

ABSTRACT

Title of Dissertation: THE ROLE OF THE $Ig\alpha/Ig\beta$ HETERODIMER IN THE
INTERNALIZATION AND INTRACELLULAR TRANSPORT
OF THE B CELL ANTIGEN RECEPTOR

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The B cell antigen receptor (BCR) consists of membrane-bound immunoglobulin (mIg), which binds extracellular antigen, and the $Ig\alpha/Ig\beta$ heterodimer ($Ig\alpha/Ig\beta$), the signaling component. Crosslinking of BCR by multivalent antigen initiates signaling, the first step in activating B cells, and leads to rapid internalization and transport of antigen to the major histocompatibility complex (MHC) class II containing compartments (MIICs). Here antigen is degraded to peptides which are loaded onto MHC class II molecules which are transported to the surface for presentation to T helper cells. Studies indicate

that BCR-triggered signaling plays a role in accelerated internalization and transport of antigen, but the mechanism is not clear. This work examines the role of the signaling component $Ig\alpha/Ig\beta$ in the efficient internalization and intracellular transport of the BCR. The effect of a lack of association between $Ig\alpha/Ig\beta$ and mIgM on internalization and intracellular transport of mIgM was studied using biochemical techniques and immunofluorescence microscopy. We demonstrated that a reduction of association with $Ig\alpha/Ig\beta$ leads to a reduction in signaling, a defect in internalization and transport to MIICs, and a decrease in antigen presentation. Thus, physical association of mIgM with $Ig\alpha/Ig\beta$ is necessary for efficient internalization and intracellular transport of mIgM. The role of the tyrosines in the immunoreceptor tyrosine-based activation motif (ITAM) of $Ig\alpha/Ig\beta$ was examined. Mutation of the N-terminal tyrosine had no significant effect on BCR signaling and antigen transport. Mutation of the C-terminal tyrosine resulted in decreased signaling and internalization and a defect in transport to MIICs. Mutating both tyrosines caused a greater decrease in BCR signaling and a greater defect in internalization and transport of antigen to MIICs than the C-terminal tyrosine mutation. The results of this study indicate that $Ig\alpha/Ig\beta$, in particular the ITAM of $Ig\alpha$, is essential for accelerated internalization and intracellular transport of BCR to the MIICs, probably by regulating these processes through signaling.

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By

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LIST OF ABBREVIATIONS

[¹²⁵ I]Fab	Iodine-labeled Fab
AP-2	Adaptor protein 2
APC	Antigen presenting cells
BAFF	B cell activating factor belonging to the tumor necrosis factor family
BCR	B cell antigen receptor
BLNK	B cell linker protein
CLIP	Class II associated invariant chain peptide
DAG	Diacylglycerol
EE	Early endosome
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
HA	Hemagglutinin
Ig α /Ig β	Ig α /Ig β heterodimer
Ii	Invariant chain
IP ₃	Inositol triphosphate
ITAM	Immunoreceptor tyrosine based activation motif
JNK	c-Jun NH ₂ -terminal kinase
LE	Late endosome

LAMP-1	Lysosomal associated membrane protein 1
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
mIg	Membrane immunoglobulin
MIIC	MHC class II containing compartment
NF κ B	Nuclear factor κ B
NFAT	Nuclear factor of activated T cells
PH	Pleckstrin homology
PI	Phosphoinositol
PI-3-K	Phosphatidylinositol-3-kinase
PIP ₂	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PLC γ 2	Phospholipase C- γ
PM	Plasma membrane
PTK	Protein tyrosine kinase
RAG	Recombination-activating gene
RSS	Recombination signal sequences
SH2	Src homology 2
wt	wild type
XL	Crosslinked

Chapter I. General Introduction

The human immune system has developed to recognize and remove foreign particles of invading organisms as well as to destroy the organisms themselves. There are two parts to this response, the innate response and the adaptive response. In the innate response macrophages and neutrophils, recognize, engulf and digest microorganisms, providing the first line of non-specific defense against invading organisms. Dendritic cells can also recognize and ingest pathogens and take up antigen by phagocytosis or nonspecifically by macropinocytosis. Natural killer cells are also part of the innate immune response. They lack antigen specific receptors but can kill tumor cells and cells infected with certain pathogens. The adaptive immune response involves B and T cells responding specifically and selectively to a wide variety of foreign antigens. The adaptive immune response also leads to lasting immunological memory, which provides increased protection against subsequent re-infection with the same pathogen. These two mechanisms are not completely separated, but interactive. For instance, macrophages that have phagocytosed bacteria or dendritic cells that have taken up antigen specifically or nonspecifically can present antigen to naive T cells, and thus activate them. Natural killer cells secrete interferon- γ , which helps activate helper T cells. Antibodies produced by B cells can opsonize a pathogen to facilitate phagocytosis and activate the complement system.

1.1 B cells

B cells are one of the lymphocytes of the immune system, along with T cells and natural killer cells, and are an important component of adaptive immunity. Their main function

is to produce and secrete soluble antibodies that recognize foreign antigens. Mammalian B cells develop from lymphoid progenitors in the bone marrow. Immature B cells leave the bone marrow and travel to the secondary lymphoid tissues which provide survival signals through BAFF (B cell activating factor belonging to the tumor necrosis factor family) and BAFF receptor interactions [1]. If they survive, these cells further develop into mature B cells. Mature B cells circulate in the blood stream and the lymphatic system and return to the secondary lymphoid tissues, where encounter with and presentation of foreign antigens occurs. Each B cell expresses antigen receptors (which are membrane-bound versions of its secreted antibody) are specific for a particular foreign antigen, and initiates B cell activation upon encountering the appropriate foreign antigen. Even though each B cell produces an antibody specific for one foreign antigen, mammals express a very large repertoire of antibodies. One method that leads to this diversity of antigen binding is the mechanism of somatic recombination of the immunoglobulin (Ig) genes which code for the proteins that make up both the membrane bound antigen receptor and the soluble antibody. There are multiple copies of each gene segment (V and J for light chains and V, J and D for heavy chains), and one gene segment of each type are randomly selected to create the variable region of the Ig [2]. Different combinations of the gene segments lead to diversity of the antigen binding region. There are sequence motifs known as recombination signal sequences (RSS) that are found adjacent to the V, D or J gene segments. RSSs consist of a heptamer of conserved nucleotides, a spacer that is 12 or 23 nucleotides, and a nonamer of conserved nucleotides. A gene segment that has an RSS with a 12 base pair spacer usually can only join with a gene segment that has an RSS with a 23 base pair spacer, which is known as

the 12/23 rule. Therefore, normally recombination can only occur so that a V gene segment joins to a D gene segment which joins to a J gene segment (for the heavy chain). It has been shown that sometimes this regulation is bypassed. In many species there are instances of a D segment joining another D segment. This unusual occurrence also leads to more diversity. During the process of recombination, proteins encoded by the recombination-activating genes (RAG-1 and RAG-2) [3] form complexes which bind to a 12 or 23 base pair spacer, which is how the 12/23 rule is accomplished. These protein complexes then bind to each other, which brings the gene segments in proximity. The DNA just beyond the gene segment is cleaved and hairpin structures are created. The DNA hairpins are then cleaved and subsequently the ends of the gene segments are joined together. Importantly, after the hairpins are cleaved and before the gene segments are joined together, additional nucleotides may be added or subtracted [4]. These nucleotides are called P-nucleotides when they are palindromic sequences at the end of the gene segment that are created by the cleavage of the hairpin. Nucleotides that are added to the single stranded DNA after hairpin cleavage are called N-nucleotides because they are nontemplate-encoded. Nonmatching bases are removed, complimentary nucleotides are added, and ligation leads to a coding joint between the gene segments. The nucleotides are added or deleted randomly, thus leading to a unique section of DNA sequence, which is yet another way that diversity of the variable region is generated. Somatic recombination occurs for both heavy and light chain genes in each B cell. The heavy and light chain proteins assemble to form the immunoglobulin, and each of their variable regions contributes to the specificity of the antigen binding site, thus leading to yet another way diversity is achieved. In the presence of T cell help, activation of a B cell

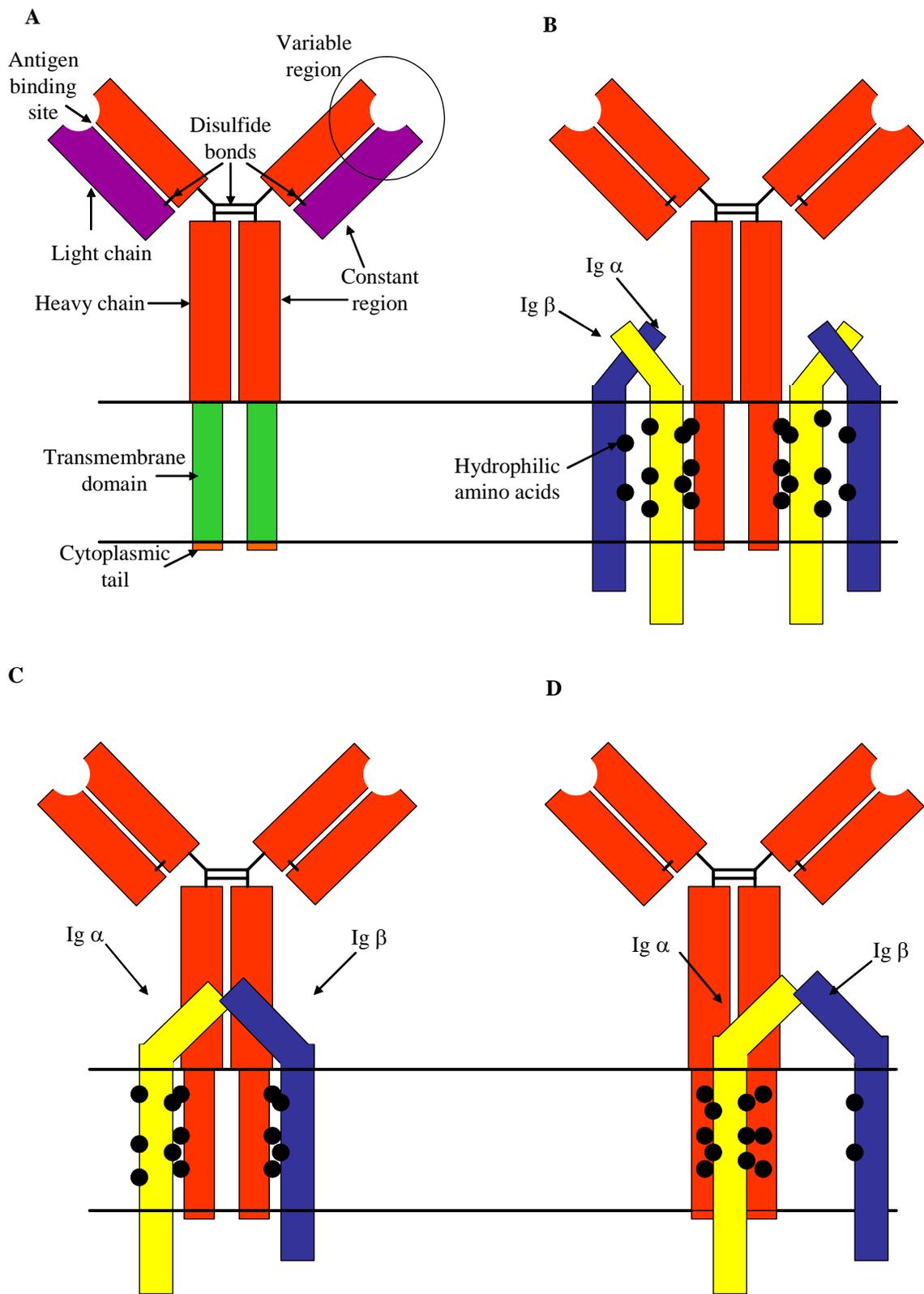
also leads to somatic hypermutation in the germinal center. This occurs after gene rearrangement, and point mutations occur in the V regions of heavy and light chain genes [5, 6]. These mutations occur at a high rate, and lead to expression of B cell receptors that are somewhat different than the original receptor. Only B cells express antigen receptors with a higher affinity for antigen will be selected to survive and differentiate into plasma cells. This process of selecting B cells with “improved” receptors is known as affinity maturation. Somatic hypermutation is yet another mechanism that leads to the large diversity of the antibody repertoire. The combination of all these mechanisms allows for the production of $\sim 10^{11}$ unique antibody molecules in a human [7].

