblotting.

2.7 SDS-PAGE and Western Immunoblotting

Samples containing SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 4% glycerol, 2.5% SDS, 0.02% bromophenol blue, 100 mM dithiothreitol (DTT) (Sigma Aldrich) were boiled in a water bath for 5 minutes and loaded into 1.5 mm thick SDS-PAGE mini-gels along with BenchMark pre-stained molecular weight standards (Invitrogen Life Technologies). The gels were run in a dual vertical mini-gel apparatus (CBS Scientific, Del Mar, California) at 25

milliamps per gel for approximately 2.5 hours. The proteins from the gels were transferred onto a BioTraceNT nitrocellulose blotting membrane (Pall Life Sciences, Pensacola, Florida) at 125 V for 1 hour in transfer buffer (20 mM Tris-HCl pH 8.0, 150 mM glycine, 20% methanol) using a Transblotter transfer apparatus (Bio-Rad Laboratories). The nitrocellulose filters were then blocked for half an hour at room temperature in Tris-buffered saline (TBS) containing 5% skim milk powder (Pacific Milk Division, Vancouver, BC). The filters were then quickly washed in TBS with 0.1% Tween 20 (TBST) (Sigma Aldrich). The filters were then incubated in primary antibody while rocking overnight at 4°C. The next day the filters were washed in TBST for 1 hour, changing the TBST every 15 minutes. They were then incubated for 1 hour in a horseradish peroxidase (HRP) conjugated secondary reagent diluted 1:10 000 in TBST. The filters were then washed in TBST 4 times at 15-minute intervals and bathed in enhanced chemiluminescence (ECL) reagent (Amersham Biosciences) for 1.5 minutes and exposed to Kodak X-Omat Blue autoradiography film (Mandel Scientific, Guelph, Ontario). The film was developed using a Kodak M35A X-OMAT Processor (Medtec Marketing Limited, Burnaby, BC).

If the filters were to be reprobed with a different primary antibody, they were washed at room temperature in TBS pH 2.0 for 20 minutes. The low pH was then neutralized by washing briefly with TBS pH 8.0. The filter could then be blocked and reprobed as described above.

2.8 Surface Expression of the BCR

2.8.1 Fluorescence activated cell sorting (FACS)

The J558 lymphoid cells were centrifuged at 1500 rpm for 5 minutes in an IEC Centra-8R tabletop centrifuge (International Equipment Company) and resuspended in 1 mL of cold sorter buffer (phosphate buffer saline (PBS) with 1% heat inactivated fetal calf serum (FCS)). One million cells were added to each Falcon 2054 FACScan tube, and the volume was adjusted to 1 mL with sorter buffer. The cells were then centrifuged at 1500 rpm for 5 minutes and resuspended in 50 μL of FITC-goat anti-IgM solution (30 μg/mL of FITC-goat anti-IgM (Jackson ImmunoResearch Laboratories, Inc.) in sorter buffer) and incubated on ice for 30 minutes. The cells were resuspended in 1 mL of sorter buffer, vortexed and another milliliter of sorter buffer was added. The cells were then centrifuged at 1500 rpm for 5 minutes in the cold

and resuspended in 1 mL of sorter buffer with 4 μg/mL of 7-Amino actinomycin D to mark the dead cells. The cells were then examined on the Becton Dickinson FACScan (BD Biosciences) according to manufacturers directions and the data was analyzed using CellQuest Software (BD Biosciences).

2.8.2 Cell surface immunofluorescence by microscopy

AtT20 cell lines were grown on glass coverslips (Sigma Aldrich) coated in poly-D-lysine (Matsuuchi, 1988) to semi-confluency. The cells were rinsed twice with phosphate buffered saline (PBS) with 20 mM glycine and fixed for 20 minutes in PBS containing 3% paraformaldehyde (BDH, Toronto, Ontario) pH 7.5. The coverslips were rinsed twice in 20 mM glycine in PBS and stained by incubating in rhodamine labeled goat anti-mouse IgM diluted 1:500 in 20 mM glycine in PBS for 30 minutes in the dark. The cells were then rinsed in PBS with 20 mM glycine and then in water. The coverslip was placed cell-side down on a slide in 3 μL of mounting media (90% glycerol, 10% PBS and 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO) (Sigma Aldrich)) (Johnson *et al.*, 1982) and sealed with nailpolish. The cells were examined using an Olympus IX70 Based DeltaVision microscope (Applied Precision, Seattle, Washington) and the data was analyzed using DeltaVision Software (Applied Precision).

CHAPTER 3

Establishing the Experimental System

3.1 Screening Antibodies for Immunoblotting and Immunoprecipitation

The immunoprecipitation experiments planned for this project required antibodies that were specific to the extracellular and cytoplasmic domains of Ig-α and Ig-β. The Matsuuchi lab had been using an Ig-α antibody, produced against the Ig-α cytoplasmic tail (described in Gold, *et al.*, 1991) as well as an Ig-β antibody that recognizes the cytoplasmic tail of Ig-β (a gift from Dr. Marcus Clark, University of Chicago, Chicago, Illinois), but two other antibodies, an Ig-α extracellular antibody and an Ig-β extracellular antibody were required. Fortunately, Abeome (Athens, Georgia) and Dr. Richard Meagher (University of Georgia, Athens, Georgia) provided the serum from several rabbits that had been immunized with a 30 amino acid peptide to the extracellular domain of mouse Ig-α or a 30 amino acid peptide to the extracellular domain of mouse Ig-β. The reactivity level of the rabbit serum to either Ig-α or Ig-β was then examined by immunoblotting. The results are shown in Table 3.1. The Abeome 689 antibody was used when an antibody to the extracellular domain of Ig-α was required because it proved to have the least amount of non-specific binding when immunoblotting. The Abeome 624 antibody was used when an antibody to the extracellular domain of Ig-β was needed. Figure 3.1 shows the specificity of the four different Ig-α and Ig-β antibodies used for this project.

Table 3.1: Reactivity of the Abeome antibodies to Ig-α or Ig-β. +, ++ and +++ represent low, medium and high levels of reactivity as determined by western blot, respectively.

Abeome antibody	Bleed Date	Reactive to domain:	Reactivity
688	11/19/02	Ig-α extracellular	+
	12/23/02	Ig-α extracellular	+
	2/20/03	Ig-α extracellular	++
	4/17/03	Ig-α extracellular	+++
	7/8/03	Ig-α extracellular	++
689	4/17/03	Ig-α extracellular	+++
	7/8/03	Ig-α extracellular	+++
690	3/20/03	Ig-α extracellular	+++
	7/8/03	Ig-α extracellular	+++
624	12/3/02	Ig-β extracellular	+++
	1/16/03	Ig-β extracellular	++
	1/27/03	Ig-β extracellular	++
	7/8/07	Ig-β extracellular	+

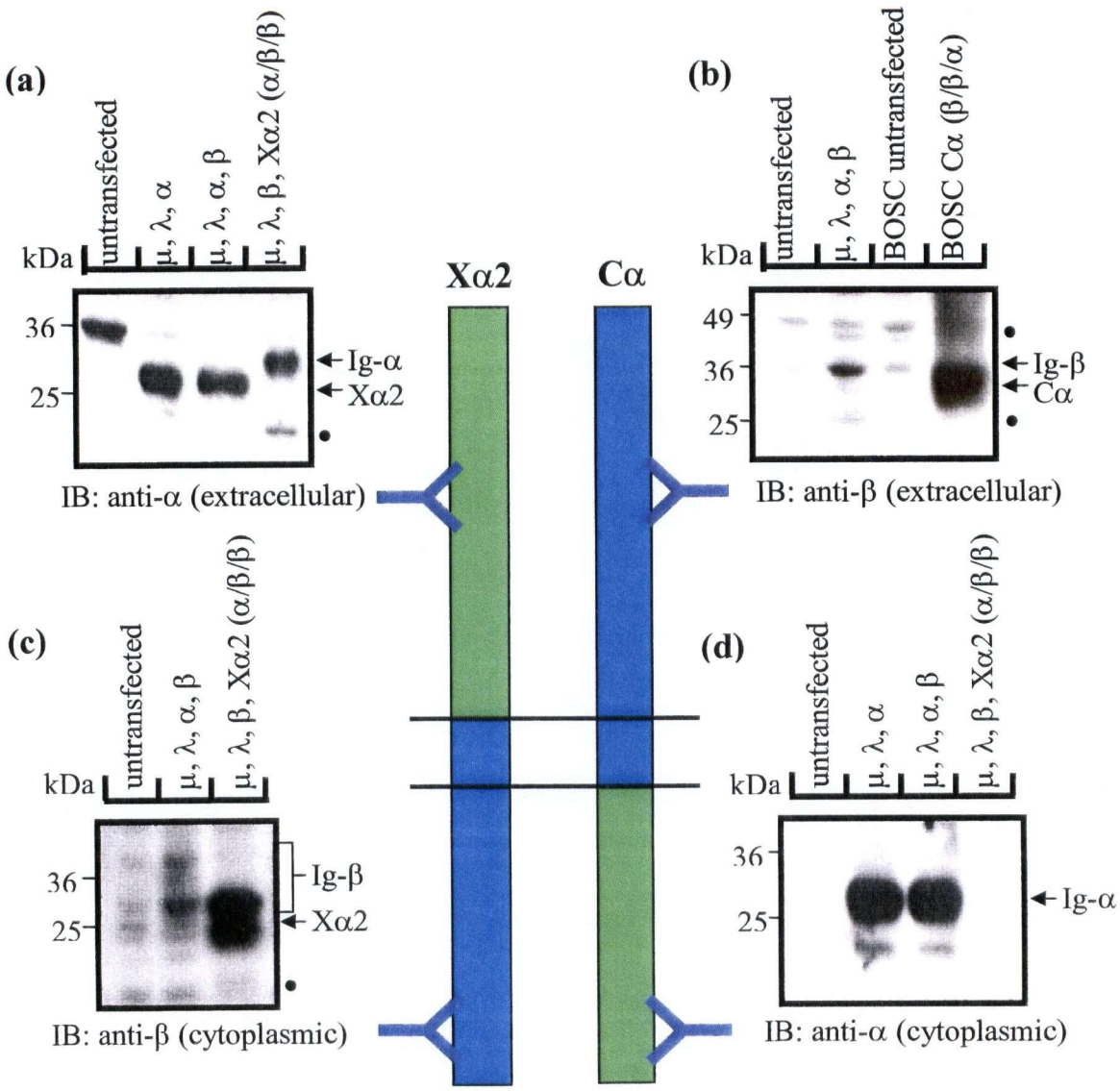


Figure 3.1 Antibody screening by immunoblotting. 30-50 μg of whole cell lysate from either cells expressing various components of the BCR or from transiently transfected BOSC cells was analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α or Ig-β antibodies **(a)** antibody reactive to the extracellular domain of Ig-α. **(b)** antibody reactive to extracellular domain of Ig-β. **(c)** antibody reactive to the cytoplamic domain of Ig-β and **(d)** antibody reactive to the cytoplasmic domain of Ig-α. μ, λ, α, β: the components of the BCR that the cell line expresses, IB: immunoblotting antibody •: background band, green colouration: Ig-α, blue colouration: Ig-β.