1.2 The B cell antigen receptor

The B cell antigen receptor (BCR) complex consists of two parts: the membrane immunoglobulin (mIg) which binds antigen and the $Ig\alpha/Ig\beta$ heterodimer ($Ig\alpha/Ig\beta$) which transduces signals. $Ig\alpha/Ig\beta$ and mIg non-covalently associate with each other in the endoplasmic reticulum and continue to associate as they are transported to the cell surface, and this association is required for the surface expression of the BCR. The mIg of the BCR is composed of two identical heavy chains and two identical light chains (Fig. 1-1). Each light chain is bound to a heavy chain by disulfide bonds, and two heavy chains are bound to each other by disulfide bonds. Both heavy and light chains have constant and variable regions. The variable regions of the heavy and light chain interact to form the antigen binding site. This allows each BCR molecule to have a unique specificity for antigen and to bind two identical ligands at its N-terminal regions at the same time. The heavy chains of mIgs have a transmembrane segment and a cytoplasmic

Figure 1-1. The B cell receptor complex.

(A) The structure of the mIg. (B) The complete BCR, including the $Ig\alpha/Ig\beta$ heterodimer. Initially it was thought that there was a heterodimer associated with each heavy chain of the mIg. Recent work has shown that only one heterodimer associates with one mIg. (B) It is still unknown whether each member of the heterodimer associates with a single heavy chain or (C) if $Ig\alpha$ associates with both heavy chains simultaneously.



tail. The transmembrane portion contains many polar amino acids, which are important for its interaction with Ig α /Ig β . The cytoplasmic tail of the mIg varies based on the isotype, but for IgM and IgD, the two isotypes found on naïve mature B cells, they are quite short, being only 3 amino acids long and do not appear to be able to transduce signal.

Ig α /Ig β and the mIg associate non-covalently, and their transmembrane domains seem to be crucial to this association. Shaw et al demonstrated that when human μ chain DNA which has a double mutation in the transmembrane domain (YS/VV) is transfected into a murine lymphoma cell line, mIgM are expressed on the surface. They examined signal transduction capability by measuring Ca²⁺ influx, and found that the YS/VV mIgM had a great defect in Ca²⁺ response compared to the wt transfected mIgM [8]. Other studies using this same cell system confirmed that the YS/VV mIgM had a defect in signaling and also showed that YS/VV mIgM failed to associate with Ig α /Ig β as wt mIgM did [9, 10]. Other researchers who transfected murine DNA into a different murine cell line demonstrated that YS/VV mIgM had a defect in signaling, measured by protein tyrosine phosphorylation and inositol production as well as Ca²⁺ influx. They also showed that YS/VV mIgM had a greatly reduced association with Ig α /Ig β compared to wt transfected mIgM [11].

As mentioned, the transmembrane domain of the mIg contains many hydrophilic residues [12]. Ig α also contains many hydrophilic residues on both sides of its putative α -helix transmembrane domain, while Ig β only has hydrophilic residues on one side [12]. This

implies that $Ig\alpha$ is in the middle of a mIg heavy chain/ $Ig\alpha/Ig\beta$ complex. Since there are two heavy chains, it was initially thought that there would be an $Ig\alpha/Ig\beta$ associated with each heavy chain. Recent work by Schamel and Reth [13] to determine the stoichiometry of the BCR showed that there is only one $Ig\alpha/Ig\beta$ heterodimer associated with each mIg. Schamel and Reth expressed FLAG epitope-tagged $Ig\alpha$ proteins in a variant of the J558L B cell line that already expressed a complete transfected BCR, including an untagged $Ig\alpha$. The tagged and untagged $Ig\alpha$ s could be differentiated on immunoblots based on molecular weight and by antibodies against the epitope tag. This allowed Schamel and Reth to observe whether one BCR complex contained both tagged and untagged $Ig\alpha$. After solubilizing the cells and isolating the BCRs by binding to its known antigen, the BCRs were then immunoprecipitated first using anti-FLAG antibodies and then anti- μ antibodies. The BCRs that were immunoprecipitated using anti-FLAG antibodies were found to only have FLAG-tagged $Ig\alpha$ s. The supernatant from the previous immunoprecipitation was then used to immunoprecipitate BCRs using anti- μ antibodies. These BCRs were found to only contain untagged $Ig\alpha$ s. The same results were obtained with an HA-tagged $Ig\alpha$ or cells expressing both FLAG and HA-tagged $Ig\alpha$ s. Sequential immunoprecipitation showed that only one type of $Ig\alpha$ protein was found in the BCRs. Schamel and Reth further assessed the stoichiometry of the BCR complex by radiolabeling the BCR-expressing J558L cells, and determining the relative amounts of radioactivity of the IgH, IgL, $Ig\alpha$ and $Ig\beta$ in isolated BCR complexes. These results indicated that each BCR contained only one $Ig\alpha/Ig\beta$ [13]. The question that remains to be answered is how the subunits of the BCR complex are arranged (Fig. 1-1B, C, and D).

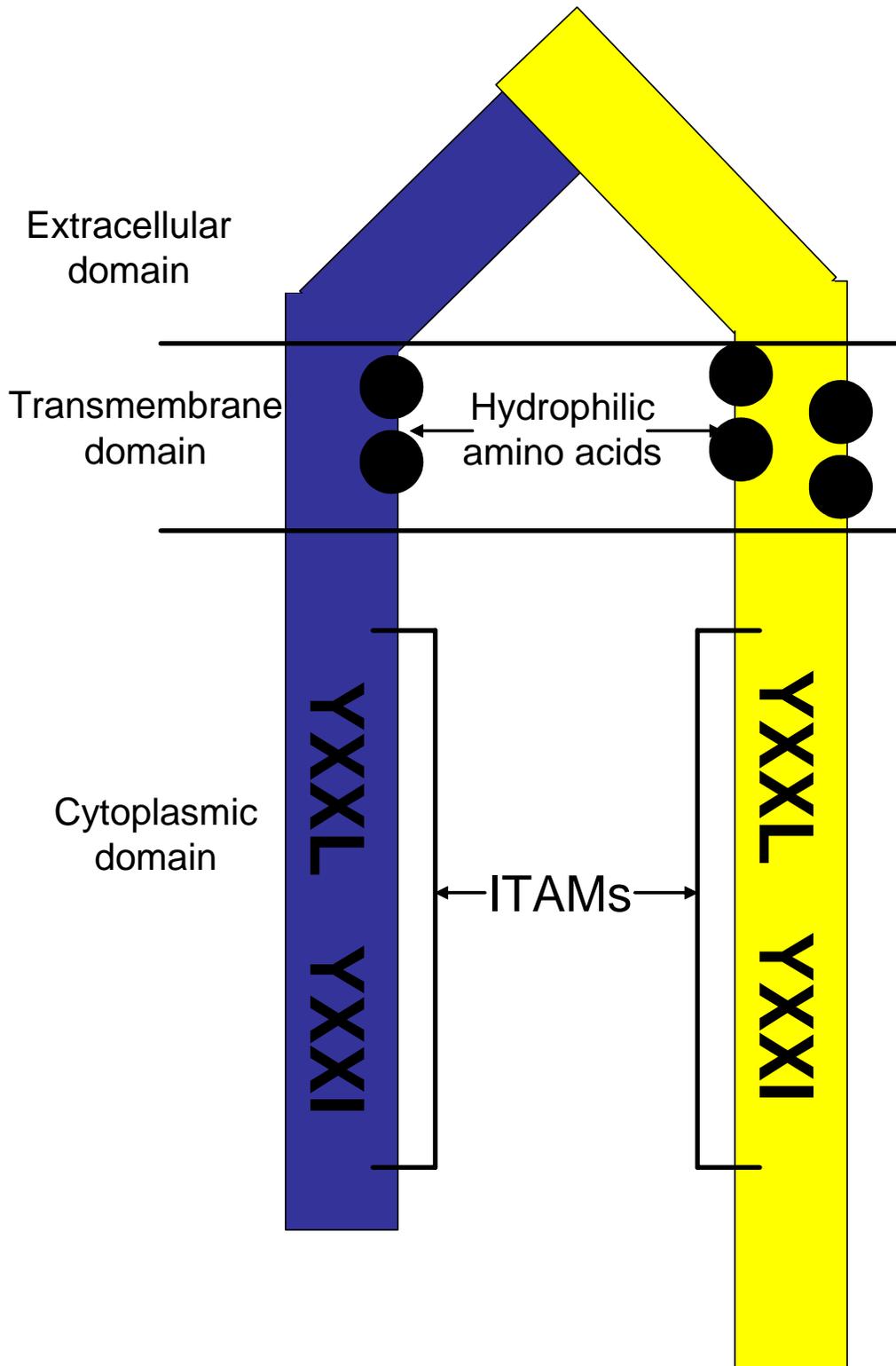
1.3 Structure of the Ig α /Ig β heterodimer

Each subunit of the Ig α /Ig β heterodimer has an N-terminal Ig-like extracellular domain, a single transmembrane domain, and a moderate size cytoplasmic domain (61 and 48 amino acids respectively) [12]. Ig α and Ig β are the products of the mb-1 [14, 15] and B29 [16, 17] genes, respectively, and are also known as CD79a and CD79b. The Ig α /Ig β proteins were discovered in a transfection experiment with a myeloma cell line (J558L) lacking μ chain. When J558L was transfected with a vector containing DNA of membrane μ chain, complete mIgM molecules assembled in the endoplasmic reticulum of the J558L μ cells, but were not transported to the surface [18, 19]. A variant of the J558L μ cells (J558L μ 3) was found to transport mIgM to the surface. In these cells, the mIgMs were found to be non-covalently associated with a disulfide-linked heterodimer of 34 kDa and 39 kDa glycoproteins [20, 21]. These molecules were not found in the original J558L μ cells. This demonstrated that these proteins were required for transport of mIgM to the cell surface. These proteins were later named Ig α and Ig β . The Ig α /Ig β heterodimer has now been found to associate with the membrane bound forms of all five Ig classes [22].

The Ig α and Ig β cytoplasmic tails each contain a consensus motif known as an immunoreceptor tyrosine-based activation motif (ITAM) (Fig. 1-2). The ITAM consists of two negatively charged amino acids and two tyrosines followed by either a leucine or an isoleucine residue (D/EX₇D/EXXYXX-LX₇YXXL/I) [23]. This motif is also found in the cytoplasmic portion of the CD3 ζ , γ , δ components of the T cell receptor complex [24] and the Fc receptor gamma chain [25]. Ig α /Ig β has been found to be the signaling

Figure 1-2. The structure of the $Ig\alpha/Ig\beta$ heterodimer.

Each member of the heterodimer consists of an extracellular, transmembrane and cytoplasmic domain. The transmembrane domain contains many hydrophilic amino acids which are thought to be important in the association with the mIg. The cytoplasmic domain contains the immunoreceptor tyrosine activation motifs (ITAMs) which are involved in signaling. It also contains the putative sorting signals (YXX Φ , where X stands for any amino acid and Φ stands for an amino acid with a bulky hydrophobic side chain).



component of the BCR complex, as will be discussed in a later section. Another interesting aspect of the ITAMs is that their key element, YXXL/I, is also a putative sorting signal. The tyrosine-based YXX ϕ sorting signal (X stands for any amino acid and ϕ stands for an amino acid with a bulky hydrophobic side chain) is found in many transmembrane proteins, such as mannose 6-phosphate receptors, transferrin receptor, lysosomal associated membrane protein-1 (LAMP-1), and HLA-DM, and has been shown to mediate internalization from the plasma membrane as well as the targeting of proteins to particular endosomal compartments [26-29]. In the ITAMs of Ig α and Ig β there are two potential YXX Φ sorting signals, one at each tyrosine (Fig. 1-2), but these have not yet been proven to be actual sorting signals.

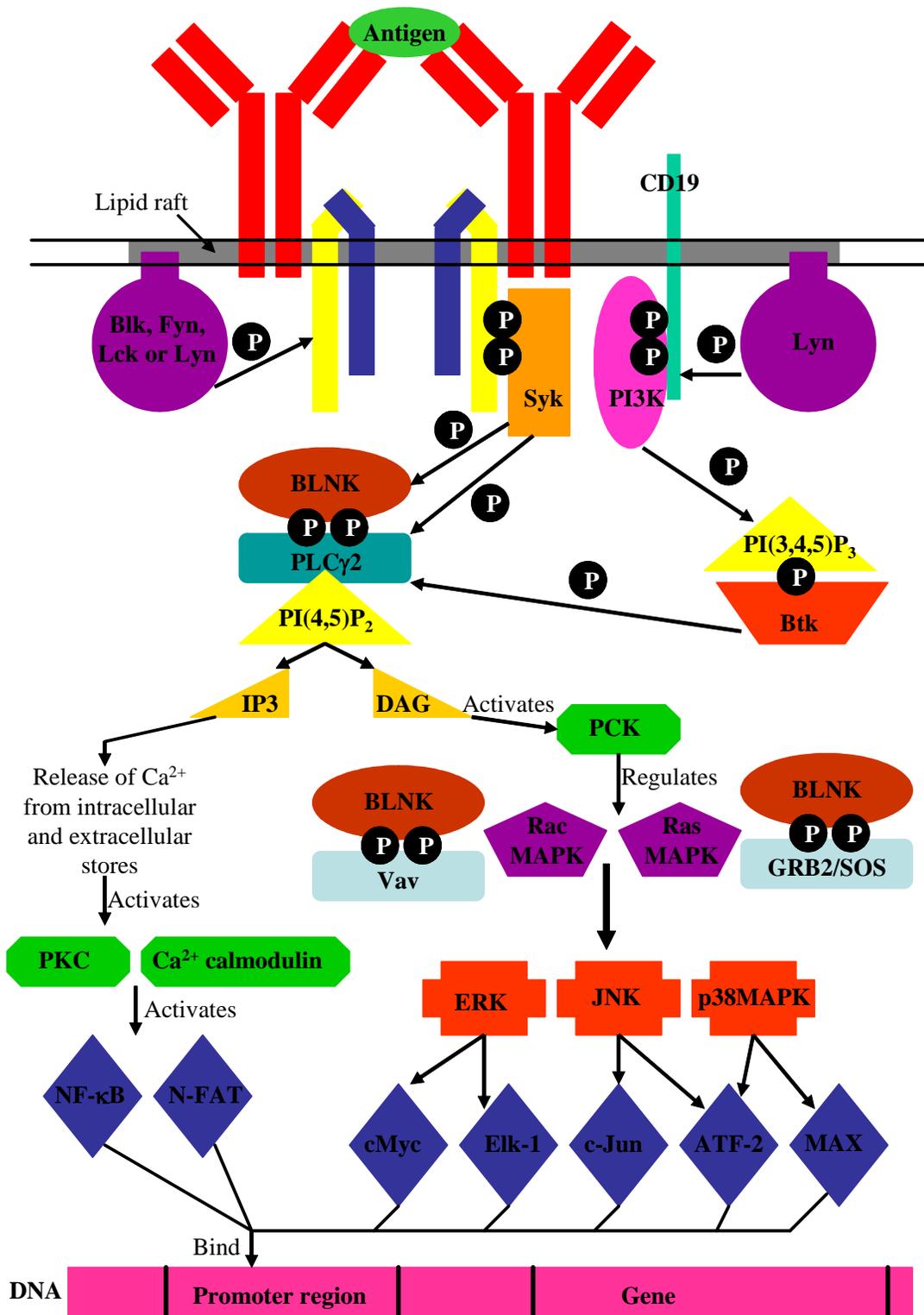
1.4 Signaling through the BCR

Binding of the BCR by multivalent antigen causes receptor aggregation and leads to activation of many effector molecules, which initiate signaling within the B cell. The first step after aggregation is phosphorylation of the ITAM tyrosine residues on Ig α and Ig β . This is accomplished by protein tyrosine kinases (PTKs), particularly the src-family kinases such as Lyn, Fyn, Blk, or Lck [30]. These PTKs are anchored in the plasma membrane due to acylation, and they have an N-terminal region that interacts with the non-phosphorylated ITAMs of resting BCR (Fig. 1-3) [31, 32]. This interaction is enhanced due the fact that ligand-aggregated BCRs move into glycosphingolipid-rich microdomains of the plasma membrane, known as lipid rafts [33-36]. It has been shown that these rafts also have increased concentrations of PTKs, which can then easily phosphorylate the BCRs that have moved into the raft [37].

Figure 1-3. The BCR signaling cascade.

Initially src-family protein tyrosine kinases phosphorylate the ITAM tyrosine residues on Ig α /Ig β . This allows Syk to associate with these phosphorylated residues via its SH2 domain. This activates Syk, which then activates BLNK which recruits PLC γ 2.

Activation of PI3K by Lyn allows PI3K to activate PI(3,4,5)P₃, which activates Btk, which in turn activates PLC γ 2. PLC γ 2 then cleaves PI(4,5)P₂ into inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ releases of Ca²⁺ from intracellular and extracellular stores which activates transcription factors such as NF- κ B and N-FAT by protein kinase C (PKC) and Ca²⁺-calmodulin. DAG and Ca²⁺ activate PKCs which then regulate the mitogen activated protein kinase (MAPK) family. The Rac and Ras MAPK pathways are activated by Vav, a guanine exchange factor, and GRB2/SOS, respectively, which associate with phosphorylated BLNK. Shown in this figure are important members of the MAPK family: extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK) and p38 MAPK. Once activated, each kinase targets particular transcription factors which it phosphorylates and therefore activates: ERK activates Elk-1 and cMyc, JNK activates c-Jun and ATF-2, and p38 MAPK activates ATF-2 and MAX. These transcription factors, along with NF- κ B and N-FAT, bind to the promoter regions of genes and leads to their transcription.



The first phosphorylation of the ITAMs is achieved by the PTKs independent of their Src homology 2 (SH2) domains, which bind phosphorylated tyrosine residues. The binding of the src-family kinase SH2 domains to the phosphorylated ITAMs amplifies ITAM phosphorylation, which leads to the recruitment and activation of other effector molecules [38]. One of these recruited effector molecules is the cytosolic tyrosine kinase Syk. It too can bind to the ITAMs through its two SH2 domains, provided both tyrosines of the ITAM are phosphorylated [39]. This binding results in the activation of Syk which leads to the initiation of several different signaling pathways. One way Syk does this is by phosphorylating the adaptor molecule B cell linker protein (BLNK), which recruits phospholipase C gamma 2 (PLC γ 2) from the cytosol through PLC γ 2's SH2 domain [40-42].

BCR aggregation also results in the phosphorylation of the BCR co-receptor molecule CD19 by Lyn. This creates binding sites for the SH2 domains of phosphatidylinositol-3-kinase (PI-3K), which in turn activates PI-3K [43-46]. PI-3K then phosphorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), producing phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P₃). PI(3,4,5)P₃ recruits Btk via its PH-domain, which activates Btk [47, 48].

After Lyn, Syk, and Btk are activated, signaling is propagated through many effector molecules. As mentioned above, PLC γ 2 is recruited to the plasma membrane by BLNK, but it must be phosphorylated by Syk and Btk for optimal activation [49-51]. Once activated, PLC γ 2 cleaves PI(4,5)P₂ into the secondary messengers inositol triphosphate

(IP₃) and diacylglycerol (DAG). IP₃ allows for the release of Ca²⁺ from intracellular and extracellular stores. This is required for the activation of transcription factors such as NF-κB and N-FAT by protein kinase C (PKC) and Ca²⁺-calmodulin [52-54]. DAG activates PKCs which then regulate the mitogen activated protein kinase (MAPK) family. The Rac and Ras MAPK pathways are activated by Vav, a guanine exchange factor, and GRB2/SOS, respectively, which associate with phosphorylated BLNK. Important members of the MAPK family are extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK) and p38 MAPK [55, 56]. Once activated, each kinase targets particular transcription factors which it phosphorylates and therefore activates. ERK activates Elk-1 and cMyc, JNK activates c-Jun and ATF-2, and p38 MAPK activates ATF-2 and MAX [56]. These transcription factors, along with NF-κB and N-FAT, bind to the promoter regions of genes whose transcription leads to activation, anergy or apoptosis of the cell.

1.5 Igα/Igβ are the signaling components of the BCR

Igα/Igβ is essential for the BCR signaling function. When the C-terminal region (transmembrane and cytoplasmic domains) of the μ heavy chain is replaced by the same regions of other proteins, such as CD8, or the cytoplasmic tail is deleted, the mIgM is expressed on the surface without association with Igα/Igβ [9, 57-59]. These mIgMs are not able to initiate tyrosine phosphorylation of cytoplasmic proteins or increase intracellular Ca²⁺ concentrations upon BCR crosslinking. Additionally, chimeric proteins of the extracellular and transmembrane domains of mIgM and the cytoplasmic tails of Igα and Igβ were able to activate Ca²⁺ flux and phosphorylation of proteins [60].

Membrane IgM with mutations in the transmembrane domain, which do not associate with Ig α /Ig β well or at all, have corresponding problems with signaling [9, 61]. One particular mutation in the hydrophilic region of the transmembrane domain which changes adjacent tyrosine and serine residues to valines (YS/VV), has been studied extensively [9-11]. This mutation in murine μ chain has been shown to have decreased association with Ig α /Ig β and reduced signal transduction [11]. The decreased signaling was characterized by decreased tyrosine phosphorylation, decreased inositol phosphate production and a smaller increase in Ca²⁺ influx. Other studies showed that the ITAMs of Ig α and Ig β are required for optimal signaling and have similar abilities to the whole cytoplasmic tails of Ig α and Ig β to trigger tyrosine kinase activation [62-64]. It was also shown that when the tyrosines of the ITAMs are mutated, signaling is greatly decreased, indicating that these residues are important for optimal signaling [62-65].