3.2 Construct Expressibility in BOSC Cells

To ensure that the mutant constructs created for this project are expressible, the plasmids described in section 2.1.3 were transfected into BOSC human fibroblast cells which are easily transiently transfected and used as a test system. Whole cell lysate was run by SDS-PAGE and immunoblotted. Figure 3.2 shows the expression of all nine constructs (Xα2, Cβ, MPβ, ΔXβ, ΔXα, MPα, Xβ, Cα and ΔαKVK) in BOSC cells.

3.3 Experimental Cell Systems

In order to examine the assembly and cell surface expression of a mutant BCR, cell lines needed to be created that expressed mIgM, Ig-β and mutant Ig-α constructs. Two different expression systems, the AtT20 non-lymphoid system and the J558 lymphoid system, were used for this purpose.

3.3.1 AtT20 non-lymphoid expression system

The AtT20 expression system is a series of non-lymphoid tissue culture cell lines (AtT20 endocrine cells) that have been previously transfected with various combinations of the BCR components. This system includes one cell line lacking Ig-α, so that mutant versions of Ig-α could be expressed.

3.3.1 J558 lymphoid expression system

The J558 expression system makes use of lymphoid tissue culture cell lines derived from the J558 plasmacytoma. Normally these cells do not express the BCR on their surface since they are antibody-secreting cells. The J558 cell lines used for this project were transfected with, and express, various components of the BCR. One of the J558 cell lines expresses mIgM and Ig-β, to which the mutant Ig-α constructs were added.

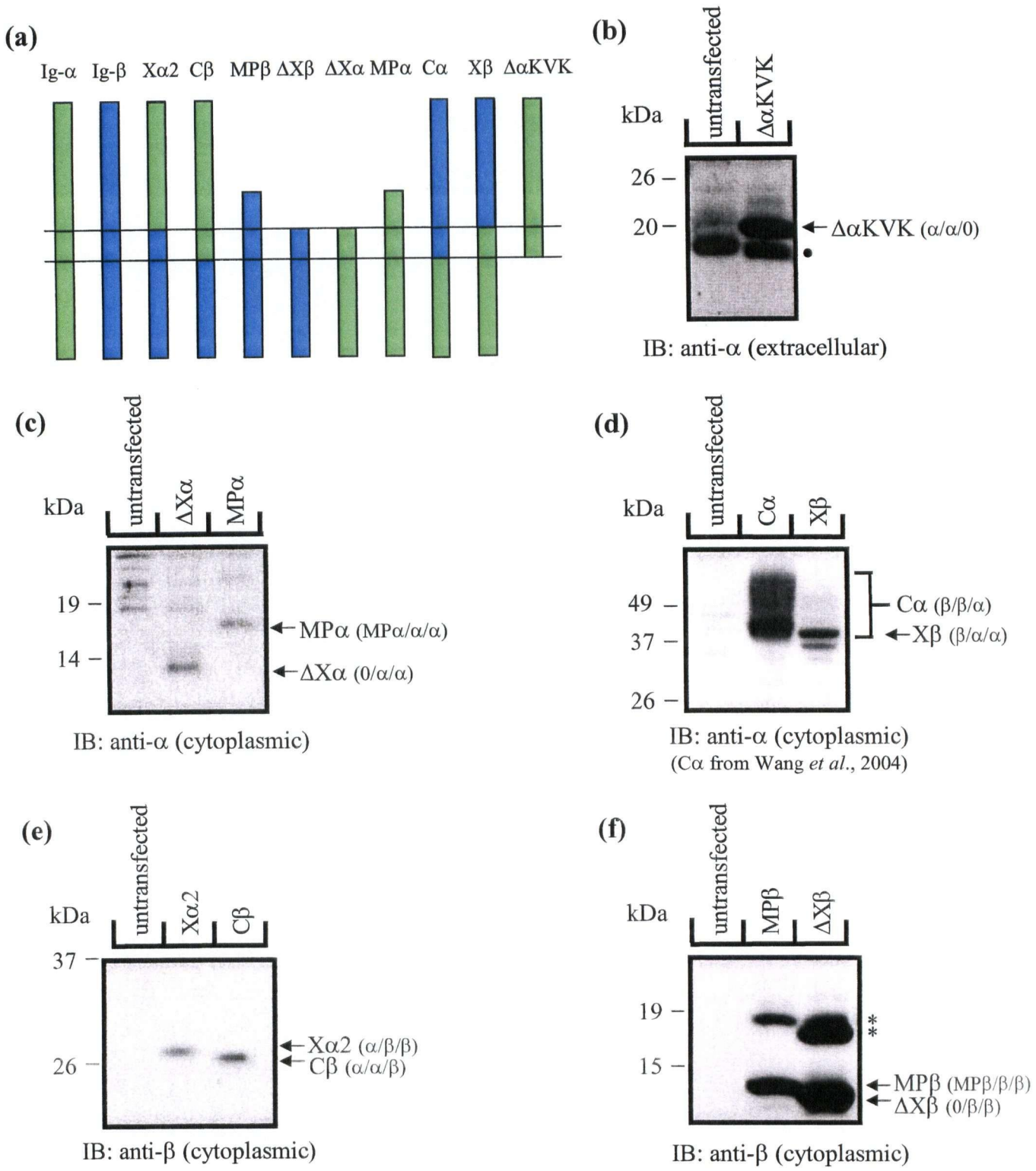


Figure 3.2: Expression of mutant Ig-α and Ig-β constructs in BOSC human fibroblast cells. 30-50 μg of whole cell lysate from transiently transfected BOSC cells was analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α or Ig-β antibodies. **(a)** schematic representation of all mutant Ig-α/β proteins (the green colouration represents Ig-α, the blue colouration represents Ig-β), **(b)** expression of ΔαKVK, **(c)** expression of ΔXα and MPα, **(d)** expression of Cα and Xβ (the various sizes are due to differential glycosylation), **(e)** expression of Xα2 and Cβ and **(f)** expression of MPβ and ΔXβ. IB: immunoblotting antibody, •: background band, *: potential homodimerization.

3.4 Expression of constructs in AtT20 and J558 cell lines

To create the cell lines needed to examine mutant BCR assembly and cell surface trafficking, five of the nine mutant constructs were successfully retrovirally infected into either the AtT20 expression system or the J558 expression system (the other four constructs will be used in future studies). J558 cells already expressing Ig-β and mIgM were infected with Cα (β/β/α), Xα2 (α/β/β) or Cβ (α/α/β) DNA (Figure 3.3 (a), (b) and (c)). Additionally, AtT20 cells already expressing Ig-β and mIgM were infected with ΔXα (0/α/α) or ΔαKVK (α/α/0) DNA (Figure 3.3 (d) and (e)). With these five mutant Ig-α constructs being expressed in cell lines already expressing the other components of the BCR, it was then possible to begin to study the assembly and cell surface trafficking of the mutant BCRs.