1.6 The importance of Ig α /Ig β signaling in development and survival of B cells

During B cell development, Ig α /Ig β heterodimer is found to express as early as the pro-B cell stage. Ig α /Ig β on the surface of pro-B cells has been shown to associate with calnexin [66] and to be able to generate signals, as measured by induction of tyrosine phosphorylation of cellular proteins [67]. The crucial role of Ig α /Ig β at the developmental stage from the pro-B cells to the pre-B cell stage has been demonstrated both in human and mouse. A human patient carrying a mutation that prevents Ig α expression lacked pre-B, immature, and mature B cells [68]. B cells of mice with either Ig α or Ig β gene knocked out do not progress past the pro-B cell stage [69, 70]. Studies using transgenic mice that express chimeras containing the extracellular and

transmembrane domains of IgM and the cytoplasmic domains of Ig α or Ig β showed that the cytoplasmic tail of either Ig α or Ig β was capable of inducing the pro- to pre-B cell transition independently [71, 72]. Kraus et al demonstrated that the Ig α ITAM tyrosines are important for the pro- to pre-B cell transition [73]. They generated transgenic mice in which the Ig α ITAM tyrosines were replaced with phenylalanines, and the Ig β cytoplasmic tail was deleted. In these mice, B cell development was completely blocked at the pro-B cell stage.

Ig α /Ig β is a part of the pre-BCR complex, which includes the membrane bound μ chains and surrogate light chains [74]. Interestingly, since this pre-BCR contains a surrogate light chain that is not polymorphic, the pre-BCR cannot bind conventional antigens, yet it has been shown to induce signals [75, 76]. This antigen-independent signaling is called tonic signaling. Although there are possible unconventional antigens for the pre-BCR [77], the results of several studies indicate that the specificity of the pre-BCR is not important for development beyond this stage [78-80]. It is possible that the extracellular domains of Ig α /Ig β were involved in ligand binding and signaling triggering that induced the development of the B cells. Bannish et al. established a chimeric protein (MAHB) that consisted of the cytoplasmic tails of Ig α and Ig β and the Lck myristoylation/palmitoylation sequence, which allowed to target the signaling components to the inner leaflet of the plasma membrane without having extracellular or transmembrane domains that may interact with ligand. [81]. B cells in the transgenic mice expressing these chimeric proteins had did progress beyond pre-B cells and had tonic signal induction that was comparable to basal signaling by unligated wt BCR. This

study showed that the only requirement for tonic signaling is the expression of Ig α /Ig β at the plasma membrane. Another study showed that although many pre-BCR complexes are assembled in the ER, these complexes are not signal competent and cannot initiate development unless transported to the cell surface [82]. It has been shown in chickens that the Ig α cytoplasmic tail is necessary and sufficient to initiate development beyond the pre-B cell stage [83]. Signaling induced by Ig α /Ig β appears to be important for development. In the transgenic mice that expressed the MAHB chimeric protein, the development of the B cells was blocked if the ITAM tyrosines of Ig α were mutated to phenylalanines [81]. In chickens, if Ig β was not present, B cell development only proceeded when the ITAM tyrosines of Ig α were intact [83]. Thus signaling mediated by Ig α /Ig β is important at this stage of development.

In mature B cells, it has been shown that after antigen-aggregated BCRs move into lipid raft domains, signaling is initiated [37]. Since it was observed that 20-30% of pre-BCR is in lipid rafts [84], it was thought that this may be one way that tonic signaling is generated, since it does not appear to be due to ligand-induced aggregation. A study utilizing the MAHB chimeric protein which was altered so that one form would target to lipid rafts while another form would target to nonraft compartment showed that the pre-BCR does not need to be localized to lipid rafts in order for tonic signaling or B cell development to occur [85].

Ig α /Ig β is important for later stages of B cell development and B cell functions as well. B cells expressing BCRs containing two Ig α cytoplasmic tails but no Ig β do develop to

mature B cells but are anergic [86]. A study using transgenic mice showed that only B cells expressing chimeras consisting of both Ig α and Ig β developed to mature B cells and were activated at the same level as B cells expressing wt BCR [87]. Mice which lack most of the Ig α cytoplasmic tail do not generate the a peripheral B cell pool as those with wt BCRs [88]. Early studies showed that a lack of BCR expression leads to cell death [89], and signaling can occur through the BCR in the absence of antigen [90]. A more recent study, using inducible ablation of the ITAM coding region of the Ig α gene, has clarified that it is the signaling through the BCR that is necessary for the survival of peripheral B cells [91]. A similar study using mutated Ig β showed like results [92]. A recent study using a B cell lymphoma cell line found that after crosslinking of the BCR, most of the mIg was internalized while 20-30% of Ig β remained on the surface [93]. These researchers speculated that the signaling component may be needed to remain on the surface in order to maintain the activation state and survival of the cell. Thus tonic signaling through the BCR appears to be important for B cell survival, but it is not clear yet how, and whether the effect is direct or indirect. The data accumulated so far demonstrated that Ig α /Ig β generated the tonic signaling are necessary for proper development of B cells from pro-B cells to mature B cells and survival of peripheral B cells.

1.7 Internalization of the BCR and processing and presentation of antigen

The BCR is constitutively internalized at a low rate in the absence of ligand binding. This constitutive internalization is dependent on the ITAM of the Ig α /Ig β [94]. These unligated BCRs have been shown to recycle through the early endosomes and return to

the cell surface [95]. Cross-linking the BCR via ligand binding leads to movement of the BCR into lipid rafts and the rearrangement of the actin cytoskeleton [36, 96, 97] and this appears to greatly enhance the internalization of the BCR. The cytoskeleton has been shown to be essential for BCR endocytosis and trafficking [98].

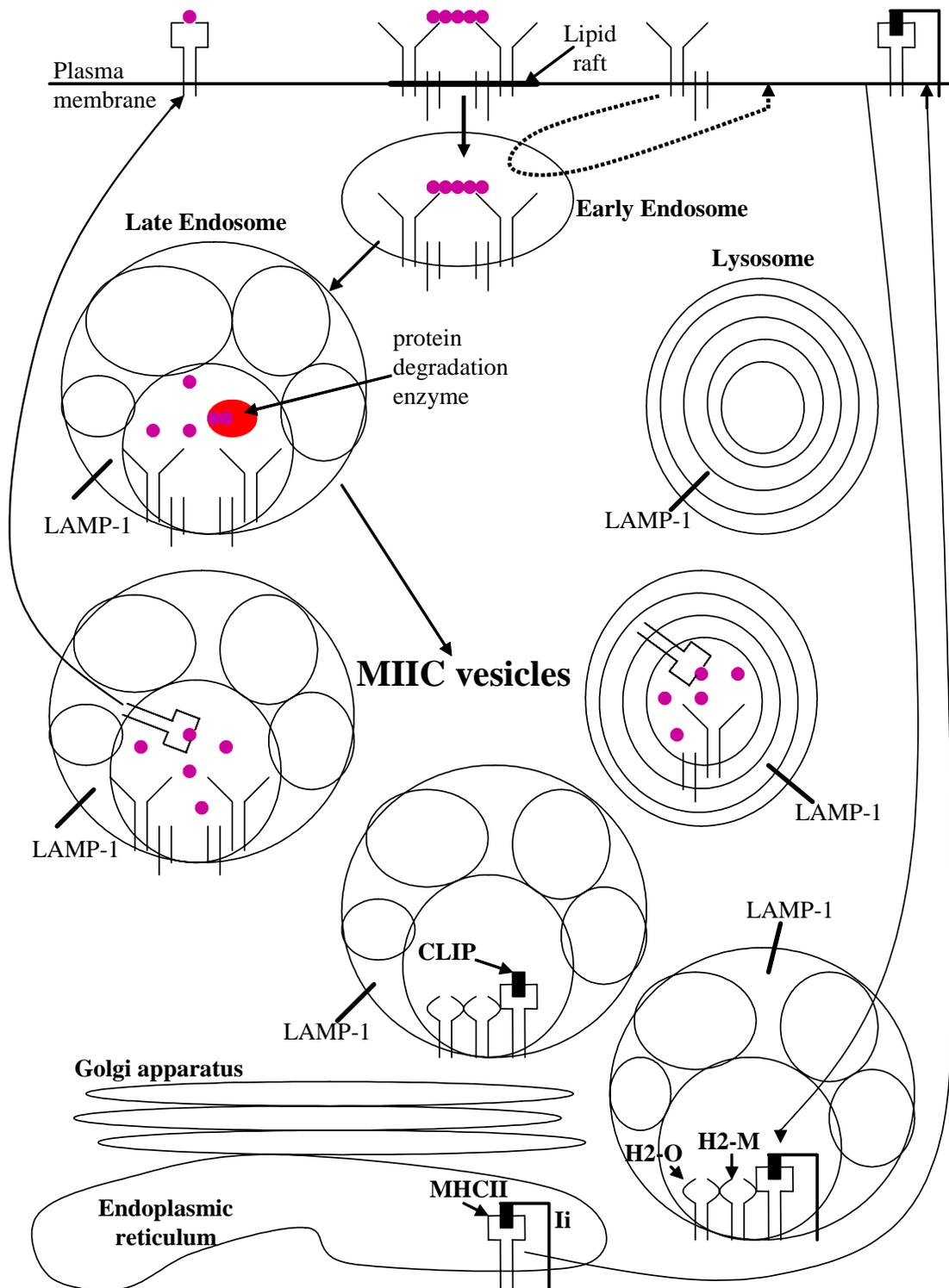
Cross-linking the BCR also leads to rapid trafficking through the endocytic pathway from early endosomes (EE) to late endosomes (LE) and finally to specialized MHC class II-containing compartments (MIIC) (Fig. 1-4) [97, 99]. In EE internalized proteins either move back to the cell surface (e.g. transferrin receptor or unligated BCR) or on to the LE [100, 101]. The low pH of endosomes allows for disassociation of proteins as well as activation of enzymes necessary for protein degradation. Both LE and lysosomes also contain lysosomal associated membrane protein-1 (LAMP-1), which is commonly used as a marker for LE and lysosomes.

The MIIC vesicles resemble LE and pre-lysosomes in that they are LAMP-1⁺, acidic and contain enzymes for protein fragmentation, such as cathepsins and thiol reductases [102-104]. Morphologically they appear as multivesicular or multilaminar vesicles. These internal vesicles have been shown to contain the majority of endocytosed antigen-aggregated receptor complexes and are rich in MHC class II [105].

The MHC class II molecules are assembled in the endoplasmic reticulum (ER) along with trimers of invariant chain (Ii). The N-terminal region of Ii is called CLIP, and it resides

Figure 1-4. The internalization and trafficking of the BCR and the processing and presentation of antigen.

BCRs crosslinked by antigen are internalized into early endosomes and trafficked to late endosomes and then MIIC vesicles. MHCII molecules are assembled in the endoplasmic reticulum and are transported to the MIIC vesicles, where processed antigen is loaded into the peptide groove. The MHCII:peptide complexes are then transported to the cell surface to be presented to T cells.



in the antigen-binding groove of the MHC class II. The C-terminal region of Ii is anchored in the membrane and contains a di-leucine trafficking motif that targets the molecules to LE [106-111]. In the MIIC cathepsin S degrades Ii until only CLIP remains in the MHC class II groove [112]. CLIP is exchanged for antigenic peptide through the action of a MHC class II-like molecule H2-M (HLA-DM in humans) [113-117]. Another MHC class II-like molecule, H2-O (HLA-DO in humans) associates with H2-M. Its role is not known, but it is thought that it may regulate H2-M and peptide loading in a pH-dependent manner [118, 119]. This would ensure that only peptides from antigens that reach late endosomes will be loaded onto the MHC class II. Once the peptide is loaded onto the MHC class II, these MHC class II:peptide complexes are rapidly exported to the cell surface to be displayed for T cells.