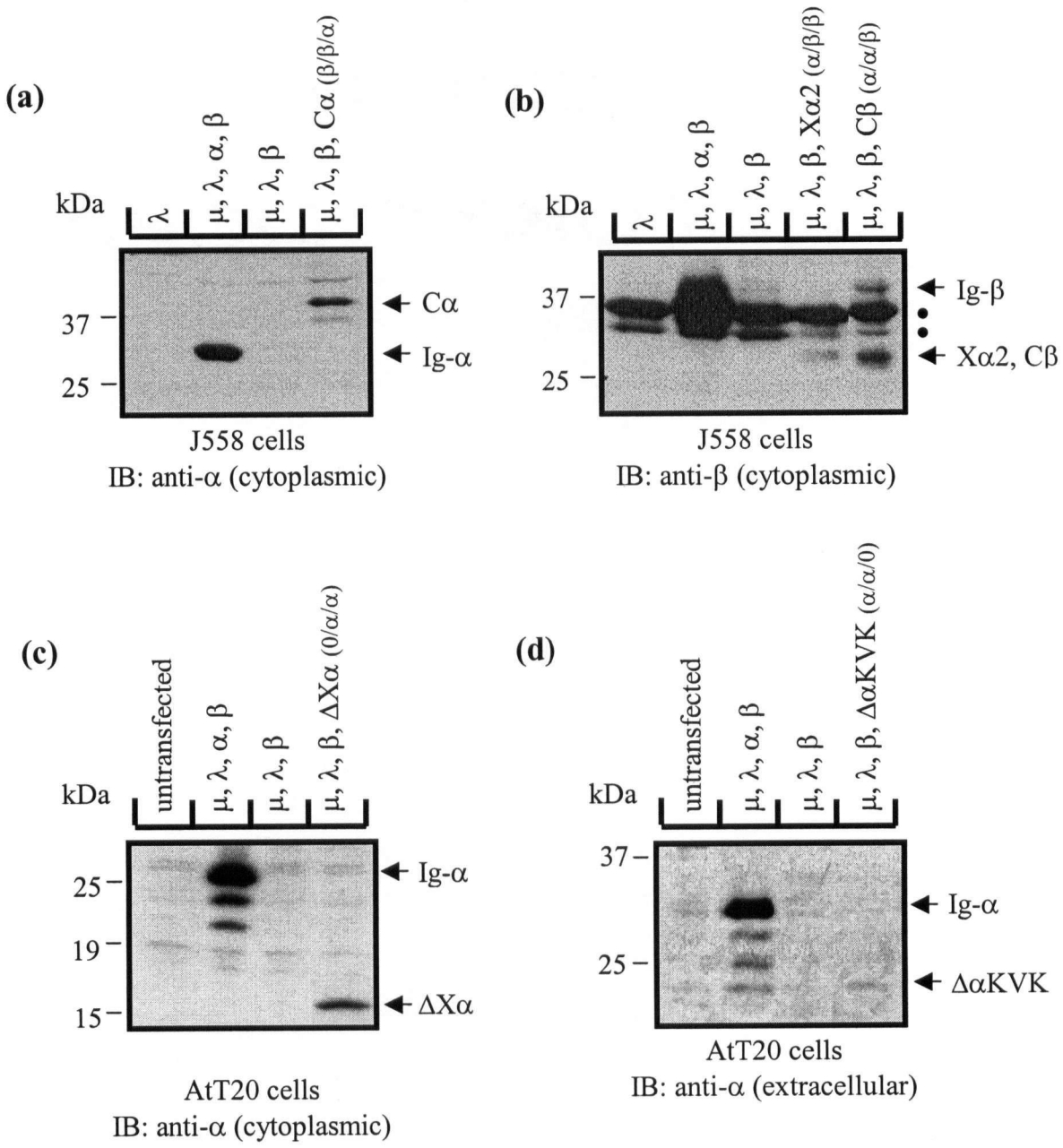


Figure 3.3: Transient expression of mutant Ig-α constructs in J558 mouse plasmacytoma cells and stable expression of mutant Ig-α constructs in AtT20 non-lymphoid cells.. Thirty-50 μg of whole cell lysate from drug selected cells were analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α or Ig-β antibodies. **(a)** expression of Cα (β/β/α) in J558 cells, **(b)** expression of Xα2 (α/β/β) and Cβ (α/α/β) in J558 cells, **(c)** expression of ΔXα (0/α/α) in AtT20 cells, **(d)** expression of ΔαKVK (α/α/0) in AtT20 cells. Labels on top of gels indicate BCR chains expressed by transfected cells. IB: immunoblotting antibody, •: background band.

CHAPTER 4

BCR Assembly and Cell Surface Trafficking

4.1 Introduction

All four components of the mIgM-containing BCR (H chains, L chains, Ig-α and Ig-β) must be expressed in the cell in order for assembly and cell surface trafficking to occur (Matsuuchi *et al.*, 1992; Venkitaraman *et al.*, 1991). However, if the cytoplasmic tails of either Ig-α or Ig-β are truncated or mutated, the mIgM BCR can still traffic to the cell surface, but B cell signalling and development are disrupted (Reichlin *et al.*, 2001, Wang *et al.*, 2004). The study by Reichlin *et al.* (2001) showed that truncation of the Ig-α or Ig-β cytoplasmic tail, leaving a heterodimer in the extracellular and transmembrane domains, allowed for BCR formation and cell surface trafficking. In support of this data, a study by Wang *et al.* (2004) showed that an Ig-β mutant that heterodimerized with Ig-α in the extracellular and transmembrane domains, but homodimerized in the cytoplasmic domain, was able to form a complete BCR and traffic to the cell surface. Both of these studies suggest that heterodimerization in the extracellular and transmembrane domains is required for BCR assembly and cell surface trafficking. This hypothesis was further examined here with five mutant Ig-α constructs (Cα (β/β/α), Xα2 (α/β/β), Cβ (α/α/β), ΔαKVK (α/α/0) and ΔXα (0/α/α)). These studies will help us to increase our understanding of which portions of the BCR are required for BCR cell surface expression during the different stages of B cell development.

4.2 BCR Assembly

4.2.1 BCR assembly with the Cα (β/β/α) construct

J558 cells expressing mIgM, Ig-β and the mutant Ig-α construct Cα (β/β/α), were used to examine whether Ig-α/β heterodimerization in the cytoplasmic domain would allow for BCR assembly. Figure 4.1(a) depicts the potential structure of the Cα BCR. In this BCR heterodimerization can only occur between the cytoplasmic domains of Ig-β and Cα.

Immunoprecipitation experiments were used to examine whether or not the Cα BCR is able to assemble into a complete BCR. The μ H chain portion of mIgM was successfully immunoprecipitated from the cells expressing Cα, but immunoblotting demonstrated that Cα does not associate with mIgM (Figure 4.1(c)). Additionally, the Cα protein was immunoprecipitated from the Cα cell lysate, but immunoblotting for Ig-β showed that Cα does not associate with Ig-β (Figure 4.1(d)). These findings suggest that heterodimerization in the cytoplasmic domain of Ig-α/β does not allow for BCR assembly.

4.2.2 BCR assembly with the Xα2 (α/β/β) construct

In a second examination, J558 cells expressing mIgM, Ig-β and the Xα2 (α/β/β) construct were used to identify whether heterodimerization in the Ig-α/β extracellular domain would allow for BCR assembly. Figure 4.2(a) shows the potential structure of the Xα2 BCR. Immunoprecipitation of the μ H chain of mIgM followed by immunoblotting for Xα2 showed that Xα2 is not able to associate with mIgM (Figure 4.2(c)). Immunoprecipitation of Ig-β followed by immunoblotting indicated that Xα2 is also not able to associate with Ig-β (Figure 4.2(d)). These findings suggest that heterodimerization solely in the extracellular domain of Ig-α/β does not allow for BCR assembly.

4.2.3 BCR assembly with the Cβ (α/α/β) construct

Due to the finding that Ig-α/β heterodimerization in the cytoplasmic or extracellular domains on their own, do not allow for BCR assembly, J558 cells, expressing mIgM, Ig-β and the Cβ (α/α/β) construct, were used to determine if heterodimerization in the extracellular **and** transmembrane domains would allow for BCR formation. Figure 4.3(a) shows the potential structure of the Cβ BCR. Immunoprecipitation of the μ H chain of mIgM followed by immunoblotting for Cβ showed that Cβ is able to interact with mIgM in these cells (Figure 4.3(c)). Immunoprecipitation of Ig-β and immunoblotting for Cβ indicated that Cβ also binds Ig-β (Figure 4.3(d)). These findings suggest that heterodimerization in the extracellular **and** transmembrane domains of Ig-α/β does allow for BCR assembly.

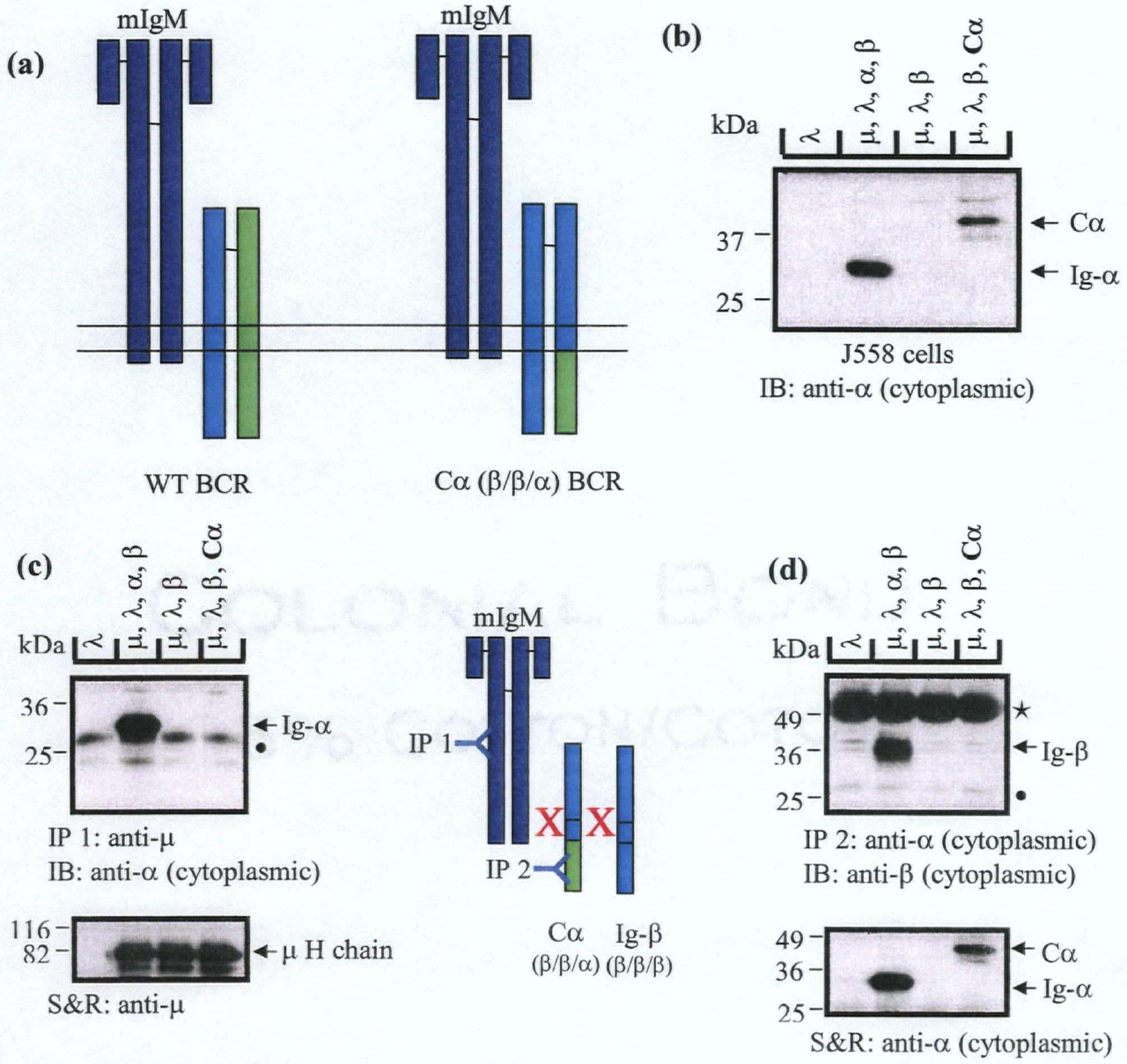


Figure 4.1: Transient expression of the Cα construct in J558 mouse plasmacytoma cells and the association of Cα with Ig-β and mIgM **(a)** diagrammatic representation of the Cα BCR to show the potential interactions that could occur between the different components. **(b)** expression of Cα in J558 cells, 30-50 μg of whole cell lysate from drug selected cells was analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α antibodies. Figure repeated from Figure 3.3(a). **(c)** association of Cα with Ig-β and **(d)** association of Cα with mIgM. J558 cells expressing Cα were lysed and BCR components were immunoprecipitated from 1000 μg of whole cell lysate using Protein A-Sepharose and μ or Ig-α specific antibodies. Immunoprecipitates were analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α or Ig-β antibodies (upper panels) and then stripped and reprobed (S&R) with μ or Ig-α antibodies (lower panels). μ, λ, α, β: the components of the BCR that the cell line expresses, IP: immunoprecipitating antibody, IB: immunoblotting antibody, S&R: immunoblotting antibody used to reprobe the filter, •: background band, ★: immunoprecipitating antibody, green colouration: Ig-α, light blue colouration: Ig-β, dark blue colouration: mIgM.

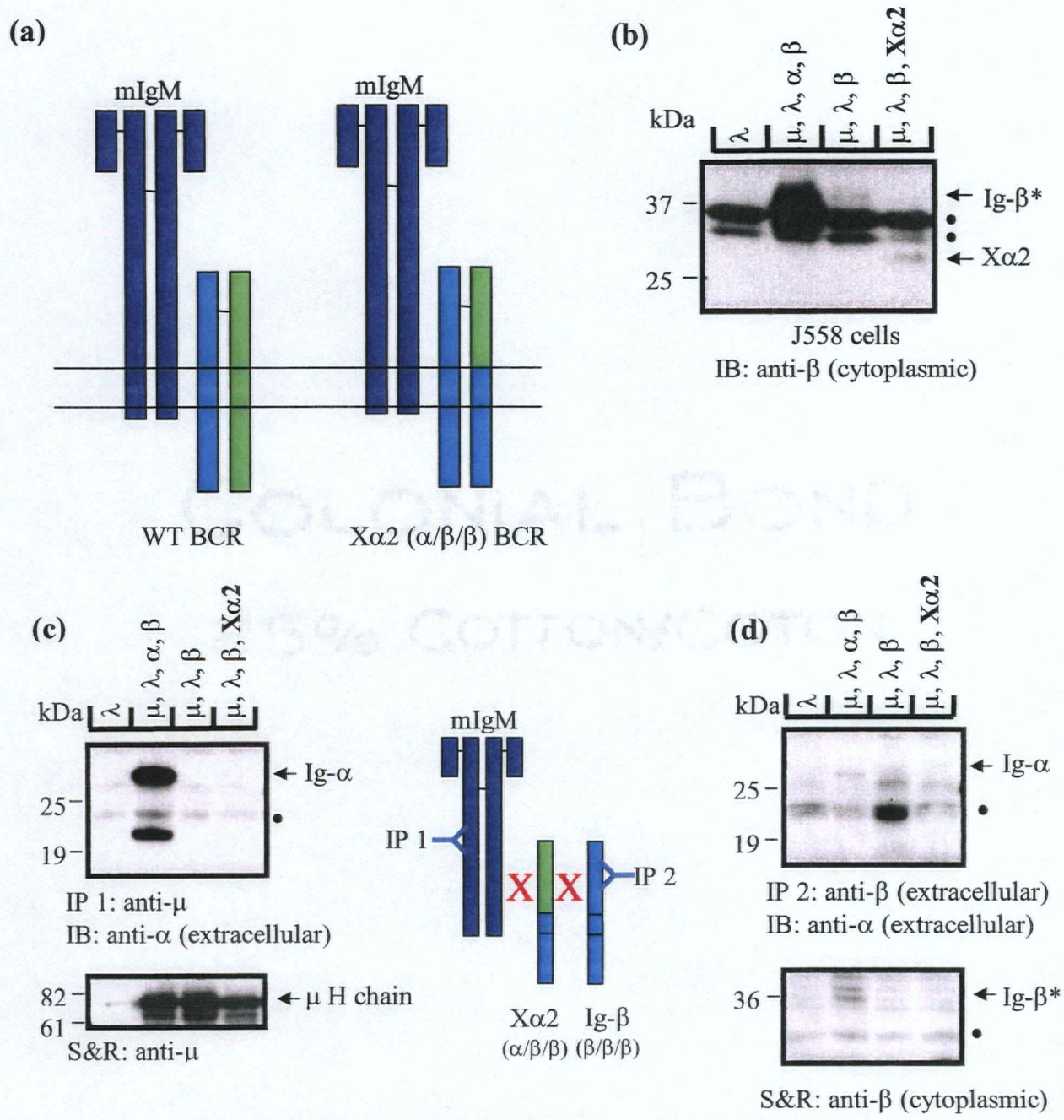


Figure 4.2: Transient expression of the Xα2 construct in J558 mouse plasmacytoma cells and the association of Xα2 with Ig-β and mIgM **(a)** diagrammatic representation of the Xα2 BCR to show the potential interactions that could occur between the different components. **(b)** expression of Xα2 in J558 cells, 30-50 μg of whole cell lysate from drug selected cells was analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-β antibodies. Figure repeated from Figure 3.3(b). **(c)** association of Xα2 with Ig-β and **(d)** association of Xα2 with mIgM. J558 cells expressing Xα2 were lysed and BCR components were immunoprecipitated from 1000 μg of whole cell lysate using Protein A-Sepharose and μ or Ig-β specific antibodies. Immunoprecipitates were analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α antibodies (upper panels) and then stripped and reprobed (S&R) with μ or Ig-β antibodies (lower panels). μ, λ, α, β: the components of the BCR that the cell line expresses, IP: immunoprecipitating antibody, IB: immunoblotting antibody, S&R: immunoblotting antibody used to reprobe the filter, •: background band, ★: immunoprecipitating antibody, green colouration: Ig-α, light blue colouration: Ig-β, dark blue colouration: mIgM. * the various sizes of Ig-β are due to a range of glycosylated forms.