1.8 The importance of the $Ig\alpha/Ig\beta$ heterodimer in internalization of the BCR and antigen processing and presentation

Previous studies have indicated the importance of $Ig\alpha/Ig\beta$ in BCR-mediated antigen processing and presentation. First, it is important to note that the complete BCR, including the $Ig\alpha/Ig\beta$ heterodimer, has been shown to move through the endocytic pathway after antigen binding [120]. The $Ig\alpha/Ig\beta$ heterodimer does not dissociate from the mIg in EE, suggesting that it is necessary for the proper trafficking of the BCR and antigen to the MIIC compartment. The $\mu(YS/VV)$ mutant, which has decreased association with $Ig\alpha/Ig\beta$, has also been shown to have a greatly reduced ability to facilitate antigen presentation [10]. Patel and Neuberger studied a mIgM mutant which

had extensive changes in its transmembrane domain. This mutant was transported to the surface without associating with Ig α /Ig β , and did not associate with Ig α /Ig β even when co-expressed in the same cell. This mutant was found to have a greatly reduced ability to facilitate antigen presentation, which was restored when the cytoplasmic tail of Ig β was added to the mutated mIgM [94].

Thus, Ig α and Ig β appeared to be important for antigen presentation, but the reason for this ability to facilitate antigen presentation was not clear. Several studies suggest Ig α /Ig β is important to proper intracellular trafficking of antigen. Bonnerot et al. showed using immunofluorescence microscopy that FcR-Ig α chimeras, which are fusion proteins of FcR extracellular and transmembrane domain and Ig α cytoplasmic tail, target antigen to MHC class II-rich compartments, but FcR-Ig β chimeras appear to target to recycled MHC class II molecules [121]. Lankar et al. examined FcR-Ig α and FcR-Ig β chimeras and found that FcR-Ig α was able to facilitate presentation of cryptic epitopes [122]. Li et al. used platelet-derived growth factor receptor-Ig α or Ig β chimeras to study the role of the cytoplasmic tails of each molecule in trafficking of the BCR. They showed that Ig β alone causes the BCR to move rapidly through the endocytic pathway to the lysosomes. Ig α alone also moves to the lysosomes, but more slowly. When both cytoplasmic tails are expressed the BCR moves quickly to the antigen loading compartments [123]. Siemasko et al. used an immunofluorescence assay to observe the fusion of LAMP-1-containing late endosomes into multivesicular MIIC. They found, also using platelet-derived growth factor receptor-Ig α /Ig β chimeras, that Ig α /Ig β is actually required for this

fusion to occur [124]. Thus, Ig α /Ig β appears to be important for proper antigen trafficking and presentation.

1.9 The relationship between BCR signaling and antigen processing and presentation

Recent evidence indicates that the signaling function of the BCR is also important for the efficiency of antigen trafficking and presentation. Previous studies have demonstrated that crosslinking the BCR with multivalent antigen, which initiates signaling, not only increases the rate and amount of internalization, but also the rate of transport of BCR to peptide loading compartments [97]. Pure and Tardelli showed that tyrosine kinase inhibitors that block BCR signaling also block ligand-induced internalization of the mIg [125]. Wagle et al demonstrated that tyrosine kinase inhibitors that block BCR signaling reduce BCR-mediated antigen internalization, decrease the accelerated transport of antigen to the MIICs and inhibit antigen presentation [126]. Overexpression of a dominant negative mutant of the tyrosine kinase Syk decreases antigen presentation [122]. Several studies have demonstrated that the tyrosines of the ITAM of Ig α are necessary for efficient antigen processing and presentation. Cassard et al. showed that a mutation of one of the tyrosines prevents constitutive internalization of the BCR [95]. Lankar et al used FcR-Ig α chimeras with mutations of the two ITAM tyrosines and found that both are required for optimum presentation of antigen [122]. Siemasko et al [124] studied a chimera that contained a wt Ig β cytoplasmic tail and an Ig α cytoplasmic tail with both ITAM tyrosines mutated. They showed that this chimera had decreased internalization and antigen presentation compared to a chimera with a wt Ig α and Ig β .

Interestingly, cells expressing the chimera with the mutated tyrosines did not have aggregation of LAMP-1⁺ compartments, whereas the wt chimera did. This aggregation is known to be an important event in efficient presentation of antigen [127], and it appears the tyrosines of the Ig α ITAM are necessary for it to occur. Previous work in our lab shows that crosslinking the BCR induces the tyrosine phosphorylation of clathrin in lipid rafts and the recruitment of clathrin to the cell surface and BCR-containing vesicles [128]. Recruitment and phosphorylation of clathrin is a necessary step in BCR internalization. Thus, the signaling function of Ig α /Ig β appears to be important for the occurrence and efficiency of antigen processing and presentation.

1.10 Statement of purpose

The purpose of this work is to examine the mechanism of the regulation of BCR internalization and transport within the cell. In the second chapter I characterized the effects of the dissociation of the ligand-binding mIgM from the signaling Ig α /Ig β . I discovered that their association is required for efficient internalization and transport of the mIgM to the MIIC. In chapter three I studied the role of the tyrosines and the putative internalization motifs of the ITAM of Ig α in internalization and transport of BCR. I determined that both tyrosines are essential for optimal internalization and transport of the BCR to MIICs, but that one tyrosine perhaps has a dominant role. I was not able to determine if the putative internalization motifs are actual targeting motifs. My results as a whole demonstrate that the signaling induced by Ig α /Ig β does have a role in the regulation of efficient internalization of BCR and intracellular transport of BCR to the MIIC.

1.11 Significance

T cell-dependent antibody responses to protein antigens require that the protein antigens are converted into peptides and the antigenic peptides are displayed in MHC class II bound forms on the surface of B cells. B cells can uptake antigen through fluid-phase pinocytosis, but this is a very inefficient method of presenting antigen compared to BCR-mediated antigen presentation. To achieve the same level of antigen presentation with fluid-phase pinocytosis as compared to BCR-internalized antigen requires 1,000 to 10,000 times more antigen [129]. Efficient antigen presentation is so important to mounting an immune response because in the body antigen is usually found in low concentrations. The BCR increases the efficiency of antigen processing and presentation by increasing the kinetics and specificity of antigen capturing, uptaking and transporting to the MHC. Without the ability of BCR to internalize and present antigen at low concentrations, a strong immune response may not occur. This dissertation will examine the molecular mechanisms and regulation of BCR-mediated antigen processing

B cell activation requires two stages of signals. The first signal required for B cell activation is the induction of the signaling cascade, which occurs after BCR is engaged with multivalent signal and is initiated with the phosphorylation of the tyrosines of the ITAMs of $Ig\alpha/Ig\beta$. The generation of the first stage of signal is regardless of whether the antigen is a T cell-dependent antigen. For T cell-dependent antigen, antigen-specific T cells provide a second activation signal, or for T cell-independent antigen, B cells will receive the second activation signal through extensive crosslinking of BCRs by antigen and through other receptors such as a Toll-like receptor. As discussed in the introduction

section, $Ig\alpha/Ig\beta$ has been shown to be important for the generation of both stages of signals. Because $Ig\alpha/Ig\beta$ contains the ITAM and putative trafficking motifs, it potentially provides a coupling mechanism for two signaling events. This study examined the role of the $Ig\alpha/Ig\beta$ heterodimer in BCR-mediated antigen uptake and transport to antigen processing compartments. I examined the effect of perturbing the interaction of mIg with $Ig\alpha/Ig\beta$ and mutations of the $Ig\alpha$ ITAM motif on the signaling and antigen transport functions of the BCR.

This study will add to our understanding of how B cells efficiently process and present antigens and how BCR-mediated signaling regulates these activities. Increasing our knowledge of the regulation of antigen processing and presentation in B cells at a molecular level could provide new strategies to manipulate or control these processes. This will allow for development of improved vaccines or therapeutics to reduce antibody production, such as for autoimmune diseases.

Chapter II: Destabilization of the B cell antigen receptor blocks signaling and antigen targeting

2.1 Abstract

The binding of antigens to the B cell antigen receptor (BCR) results in the initiation of signaling cascades and the internalization of the antigens for processing and presentation. Recent studies by others [130-132] indicate that antigen binding destabilizes the BCR as a mechanism to down regulate B cell responses. Two point mutations in the transmembrane domain of mouse mIgM (YS to VV) weaken the interaction of mIgM with Ig α /Ig β heterodimer, resulting in a destabilized BCR. Here we show that μ YS/VV BCR is defective in signaling and does not target antigens to late endocytic compartments for processing and presentation. Significantly, coligation of the μ YS/VV BCR with an endogenous wild type IgG_{2a} BCR interferes with both signaling and antigen-targeting functions of the endogenous BCR. Thus, the destabilized BCR has a dominant effect, down regulating the function of stable wild type BCR. The ability of the destabilized BCR to influence the stable BCR may play an important role in turning off B cell responses for antigen-driven anergy and tolerance.

2.2 Introduction

The B cell antigen receptor (BCR) plays two major roles in B cell activation: transducing signals for initiation of signaling cascades and internalizing antigen for antigen processing and presentation. Both BCR signaling and BCR-mediated antigen processing and presentation are required for the induction of T cell-dependent antibody responses and the generation of memory B cells. In mature resting B cells, the binding of antigens

to the BCR initiates signaling cascades to promote B cells to enter the cell cycle [30, 133, 134]. Subsequently, the BCR internalizes the antigen and delivers it to an endocytic compartment that contains both newly synthesized major histocompatibility complexes (MHC) class II and DM, a class II-like molecule that catalyzes peptide exchanges, for processing and peptide-loading [97, 99, 135]. This compartment has been termed an MHC class II-containing compartment (MIIC). The resulting class II-peptide complexes are presented on the surface of B cells for recognition of T helper cells [129, 136, 137].

The signaling and antigen-targeting functions of the BCR appear to be interrelated, and the activation of the BCR increases the antigen-presenting ability of B cells by accelerating the intracellular trafficking of the BCR and increasing the expression level of co-stimulating molecules like B7 [138, 139]. Early studies showed that the BCR constitutively internalizes and moves through the endocytic pathway at a low rate. Cross-linking the BCR, which initiates signaling cascades, increases the internalization rate and accelerates the targeting of the BCR to the MIIC [97]. In B cell lymphoma A20 cells, cross-linking the BCR has been shown to induce the redistribution and aggregation of late endosomes [127]. Tyrosine kinase inhibitors that block BCR signaling inhibit the accelerated antigen transport [125, 126], indicating that BCR-mediated signaling regulates BCR-mediated antigen transport. The regulatory function of BCR-mediated signaling is further demonstrated by B cells over-expressing the negative Syk mutant where both BCR-mediated signaling and BCR-facilitated antigen presentation are inhibited [122]. Thus, BCR signaling appears to modulate its antigen-targeting function in order to coordinate B cell responses. Even though both the BCR signaling and

trafficking pathways have been well characterized, how BCR signaling regulates BCR-mediated antigen processing remains unclear.

The BCR is a multi-component complex consisting of membrane immunoglobulin (mIg) and $Ig\alpha/Ig\beta$ heterodimer ($Ig\alpha/Ig\beta$) [12]. Membrane Ig serves as the ligand-binding domain for BCR. $Ig\alpha/Ig\beta$ that contains immunoreceptor tyrosine-based activation motifs (ITAMs) provides the BCR with signaling transducing ability. Besides its signaling function, $Ig\alpha/Ig\beta$ has been shown to play important roles in BCR-mediated antigen processing. A mutated human-murine chimeric mIgM that lacks association with $Ig\alpha/Ig\beta$ is able to internalize antigens, but fails to facilitate antigen processing [8, 10], indicating an essential role of $Ig\alpha/Ig\beta$ in BCR-mediated antigen processing. FcR chimeric proteins containing the cytoplasmic tail of either $Ig\alpha$ or $Ig\beta$ all can internalize antigens. However, the internalization of antigens by the FcR-chimera containing the cytoplasmic tail of $Ig\alpha$ but not $Ig\beta$ leads to the presentation of cryptic epitopes [121], suggesting that the cytoplasmic tails of $Ig\alpha$ and $Ig\beta$ target antigens to different processing compartments. Using chimeric proteins consisting of the extracellular and transmembrane domains of the platelet-derived growth factor receptor, Siemasko et al. [124] showed that only the chimera complex containing both $Ig\alpha$ and $Ig\beta$ chimeric proteins can facilitate antigen processing to a level similar to the wild type (wt) BCR, indicating that both $Ig\alpha$ and $Ig\beta$ are required for BCR-mediated antigen processing and presentation.