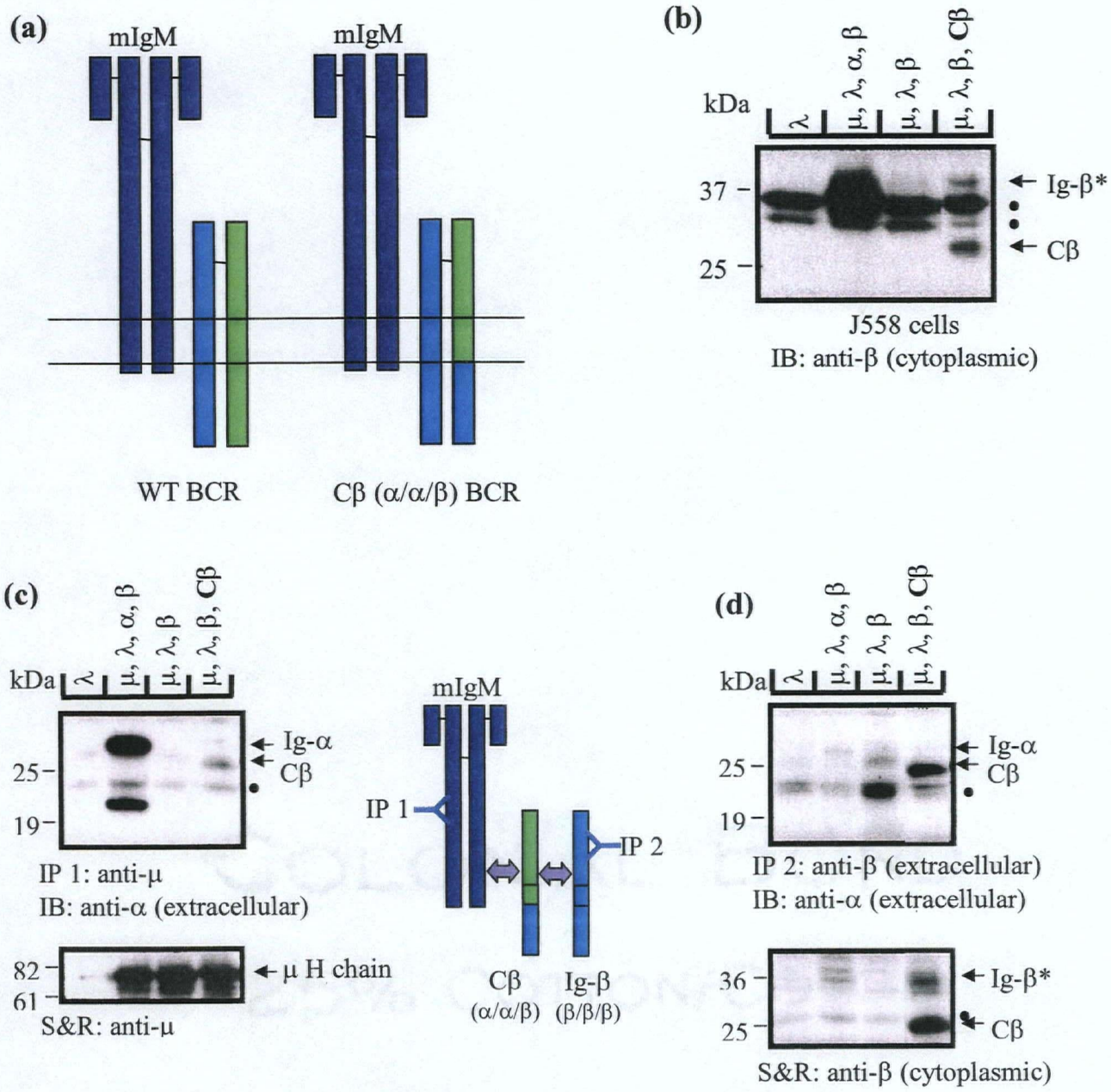


Figure 4.3: Transient expression of the Cβ construct in J558 mouse plasmacytoma cells and the association of Cβ with Ig-β and mIgM **(a)** diagrammatic representation of the Cβ BCR to show the potential interactions that could occur between the different components. **(b)** expression of Cβ in J558 cells, 30-50 μg of whole cell lysate from drug selected cells was analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-β antibodies. Figure repeated from Figure 3.3(b). **(c)** association of Cβ with Ig-β and **(d)** association of Cβ with mIgM. J558 cells expressing Cβ were lysed and BCR components were immunoprecipitated from 1000 μg of whole cell lysate using Protein A-Sepharose and μ or Ig-β specific antibodies. Immunoprecipitates were analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α antibodies (upper panels) and then stripped and reprobed (S&R) with μ or Ig-β antibodies (lower panels). μ, λ, α, β: the components of the BCR that the cell line expresses, IP: immunoprecipitating antibody, IB: immunoblotting antibody, S&R: immunoblotting antibody used to reprobe the filter, •: background band, ★: immunoprecipitating antibody, green colouration: Ig-α, light blue colouration: Ig-β, dark blue colouration: mIgM. * the various sizes of Ig-β are due to a range of glycosylated forms.

4.2.4 BCR assembly with the ΔαKVK (α/α/0) construct

AtT20 cells expressing mIgM, Ig-β and ΔαKVK (α/α/0) were used to determine whether BCR assembly can occur with Ig-α/β heterodimerization in the extracellular and transmembane domains, but without cytoplasmic dimerization. The ΔαKVK construct is truncated in the cytoplasmic domain and has only three cytoplasmic amino acids, lysine, valine, lysine. These are the same amino acids that are in the cytoplasmic tail of the μ H chain of mIgM. A diagram of the potential structure of the ΔαKVK BCR is shown in Figure 4.4(a). Immunoprecipitation of the μ H chain followed by immunoblotting for ΔαKVK showed that ΔαKVK is able to associate with mIgM (Figure 4.4(c)). Immunoprecipitation of Ig-β and immunoblotting for ΔαKVK showed that ΔαKVK is not able to associate with Ig-β (Figure 4.4(d)). As the structures of Cβ and ΔαKVK are similar, with the only difference being that Cβ has an Ig-β cytoplasmic tail, this data indicates that dimerization in the cytoplasmic domain and heterodimerization in the extracellular and transmembrane domains of Ig-α/β is needed for proper BCR assembly.

4.2.5 BCR assembly with the ΔXα (0/α/α) construct

AtT20 cells expressing mIgM, Ig-β and ΔXα were used to determine whether BCR assembly can occur with heterodimerization in the transmembrane and cytoplasmic domains. Figure 4.5(a) diagrams the potential structure of the ΔXα BCR. Immunoprecipitation of the μ H chain of mIgM followed by immunoblotting for ΔXα showed that ΔXα is able to associate with mIgM (Figure 4.5(c)). Immunoprecipitation of Ig-β with immunoblotting for ΔXα showed that ΔXα is not able to associate with Ig-β. Therefore, as with ΔαKVK, only the interaction between mIgM and ΔXα occurs, and Ig-β is not able to associate to form a complete BCR.

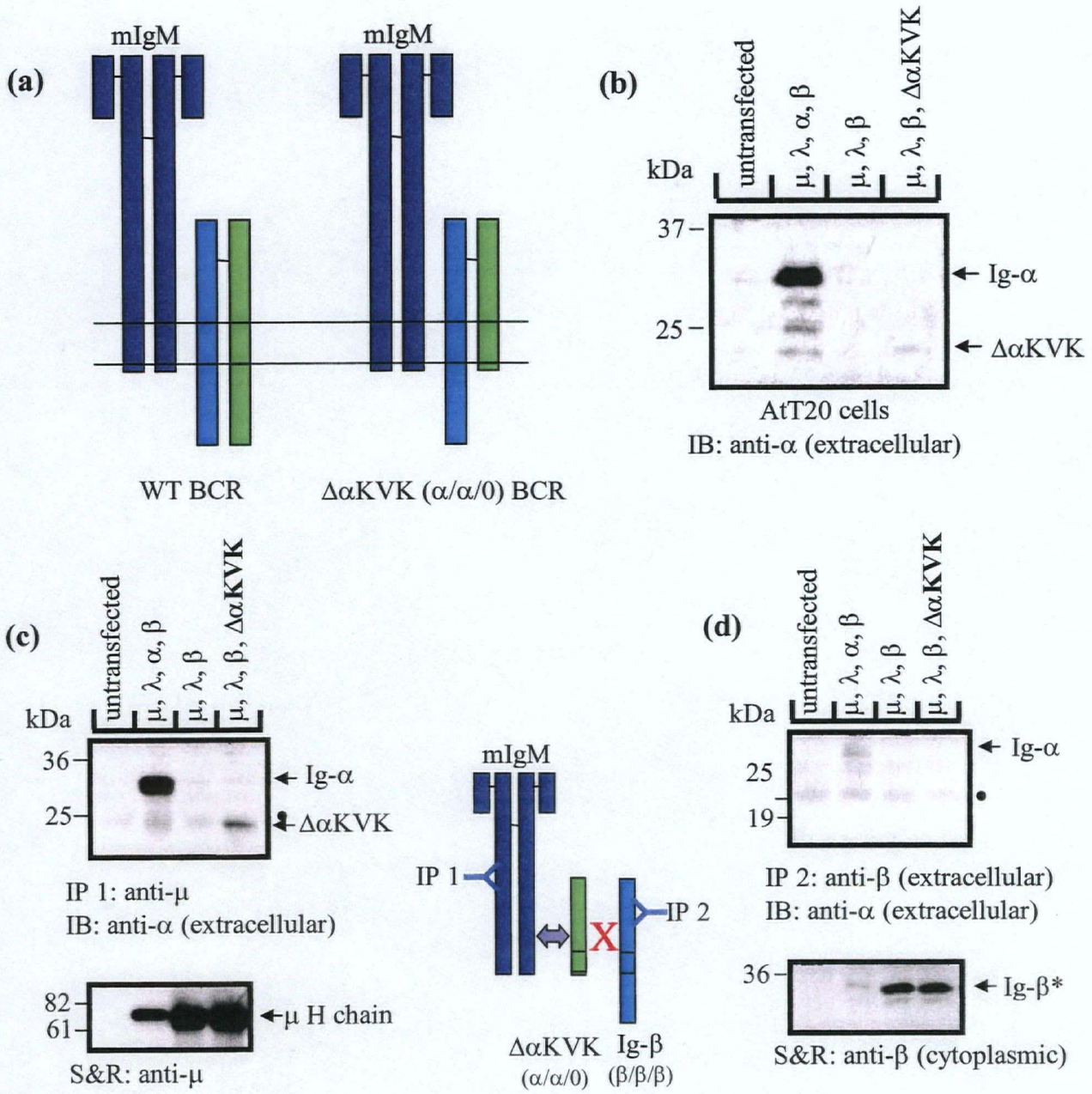


Figure 4.4: Stable expression of ΔαKVK in AtT20 non-lymphoid cells and the association of ΔαKVK with Ig-β and mIgM **(a)** diagrammatic representation of the ΔαKVK BCR to show the potential interactions that could occur between the different components. **(b)** expression of ΔαKVK in AtT20 cells, 30-50 μg of whole cell lysate from drug selected cells was analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α antibodies. Figure repeated from Figure 3.3(d). **(c)** association of ΔαKVK with Ig-β and **(d)** association of ΔαKVK with mIgM. AtT20 cells expressing ΔαKVK were lysed and BCR components were immunoprecipitated from 1000 μg of whole cell lysate using Protein A-Sepharose and μ or Ig-β specific antibodies. Immunoprecipitates were analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α antibodies (upper panels) and then stripped and reprobed (S&R) with μ or Ig-β antibodies (lower panels). μ, λ, α, β: the components of the BCR that the cell line expresses, IP: immunoprecipitating antibody, IB: immunoblotting antibody, S&R: immunoblotting antibody used to reprobe the filter, •: background band, ★: immunoprecipitating antibody, green colouration: Ig-α, light blue colouration: Ig-β, dark blue colouration: mIgM. * the various sizes of Ig-β are due to a range of glycosylated forms.

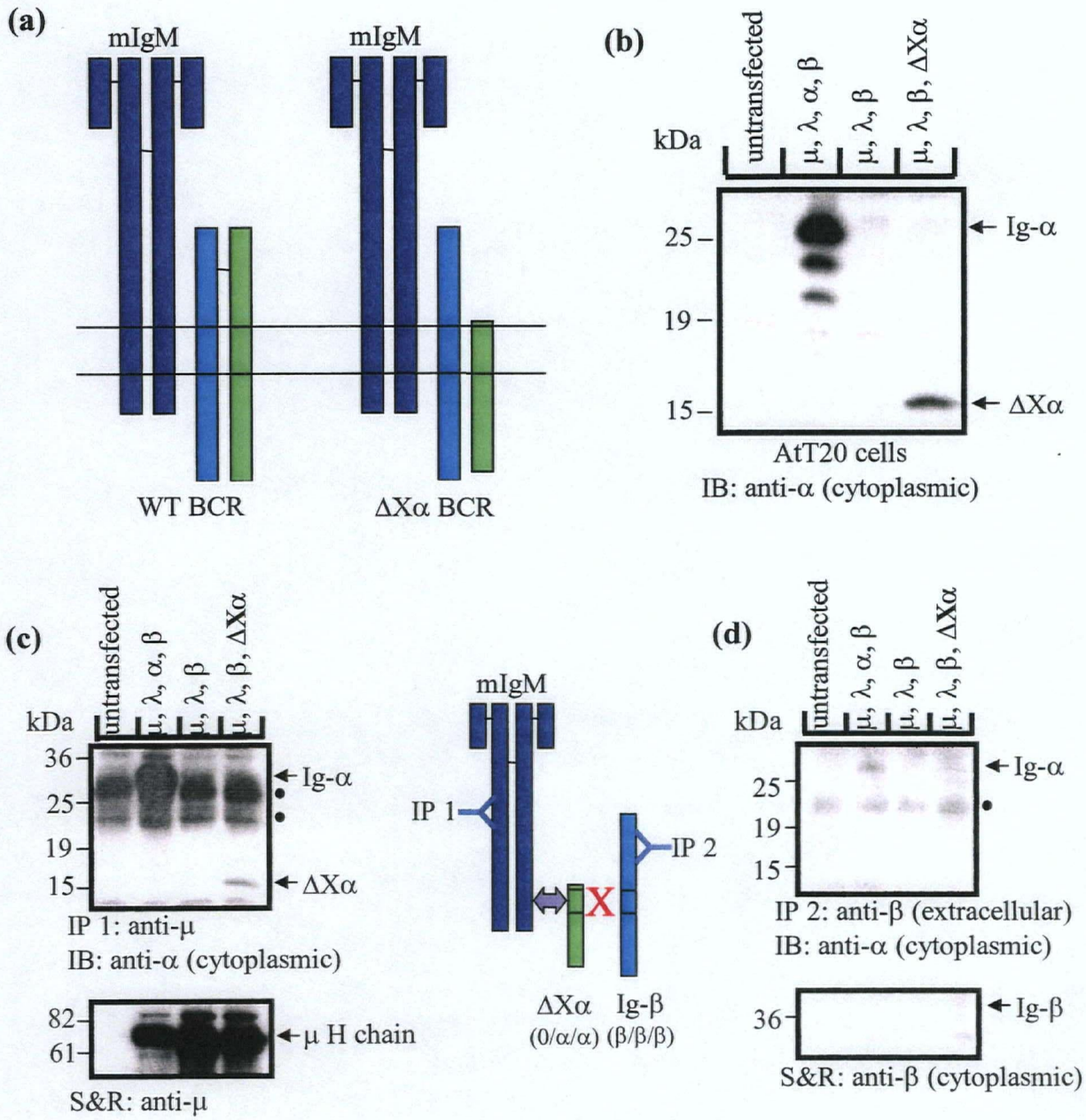


Figure 4.5: Stable expression of ΔXα in AtT20 non-lymphoid cells and the association of ΔXα with Ig-β and mIgM **(a)** diagrammatic representation of the ΔXα BCR to show the potential interactions that could occur between the different components. **(b)** expression of ΔXα in AtT20 cells, 30-50 μg of whole cell lysate from drug selected cells was analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α antibodies. Figure repeated from Figure 3.3(c). **(c)** association of ΔXα with Ig-β and **(d)** association of ΔXα with mIgM. AtT20 cells expressing ΔXα were lysed and BCR components were immunoprecipitated from 1000 μg of whole cell lysate using Protein A-Sepharose and μ or Ig-β specific antibodies. Immunoprecipitates were analyzed by SDS-PAGE. Proteins from the gels were transferred to nitrocellulose and immunoblotted with Ig-α antibodies (upper panels) and then stripped and reprobed (S&R) with μ or Ig-β antibodies (lower panels). μ, λ, α, β: the components of the BCR that the cell line expresses, IP: immunoprecipitating antibody, IB: immunoblotting antibody, S&R: immunoblotting antibody used to reprobe the filter, •: background band, ★: immunoprecipitating antibody, green colouration: Ig-α, light blue colouration: Ig-β, dark blue colouration: mIgM.

4.3 BCR Cell Surface Expression

If all the components of the BCR are able to interact with one another within a cell, the BCR will assemble and traffic to the cell surface. Cell surface expression of the BCR can be determined using a fluorescently tagged antibody to the μ H chain of mIgM and employing either fluorescent activated cell sorting (FACS) or microscopy. If the BCR is expressed on the cell surface, then the surface of the cell will be fluorescently labeled.

FACS was used to determine whether or not the Cα, Xα2 and Cβ BCRs are expressed on the surface of J558 cells. Figure 4.6 shows the FACS data for the cells that were expressing one of these three constructs. In the WT BCR panel the two clusters of data points represent either cells that were fluorescently labeled and were expressing the BCR on their cell surface (lower right quadrant) or cells that were not (lower left quadrant). The upper quadrants represent dead cells. Of the three constructs tested by this method, the only one that had data points in the lower right quadrant was Cβ (α/α/β). This indicates that BCRs containing Cβ are expressed on the cell surface while the BCRs containing Cα or Xα2 are not expressed on the cell surface.

Immunofluorescence microscopy was used to visualize BCR cell surface expression in adherent AtT20 cells. BCR surface expression was seen in the cells expressing the WT BCR, but neither of the cell lines expressing the ΔαKVK BCR or the ΔXα BCR had any BCR surface expression (data not shown).

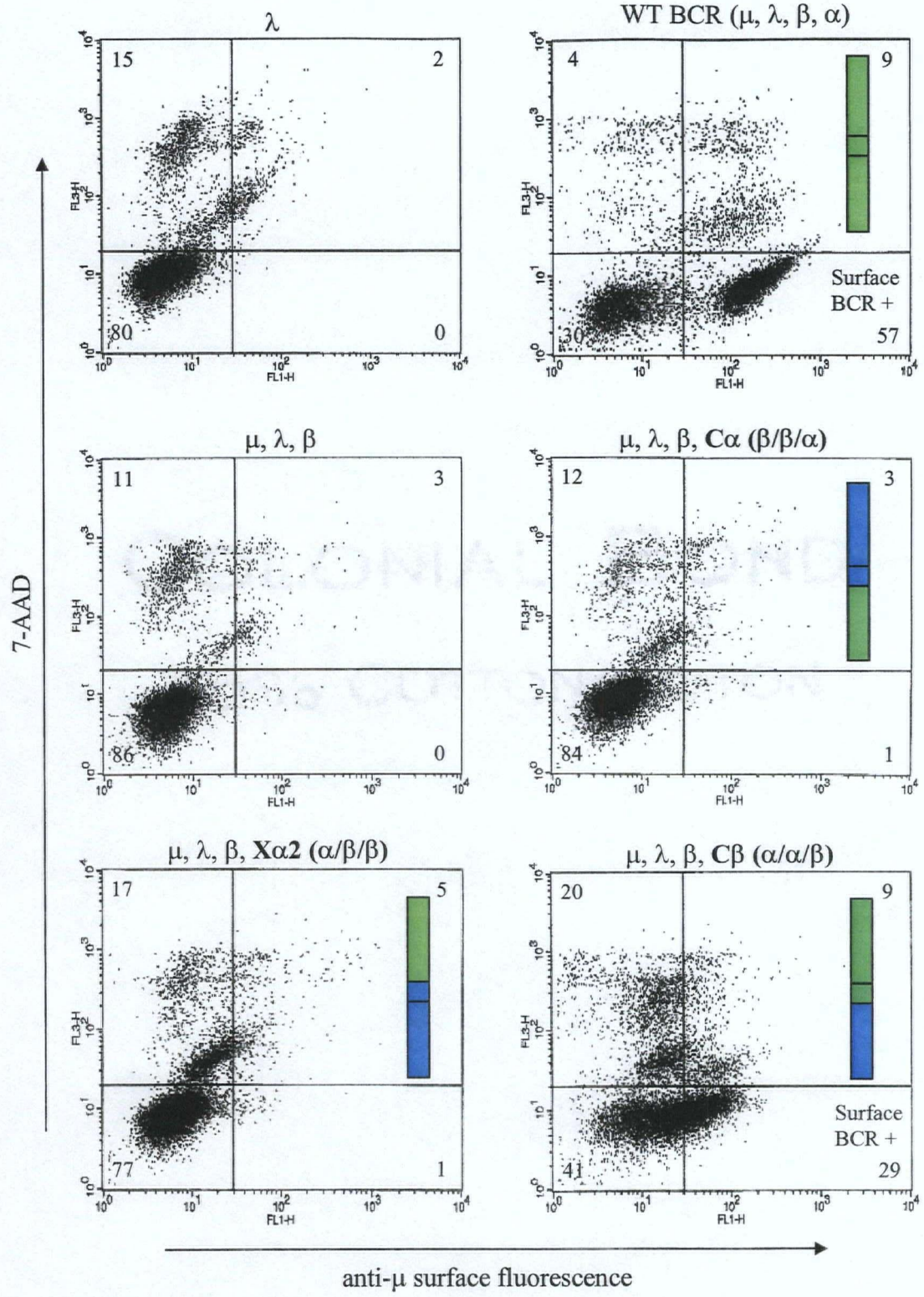


Figure 4.6: Fluorescence activated cell sorting (FACS) of J558 cells expressing Cα, Xα2 or Cβ. J558 cells expressing mutant Ig-α constructs were stained with FITC-goat anti-IgM to label the μ H chain on the cell surface, and with 7-Amino actinomycin D (7-AAD) to mark the dead cells. The cells were then analyzed by FACS. μ, λ, α, β: the components of the BCR that the cell line expresses. Live cells expressing the BCR on their cell surface are represented in the lower right quadrant. The numbers represent the percent of cells in each quadrant. Green colouration represents Ig-α, light blue colouration represents Ig-β.