Ig α /Ig β non-covalently associates with mIg. This non-covalent interaction bridges the binding of antigens to the phosphorylation of cytoplasmic tails of Ig α /Ig β and the activation of signaling cascades. Vilen et al. [132] showed that the binding of moderate- to low-affinity antigens to the BCR induces the disassociation of Ig α /Ig β from mIg, resulting in a destabilized receptor and the unresponsiveness of B cells to antigens [130-132]. This indicates that the interaction of Ig α /Ig β with mIg can be modulated by antigen binding to induce the desensitization of the BCR, and suggests that destabilizing the BCR is a mechanism to turn off B cell responses. Introducing mutations into the transmembrane domain of mIg disrupts the interaction of Ig α /Ig β with mIg and interferes with signaling and antigen-processing functions of the BCR [8, 11]. Thus the mutated BCR provides a model for the function of a destabilized BCR. Here, we analyzed the function of a mouse mIgM containing two point mutations YS to VV that interfere with the interaction between mIgM and Ig α /Ig β . We studied the effects of the receptor's stability on both its signaling and antigen-targeting functions, and the effect of the destabilized BCR on the functions of the coexpressed wt BCR. Our studies provide evidence that the stability of the BCR complex is essential for both its signaling and antigen-targeting functions, and the destabilized BCR interferes the function of the stable BCR when they are coligated.

2.3 Materials and Methods

Cell culture and antibodies

The B cell lymphoma, A20 IIA1.6, is an H-2^d, IgM⁻, IgG_{2a}⁺ and FcR⁻ cell line. The A20 cells were cultured in DMEM supplemented as described [140] and containing 10% FCS. The rat hybridoma 1D4B producing a LAMP-1-specific mAb was obtained from the Development Studies Hybridoma Bank (Iowa City, IA). Goat antibodies specific for rat IgG, mouse IgG+M (anti-IgG+M), rabbit anti-mouse μ (anti- μ), Fab fragment of goat anti-mouse μ chain antibody (Fab-anti- μ), and their FITC and TRITC-conjugates were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Tyrosine phosphate-specific mAb, 4G10, was from Upstate Biotechnology (Waltham, MA). Light chain, λ_1 - and γ_{2a} -specific antibodies, and their TRITC-conjugates were from Southern Biotechnology Associates Inc. (Birmingham, AL). Myoglobin and PP2 were purchased from Sigma (St. Louis, MO).

Plasmid construction and transfection

Plasmid p μ containing a functionally rearranged genomic clone of the murine μ gene, p μ YS/VV that contains two point mutations in the transmembrane domain of mIgM, and pRSV λ_1 containing a gene that encodes murine λ_1 light chain were kindly provided by Dr. Anthony L. DeFranco at University of California, San Francisco [11]. The mutation YS to VV was verified by DNA sequencing. Plasmids p μ and pRSV λ_1 were cotransfected into A20 cells by electroporation. Drug resistance was conferred to cell lines by cotransfection with pSV2neo (Clontech Laboratories, Inc., Palo Alto CA) coding for G418 resistance. Stable drug-resistant clones were selected in medium containing

0.75 mg/ml G418. Drug-resistant clones were screened for expression of μ and λ_1 by flow cytometry.

Surface biotinylation

A20 cells were washed at 4°C with HBSS lacking phosphate and containing 20 mM Na HEPES, pH 7.4, and incubated in the same buffer containing 0.2 mg/ml sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce, Rockford, IL) for 15 min at 4°C. After 15 min incubation, a freshly made biotin solution was added, and the incubation was extended for another 15 min at 4°C. The cells then were washed with DMEM containing 6 mg/ml BSA and 20 mM MOPS, pH 7.4 (DME/BSA) at 4°C and chased at 37°C for varying lengths of time.

Coimmunoprecipitation

Surface-biotinylated cells were lysed in 1% digitonin (Calbiochem, San Diego, CA) lysis buffer (1% digitonin, 10 mM triethanolamine, pH 7.5, 150 mM NaCl, 1 mM EDTA) [20]. Membrane IgM was immunoprecipitated from the lysate using anti- μ antibody conjugated to Agarose beads (Sigma, St. Louis, MO). The immunoprecipitates were analyzed by SDS-PAGE and Western blotting. Biotinylated proteins were visualized using HRP-conjugated streptavidin (HRP-streptavidin). The Ig α in the anti- μ immunoprecipitates was verified by blotting the same blot with anti-Ig α antibody. The amounts of the biotinylated Ig α and μ -chain were quantified using densitometry and the ratio of the biotinylated Ig α to the biotinylated μ -chain at each time point was calculated. The data were plotted as percentages of the ratio of the Ig α to wt μ at time 0.

Analysis of Tyrosine Phosphorylated Proteins

Cells were incubated with either anti- μ (20 $\mu\text{g/ml}$) or anti- γ_{2a} (20 $\mu\text{g/ml}$) or anti-IgG+M antibody (20 $\mu\text{g/ml}$) for 30 min at 4°C. The cells were then warmed to 37°C for 2 min. Subsequently, the cells were rapidly centrifuged and lysed with lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM Na₃VO₄, and protease inhibitors). The cell lysates were analyzed by SDS-PAGE and Western blotting. Blots were probed for the presence of phosphorylated tyrosines using a mAb, 4G10. Blots were visualized with ECL (NEN Life Science Products, Boston, MA).

Antigen presenting assay

Myoglobin conjugated to goat anti-mouse μ -chain antibody was generated as previously described [141]. Briefly, myoglobin (Sigma, St. Louis, MO) was incubated at a 1:1 molar ratio with 2-iminothiolane HCl (Pierce, Rockford, IL) in a thiolation buffer (100 mM PO₄, pH 8, 50 mM NaCl and 1 mM EDTA) for 30 min at 4°C. Thiolated myoglobin was isolated using Thiopropyl Sepharose 6B beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Goat anti-mouse μ -chain antibody was incubated with a 1:10 molar ratio excess of LC-SPDP (Pierce, Rockford, IL) in a conjugation buffer (10 mM PO₄, pH 8, 150 mM NaCl) for 45 min at room temperature. The reactive antibody was then incubated with a 10:1 molar excess of thiolated myoglobin at room temperature overnight. The myoglobin conjugate was purified using Centricon YM100 with 100 kDa molecular weight cut-off (Millipore, Bedford, MA). The concentration of myoglobin in the conjugate was determined using the optical density of myoglobin at 525 nm.

A20 cells expressing either wt μ or μ YS/VV (5×10^4) as antigen-presenting cells and myoglobin-specific T cell hybridoma (5×10^4) were co-cultured with graded doses of myoglobin conjugated to the anti- μ antibody for 24 h. Culture supernatants were removed and tested for their IL-2 content by the ability to maintain the growth of the IL-2-dependent T cell line, CTLL [142]. The proliferation of CTLL was measured by incorporation of [3 H]thymidine. All assays were done in triplicate.

Internalization assay

Fab-anti- μ was iodinated to a specific activity of 1.0 to 1.5×10^7 cpm/ μ g [143]. More than 95% of the [125 I]-Fab was precipitated by 10% trichloroacetic acid, indicating little or no free 125 I. Cells were incubated with [125 I]Fab-anti- μ in the presence or absence of anti- μ (20 μ g/ml) or anti- γ_{2a} (20 μ g/ml) or anti-IgG+M (20 μ g/ml) antibody in DME/BSA on ice for 1 h. After extensive washing at 4°C, the cells were chased at 37°C for varying lengths of time. At the end of each time period, the incubation media were collected and counted as the fraction of [125 I]-Fab-anti- μ released from the cells. [125 I]-Fab-anti- μ on the cell surface was removed by incubating the cells in a low pH solution (20 mM HCl, 150 mM NaCl) for 15 min on ice. [125 I]-Fab-anti- μ associated with the cells after stripping was counted as internalized. The radioactivity of released, surface, and internal fractions was counted, calculated and expressed as percentages of the total radioactivity initially associated with the cells.

Immunofluorescence microscopy

To label mIgM, A20 cells were incubated with FITC-Fab-anti- μ for 40 min at 4°C on polylysine-coated slides (Sigma, St. Louis, MO) in the presence or absence of anti- μ (20 μ g/ml) or anti- γ_{2a} (20 μ g/ml) or anti-IgG+M (20 μ g/ml) antibody. To label mIgG_{2a}, cells were incubated with TRITC-anti- γ_{2a} antibody at 4°C for 40 min. The cells were then washed at 4°C and chased for varying lengths of time at 37°C. After the chase, the cells were fixed by incubating them with 4% paraformaldehyde in PBS for 20 min. For LAMP-1 staining, after the chase, cells were fixed and incubated with a permeabilization buffer (5% FCS, 0.05% saponin, 10 mM glycine, 10 mM HEPES, pH 7.4) for 15 min. They were then incubated with 1D4B mAb in the permeabilization buffer for 1 h, washed, and incubated with TRITC-goat-anti-rat IgG for 30 min. The cells were washed, post-fixed and mounted with Gel/Mount (Biomedex, Foster City, CA). Double immunofluorescence analyses were carried out on a scanning laser confocal microscope (Zeiss LSM 510). Images were acquired using a 100X oil immersion objective, and cropped using Photoshop (Adobe, Mountain View, CA). Optical sections from the middle of cells were selected.

2.4 Results

The mutation YS to VV destabilizes the BCR

We expressed a wild type (wt μ) or a mutated murine μ -chain in murine B cell lymphoma A20 cells that express an endogenous IgG_{2a} BCR. The mutated μ -chain contains two point mutations changing the neighboring tyrosine and serine residues in the C-terminal hydrophilic patch of the transmembrane region to valines (μ YS/VV) (Fig. 2-1A). The

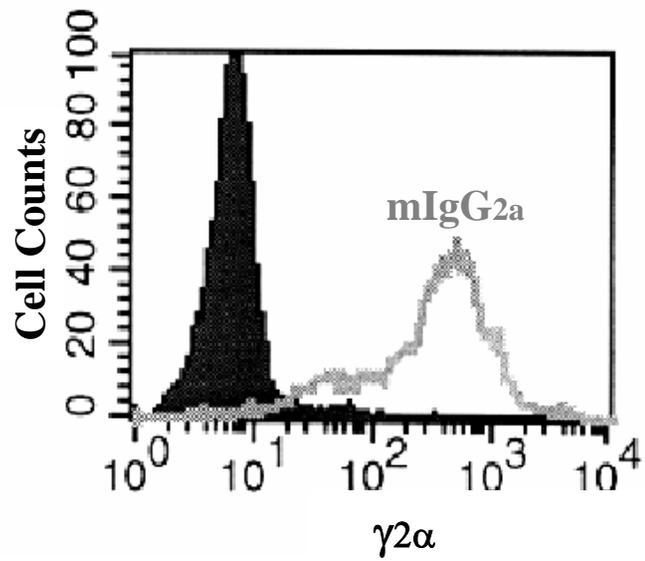
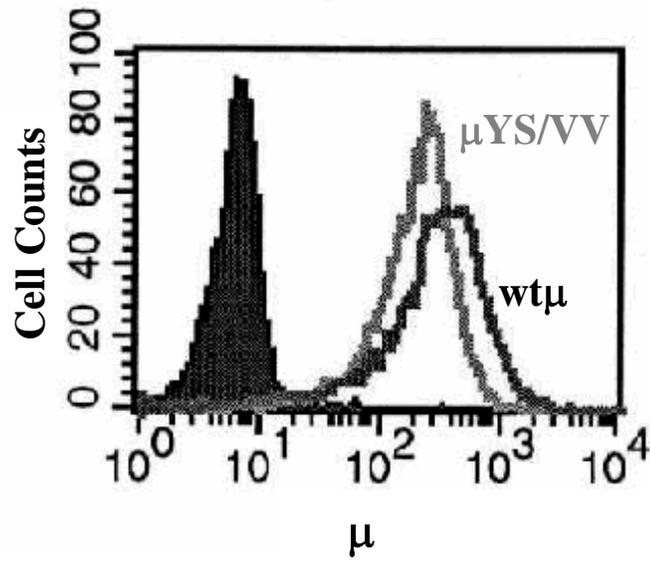
Figure 2-1. Cell surface expression of wt μ and μ YS/VV on transfected A20 cells.

(A) Sequence of the transmembrane domain of wt and mutated mouse μ -chains. The single letter amino acid code is used. Amino acids are numbered from the N-terminus to the C-terminus, and introduced mutations are marked by bold letters. The underlined threonine is an alanine in human μ and is the only difference between the μ transmembrane domains of the two species. (B) FACScan analysis of transfected mIgM. The A20 cells and transfectants expressing the indicated form of mIgM were stained with FITC-conjugated goat anti-mouse μ or TRITC-conjugated goat anti-mouse γ_{2a} antibody and analyzed by flow cytometry.

A

	1	25
<i>wt</i> μ		
	LWTTASTFIVLFLLSLFYSTTVTLF	
<i>μYS/VV</i>	LWTTASTFIVLFLLSLFVVTTVTLF	

B

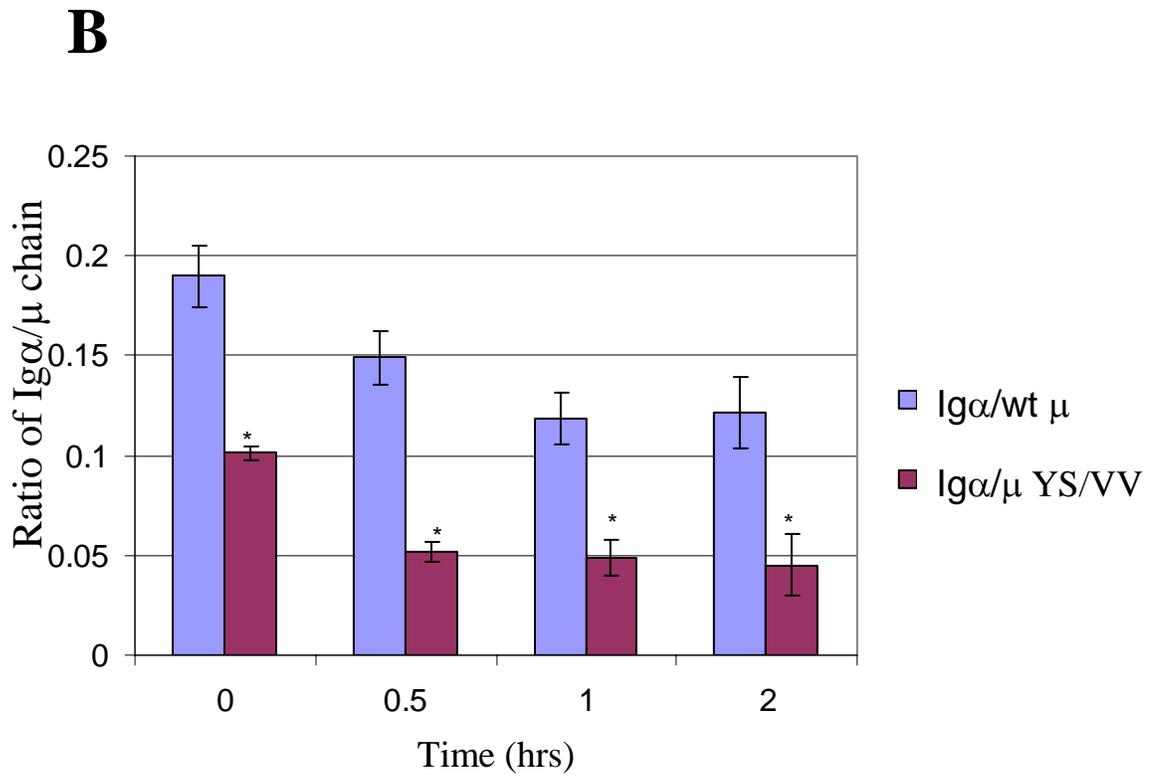
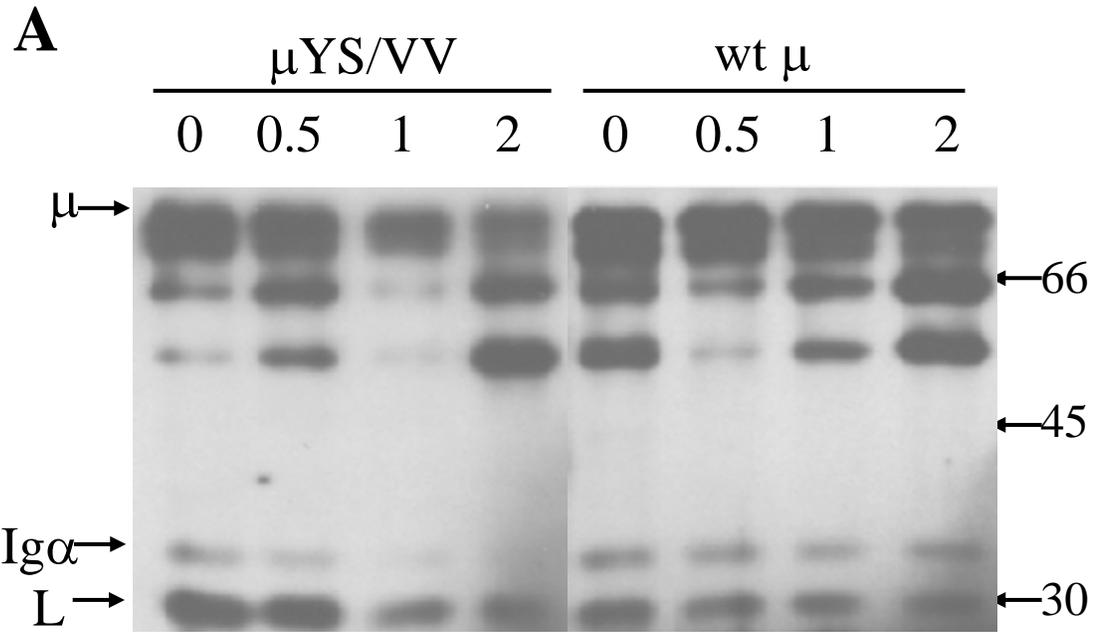


levels of surface expression of μ -chains in transfected clone lines were analyzed using flow cytometry. Clones expressing wt μ or μ YS/VV at a level similar to that of mIgG_{2a} were chosen for further analysis (Fig. 2-1B).

The stability of the μ YS/VV- or wt μ -containing BCR on the plasma membrane was analyzed. The cell surface protein was biotinylated at 4°C, and then chased at 37°C for varying lengths of time. The cells were lysed in a digitonin lysis buffer to preserve the non-covalent interaction between mIg and Ig α /Ig β . The cell lysates were subjected to immunoprecipitation using anti- μ antibody conjugated to Agarose beads. The immunoprecipitates were analyzed by SDS-PAGE and Western blotting. The biotinylated proteins were visualized by HRP-conjugated streptavidin (Fig. 2-2A). The existence of Ig α /Ig β in the immunoprecipitates was verified by reblotting the same blot with Ig α -specific antibodies (data not shown). The amount of biotinylated Ig α in the immunoprecipitates was quantified by densitometry, and the ratio of the biotinylated Ig α to the biotinylated μ -chain was calculated. Shown are the ratios of the coimmunoprecipitated biotinylated Ig α to biotinylated μ chain at each time point (Fig. 2-2B). Biotinylated Ig α was coimmunoprecipitated with wt μ -chain through the 2 h of chase time in A20 cells expressing wt μ chains (Fig. 2-2). The amount of surface biotinylated Ig α in the coimmunoprecipitates gradually decreased with time. In μ YS/VV expressing cells, the amount of biotinylated Ig α coimmunoprecipitated with μ YS/VV was significant less than what was seen with wt μ at all the time point (Fig. 2-2). Thus, μ YS/VV BCR is relatively unstable.

Figure 2-2. Co-immunoprecipitation of Ig α with mIgM.

(A) The surface of A20 cells were biotinylated at 4°C, then washed and chased at 37°C for the times indicated. The cells were lysed in 1% digitonin lysis buffer. Membrane IgM was immunoprecipitated from the cell lysates with anti- μ antibody conjugated to Agarose beads. The immunoprecipitates were analyzed by SDS-PAGE and Western blotting. The immunoprecipitates from cell lysates generated from an equal number of cells were loaded onto each well of gels. The biotinylated mIgM and Ig α were detected using streptavidin-HRP. The blots were analyzed by densitometry. A representative blot is shown. (B) The amounts of the biotinylated Ig α and μ -chain at each time point were quantified using densitometry and the ratio of the biotinylated Ig α to the biotinylated μ -chain was calculated. The average of the results of three independent experiments is shown. * indicates that there is a significant difference between the amounts of surface biotinylated Ig α coimmunoprecipitated with wt mIgM and with mIgM containing μ YS/VV ($p < 0.01$).



The μ YS/VV BCR is defective in signaling and coligating the μ YS/VV BCR with the endogenous BCR dampens the endogenous BCR signaling

The ability of transfected μ -chains to initiate signaling was analyzed by measuring tyrosine phosphorylation of cellular proteins following BCR cross-linking. Cells were incubated with or without a μ -chain-specific antibody (Fig. 2-3A) at 4°C for 10 min, and then warmed to 37°C for 2 min. The cells were lysed and analyzed using SDS-PAGE and Western blot probing for phosphorylated proteins. Cross-linking wt μ induced protein tyrosine phosphorylation at a level similar to that induced by cross-linking the endogenous wt IgG_{2a} BCR (Fig. 2-4), indicating that the signaling function of transfected wt μ BCR is equivalent to the endogenous BCR. In contrast, the level of protein tyrosine phosphorylation induced through μ YS/VV was much lower than those induced through the wt μ or endogenous BCR (Fig. 2-4). Thus, the mutation YS to VV reduces the ability of BCR to initiate protein tyrosine phosphorylation.

Next, we asked whether the μ YS/VV BCR affects the signaling function of endogenous wt BCR when either cross-linked independently or coligated. To cross-link the endogenous BCR independently, cells were treated with an antibody specific for mIgG_{2a} (Fig. 2-3B). In μ YS/VV-expressing cells, cross-linking mIgG_{2a} induced tyrosine phosphorylation of a spectrum of proteins at levels similar to those seen in wt μ -expressing cells (Fig. 2-4), indicating that the defective BCR does not affect the signaling function of the endogenous BCR when the endogenous BCR is cross-linked independently.

Figure 2-3. Cross-linking and coligation of the transfected and endogenous BCRs.

Depicted are the different cross-linking antibodies used to assess functions of the transfected BCR and the endogenous BCR. (A) The transfected BCRs are cross-linked by anti- μ antibody independently of the endogenous BCR. (B) The endogenous BCRs are cross-linked by anti- γ_{2a} antibody independently of the transfected BCR. (C) The transfected and endogenous BCRs are coligated by anti-IgG+M antibody.