CHAPTER 5

Discussion

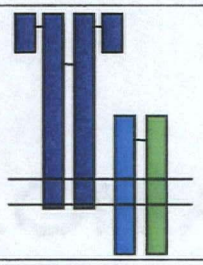
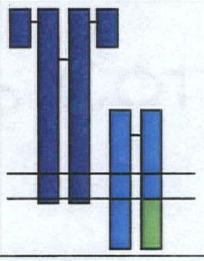
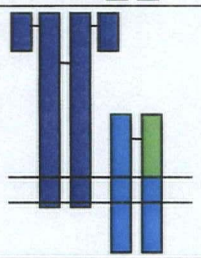
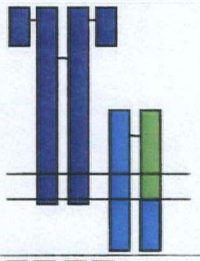
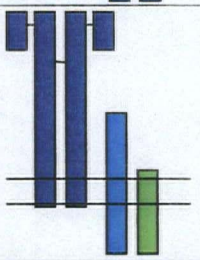
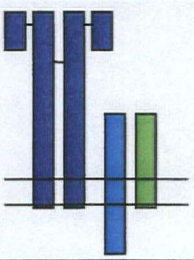
5.1 Summary of results and discussion of future directions

All four components of the mIgM-containing BCR must be expressed in a cell in order for BCR assembly and cell surface trafficking to occur (Matsuuchi *et al.*, 1992; Venkitaraman *et al.*, 1991). If the BCR is not able to be expressed on the cell surface, B cell development does not occur. For example, a deletion of Ig-α or Ig-β prevents B cell development past the pro-B cell stage (Pelanda *et al.*, 2002). Although it is recognized that all four components of the BCR are required for BCR cell surface expression, few studies have examined which portions of the BCR are important for receptor assembly and cell surface trafficking. This knowledge will allow future researchers to study BCR signalling and, the role of the BCR in B cell development and B cell leukemias.

This study has indicated that for BCR assembly and cell surface expression to occur the Ig-α/β proteins must heterodimerize in the extracellular and transmembrane domains and, potentially, dimerize in the cytoplasmic domain. This was determined by creating, seven new Ig-α/β mutant constructs (Xα2, Cβ, ΔXα, ΔXβ, MPα, MPβ and Xβ), altering one Ig-α construct (ΔαKVK) and using one construct that had been previously used as an Ig-β mutant construct (Cα). Five of these constructs (Cα, Xα2, Cβ, ΔXα and ΔαKVK) were expressed in cell lines already expressing mIgM and Ig-β. Immunoprecipitation and cell surface fluorescence studies were used to determine whether or not these mutant Ig-α constructs were able to associate with mIgM and Ig-β and if the mutant BCR was able to traffic to the cell surface. The results of these studies are summarized in Table 5.1.

Immunoprecipitation experiments showed that constructs that heterodimerized with Ig-β in the transmembrane domain (Cβ (α/α/β), ΔαKVK (α/α/0) and ΔXα (0/α/α)) were able to associate with mIgM, but not with Ig-β. Since ΔαKVK (α/α/0) is truncated cytoplasmically and ΔXα is truncated extracellularly and neither construct contains any extraneous cytoplasmic or

Table 5.1: Summary of results. The green colouration represents Ig-α, the light blue colouration represents Ig-β and dark blue colouration represents mIgM. EX: the extracellular domain, TM: the transmembrane domain, CY: the cytoplasmic domain, MP: membrane proximal region of the extracellular domain.

Mutant Ig-α BCR	Mutant Ig-α Description	Potential BCR Structure	Mutant Ig-α association with mIgM	Mutant Ig-α association with Ig-β	BCR cell surface expression
WT Ig-α (α/α/α)	WT Ig-α		yes	yes	yes
Cα (β/β/α)	EX: Ig-β TM: Ig-β CY: Ig-α		no	no	no
Xα (α/β/β)	EX: Ig-α TM: Ig-β CY: Ig-β		no	no	no
Cβ (α/α/β)	EX: Ig-α TM: Ig-α CY: Ig-β		yes	yes	yes
ΔXα (0/α/α)	EX: none TM: Ig-α CY: Ig-α		yes	no	no
ΔαKVK (α/α/0)	EX: Ig-α TM: Ig-α CY: none		yes	no	no

extracellular amino acids in the truncated region, we can infer that the association between mIgM and Ig-α is through the transmembrane domain. A mutant that is made up of only the Ig-α transmembrane domain would confirm this finding.

The Cβ (α/α/β) chimeric protein is the only construct tested that is able to associate with WT Ig-β and mIgM to form a complete BCR. It forms a heterodimer with Ig-β in the extracellular and transmembrane domains indicating that heterodimerization in those regions is necessary for BCR assembly. But, the lack of association between ΔαKVK (α/α/0) and Ig-β is puzzling because the only difference between ΔαKVK and Cβ is that Cβ has an Ig-β cytoplasmic tail, rather than being truncated cytoplasmically. Previous studies have shown that truncation of the Ig-α or Ig-β cytoplasmic tail allows for complete BCR formation and cell surface trafficking (Reichlin *et al.*, 2001). These unexpected results could indicate that the three amino acids in the cytoplasmic tail of ΔαKVK are disrupting its association with Ig-β. The three amino acids (lys, val, lys) are the same three amino acids that compose the cytoplasmic tail of the μ H chain, and they may be preventing ΔαKVK from associating with Ig-β. Alternatively, the construct used by Reichlen *et al.* (2001) is truncated at amino acid 181 of Ig-α (Torres *et al.*, 1996), therefore there are 21 cytoplasmic Ig-α amino acids in that construct, whereas ΔαKVK has none. Those extra amino acids may be enough to allow that construct to associate with Ig-β. A new ΔαKVK construct with a different cytoplasmic tail will confirm whether my findings are due to interference by the KVK cytoplasmic tail, or whether a cytoplasmic tail (being either Ig-α or Ig-β) is required for Ig-α to associate with Ig-β.

In the future the constructs that were created as a part of this project, along with additional constructs, can be used to determine which portions of the extracellular domain are necessary for BCR assembly and cell surface trafficking. The MPα and MPβ constructs will help determine if the membrane proximal region of the extracellular domain will allow BCR assembly and cell surface trafficking. Ig-β constructs, such as MPβ, which are lacking the IG region of the extracellular domain have a deletion is in a very similar region to an alternatively spliced form of the human Ig-β gene (Koyama *et al.*, 1995). This alternatively spliced form has been found to be expressed in some human B cell chronic lymphocytic leukemias (BCLLs) (Cragg *et al.*, 2002). Understanding how the splicing mutation and other mutations affect BCR assembly and cell

surface trafficking is an important part in understanding more about how low levels of BCR cell surface expression affect B cell chronic lymphocytic leukemia (BCLL) formation.

The signalling abilities of the BCR were not examined in this study, but the mutant BCRs that are found to traffic to the cell surface can be used for signalling studies. It may be that BCRs with only Ig-α or Ig-β cytoplasmic tails are not able to interact with the same signalling molecules as those with both Ig-α and Ig-β cytoplasmic tails. This is a likely scenario because although Ig-α and Ig-β both have ITAM motifs in their cytoplasmic domains, Ig-α has two other tyrosine residues that can recruit different B cell proteins (Clark *et al.*, 1992). Signalling studies performed using the J558 lymphoid cell lines would give an indication of how the mutant BCRs would function *in vivo* because the B cell derived cell line should express the proteins normally expressed in a B cell.

And if mutant BCRs signal differently than WT BCRs, it would be expected that B cell development would also be affected. Developmental studies using mouse models have already shown that the cytoplasmic tails of Ig-α and Ig-β have different functions during B cell development. Truncation of the cytoplasmic tails of either Ig-α or Ig-β demonstrated that the Ig-α cytoplasmic tail allows B cell development to a later stage than the Ig-β cytoplasmic tail (Reichlin *et al.*, 2001). Also, having a BCR with two Ig-α cytoplasmic tails allowed the B cell to proceed further developmentally than if it expressed just one Ig-α cytoplasmic tail (Wang *et al.*, 2004). These studies could be taken further to examine which cytoplasmic regions and amino acid residues are important for different aspects of B cell development.

Developmental studies can also be used to model BCLLs. If a BCR is able to traffic to the cell surface, but surface expression or signalling is reduced, the B cell becomes nonfunctional and long-lived, potentially developing into a BCLL (Gordon *et al.*, 2003) Reichlin *et al.*, (2004) have found that if a B cell is allowed to mature prior to inducing a BCR mutation that creates a BCR with two Ig-α cytoplasmic tails, the cell become anergic and long-lived, likely due to lower BCR cell surface expression. Similar BCR mutations may be one of the ways that BCLLs develop. Cragg *et al.* (2002) looked at cells from BCLLs and showed that they tend to express mRNA from a differentially spliced version of the Ig-β gene. The resulting protein is lacking a portion of its extracellular domain. They propose that the mutant protein is acting as a negative

regulator of the BCR and preventing functional levels of signalling from occurring, resulting in the long-lived anergic cell characteristic of a BCLL. In order to further understand B cell leukemias, studies need to be performed that examine which regions of the BCR protein are important for BCR assembly, trafficking and signalling during B cell development. This can be performed using the experimental systems employed for this project as well as using mouse models. This will give significant insight into how BCLLs develop and how they can be cured. Additionally, modeling common BCLL mutations in mice may demonstrate how BCLLs arise and persist in humans.

5.2 Further discussion

BCR assembly and trafficking to the cell surface is dependent upon the proper association of all four components of the BCR. This study has shown that heterodimerization between Ig-α and Ig-β in the extracellular and transmembrane domains is necessary for BCR assembly. Assembly of the BCR within the cell is dependent upon chaperone proteins that retain BCR components in the ER until assembly is complete (Matsuuchi *et al.*, 1992; Venkitaraman *et al.*, 1991). The sequence of events leading to BCR assembly by chaperone proteins may require Ig-α/β heterodimerization in the extracellular domain and the chaperone proteins bound to Ig-α or Ig-β may remain bound until disulfide bond formation occurs between Ig-α and Ig-β. Disulfide bonds could potentially occur between homodimers, but this may be prevented due to conformational differences or due to interactions between the glycosylations of the proteins. Ig-α and Ig-β have a different number of extracellular glycosylation sites, two and four, respectively, and these may assist in disulfide bond formation between heterodimers.

This study has shown that the interaction between mIgM and Ig-α appears to occur in the transmembrane domain. As mentioned, mIgM contains nine polar amino acids in its transmembrane domain. This is unusual for a membrane spanning protein and suggests that these amino acids are likely interacting with another transmembrane protein (Reth, 1992). Taking into consideration that both Ig-α and Ig-β also have polar amino acids in their transmembrane region it is likely that Ig-α and/or Ig-β shield the polar amino acids of mIgM from the hydrophobic lipid bilayer by way of hydrogen bonding between the residues. Additional interactions between Ig-α/β and mIgM may occur by way of extracellular

glycosylations. A study done by Li *et al.* (1998) indicated that the secreted form of IgM that is lacking the transmembrane domain is still able to associate with Ig-α and Ig-β and, when IgM is deglycosylated Ig-α/β binding is reduced.

The formation of the Ig-α/β heterodimer seems to require heterodimerization in the extracellular and transmembrane domains. The extracellular interactions are through disulfide bonds and potentially through other non-covalent interactions or contact between glycosylations. The heterodimerization in the transmembrane domain is conceivably by way of hydrogen bonding between two particular amino acid residues. The fifth residue in the Ig-α transmembrane region is glutamic acid, a negatively charged polar amino acid. This amino acid could be interacting with the sixth amino acid in the Ig-β transmembrane domain, a glutamine residue. Glutamic acid and glutamine are both strongly polar residues and should form strong hydrogen bonds. This bond is strengthened in the transmembrane environment, which does not contain water molecules that compete with these residues for hydrogen bonding (Partridge *et al.*, 2002).

The experiments showing that Ig-α mutant constructs can associate with mIgM prior to binding to Ig-β is contrary to the belief that the human Ig-α/β heterodimer must form before associating with human mIgM (Brouns *et al.*, 1995), but supports data by Matsuuchi and Foy (2001) who showed that murine Ig-α will associate with mIgM in the absence of Ig-β. These findings may indicate that the mouse BCR forms differently than the human BCR, but further studies are needed to confirm this finding because the truncated Ig-α proteins, ΔαKVK (α/α/0) and ΔXα (0/α/α) may interact differently with truncated Ig-β proteins than they do with WT Ig-β. In the future, construct combinations pairing ΔαKVK (α/α/0) and ΔβKVK (β/β/0) or pairing Solα (α/0/0) and Solβ (β/0/0) may indicate whether Ig-α/β heterodimerize before interacting with mIgM or if heterodimerization occurs after either Ig-α or Ig-β interact with mIgM.

Overall, I have found that the extracellular and transmembrane domains of Ig-α/β need to be heterodimeric in order for BCR assembly and cell surface trafficking to occur. Dimerization in the cytoplasmic domain may also be required, but additional studies will need to be performed in order to confirm this finding. The experiments performed as a part of this study should be the beginning of many avenues of research for the Matsuuchi lab, including studying BCR assembly

and signalling by creating mutant BCRs and examining the role of the BCR in B cell development using mouse models.

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APPENDIX

Ig-α sequence (sequence from NCBI online database, sequence number gi6680891 Mus musculus CD79A antigen, mRNA).

Interprotein disulfide bonds between cysteine residues 50 and 101, disulfide bonding with Ig-β at cysteine residue 113 and glycosylation sites at amino acids 58 and 68 (Sakaguchi *et al.*, 1988, Hombach *et al.*, 1988).

1 tgggagacgatgccaggggtctagaagccctcagagccctgcctctcctcctcttcttg 60
1 M P G G L E A L R A L P L L L F L
Signal Peptide (aa 1-22)

61 tcatacgcctgtttgggtcccggatgccaggccctgcgggtagaaggggtccaccatcc 120
18 S Y A C L G P G C Q A L R V E G G P P S
NT region (aa 1-34)

121 ctgacggtgaacttgggcgaggaggcccgcctcacctgtgaaaacaatggcaggaaccct 180
38 L T V N L G E E A R L T C E N N G R N P

181 aatatcacatggtggttcagccttcagtctaacatcacatggcccccagtgccactgggt 240
58 N I T W W F S L Q S N I T W P P V P L G
IG region (aa 35-120)

241 cctggccagggtaccacaggccagctgttcttccccgaagtaaacaagaacacaggggct 300
78 P G Q G T T G Q L F F P E V N K N T G A

301 tgtactgggtgccaagtgatagaaaacaacatattaaaacgctcctgtggtacttacctc 360
98 C T G C Q V I E N N I L K R S C G T Y L

cgcgtgcgcaatccagtccctaggcccttcctggacatgggggaaggtaccaagaaccgc 420
118 R V R N P V P R P F L D M G E G T K N R
MP region (aa 121-137)

421 atcatcacagcagaagggatcatcttgctgttctgtgcagtggtgccagggacgctgctg 480
138 I I T A E G I I L L F C A V V P G T L L
TM domain (aa 138-159)

481 ctattcaggaaacggtggcaaaatgagaagtttggggtggacatgccagatgactatgaa 540
158 L F R K R W Q N E K F G V D M P D D Y E

541 gatgaaaatctctatgagggcctgaaccttgatgactgttctatgtatgaggacatctcc 600
178 D E N L Y E G L N L D D C S M Y E D I S
Cytoplasmic domain (aa 160-220)

601 aggggactccagggcacctaccaggatgtgggcaacctccacattggagatgcccagctg 660
198 R G L Q G T Y Q D V G N L H I G D A Q L

661 gaaaagccatgactgacatgtcccacccttccctgcctgccatatgtctgactccagcat 720
218 E K P ^^^

Ig-β sequence (sequence from NCBI online database, sequence number gi6680374 Mus musculus CD79B antigen, mRNA).

Interprotein disulfide bonds between cysteine residues 43 and 124 and between 65 and 120, disulfide bonding with Ig-α at cysteine residue 135 and glycosylation sites at amino acids 68, 99, and 130 (Hermanson *et al.*, 1988).

181 gaccatggccacactggtgctgtcttccatgccctgccactggctgttgttcctgctgct 240
1 M A T L V L S S M P C H W L L F L L L
Signal Peptide (aa 1-25)

241 gctcttctcaggtgagccggtaccagcaatgacaagcagtgacctgccactgaatttcca 300
20 L F S G E P V P A M T S S D L P L N F Q
NT region (aa 1-49)

301 aggaagcccttgttcccagatctggcagcacccgaggtttgcagccaaaaagcggagctc 360
40 G S P C S Q I W Q H P R F A A K K R S S

361 catggtgaagtttcactgctacacaaaccactcaggtgcactgacctggttccgaaagcg 420
60 M V K F H C Y T N H S G A L T W F R K R

421 agggagccagcagccccaggaactggtctcagaagagggacgcattgtgcagacccagaa 480
80 G S Q Q P Q E L V S E E G R I V Q T Q N

481 tggctctgtctacaccctcactatccaaaacatccagtacgaggataatggtatctactt 540
100 G S V Y T L T I Q N I Q Y E D N G I Y F
IG region (aa 50-142)

541 ctgcaagcagaaatgtgacagcgccaaccataatgtcaccgacagctgtggcacggaact 600
120 C K Q K C D S A N H N V T D S C G T E L

601 tctagtcttaggattcagcacgttggaccaactgaagcggcggaacacactgaaagatgg 660
140 L V L G F S T L D Q L K R R N T L K D G
MP region (aa 143-158)

661 cattatcttgatccagaccctcctcatcatcctcttcatcattgtgcccatcttcctgct 720
160 I I L I Q T L L I I L F I I V P I F L L
TM domain (aa 159-179)

721 acttgacaaggatgacggcaaggctgggatggaggaagatcacacctatgagggcttgaa 780
180 L D K D D G K A G M E E D H T Y E G L N

781 cattgaccagacagccacctatgaagacatagtgactcttcggacaggggaggtaaagtg 840
200 I D Q T A T Y E D I V T L R T G E V K W
Cytoplasmic domain (aa 180-228)

841 gtcggtaggagagcatccaggccaggaatgagggtcaccttcatcctgctcaactcttgg 900
220 S V G E H P G Q E ^^^