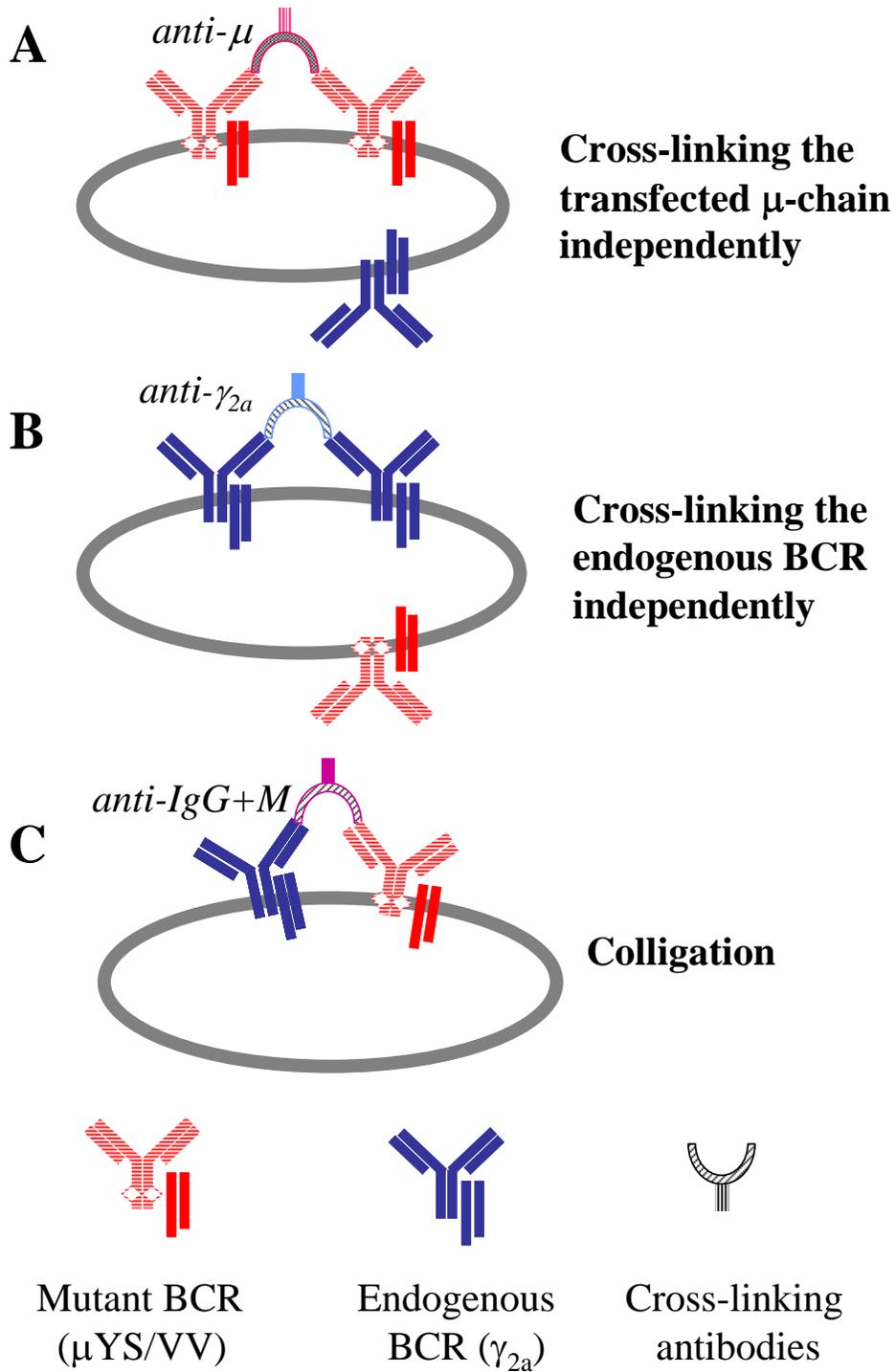
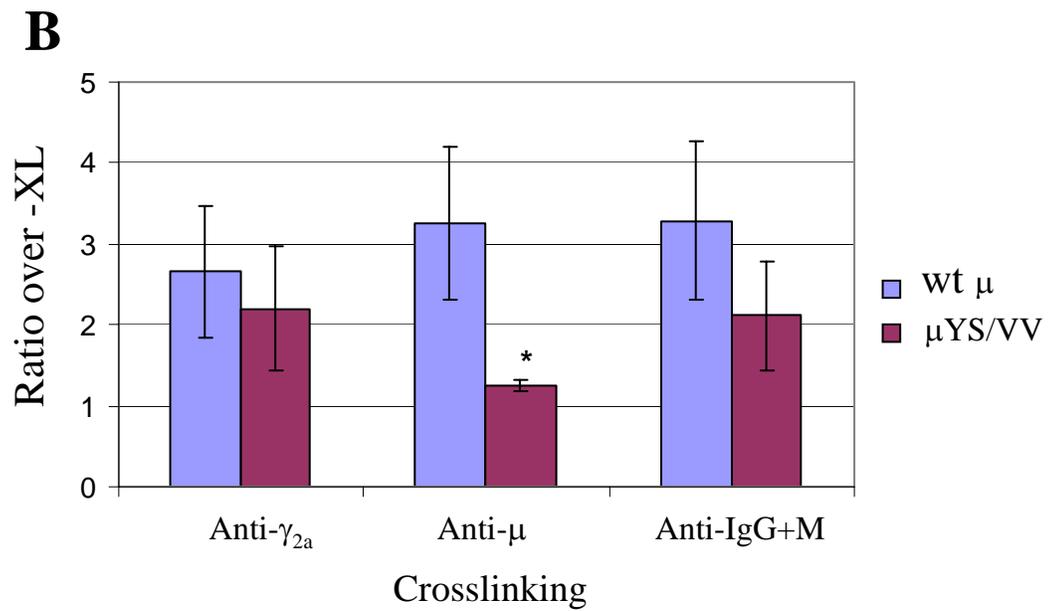
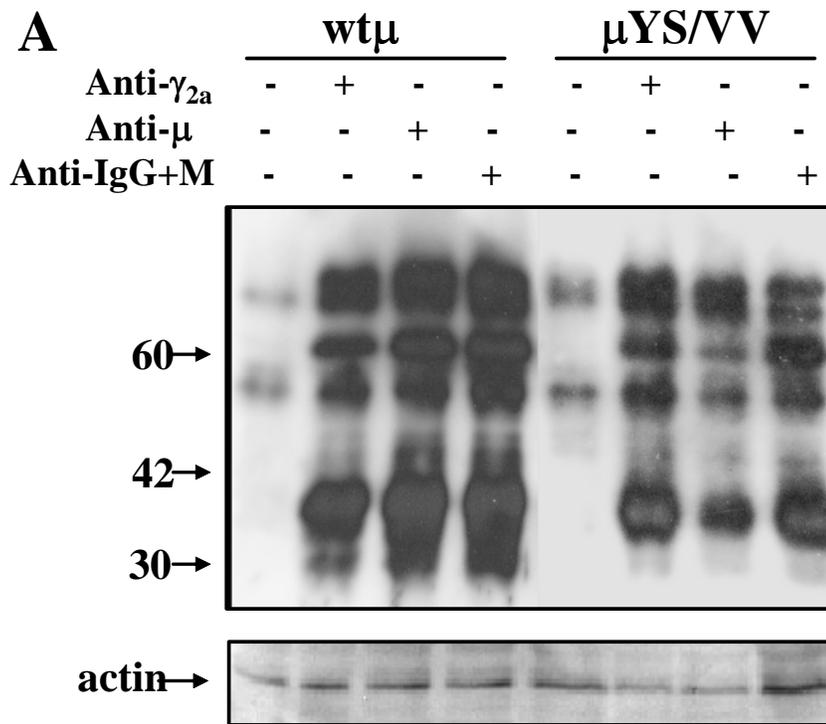


Figure 2-4. Protein tyrosine phosphorylation induced by transfected μ -chains.

(A) Transfected A20 cells were incubated with medium alone, different cross-linking or coligation antibodies at 4°C for 30 min and warmed to 37°C for 2 min. The cells were immediately lysed, and the cell lysates were analyzed using SDS-PAGE and Western blotting. The cell lysates generated from an equal number of cells were loaded onto each well of gels. Blots were probed with mAb 4G10 and an HRP-conjugated secondary antibody for detecting phosphorylated tyrosines. The blots were stripped and probed with anti-actin antibody for loading controls. Shown is a representative blot of four independent experiments. (B) The amount of protein tyrosine phosphorylation was quantified using densitometry. The ratio of amount of protein tyrosine phosphorylation for each crosslinking or coligation condition over the amount of protein phosphorylation of non-stimulated cells was calculated. The average of the results of four independent experiments is shown. * indicates that there is a significant difference between the values for wt μ and μ YS/VV for the anti- μ crosslinking and between the values for μ YS/VV and for the anti-IgG and anti- μ conditions ($p < 0.01$).



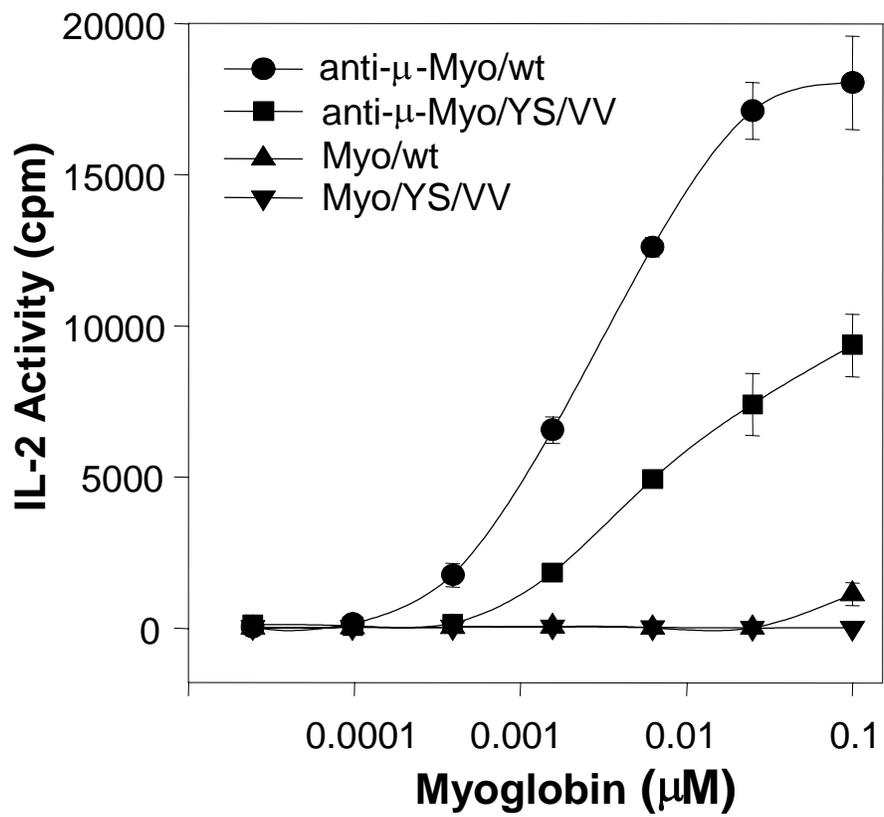
The μ YS/VV and endogenous BCRs were coligated using a polyclonal anti-mouse IgG+M antibody (Fig. 2-3C). Previous work in our lab showed that the anti-mouse IgG+M antibody binds to both mIgM and mIgG_{2a} as shown by immunofluorescence microscopy using non-transfected A20 cells and CH27 cells expressing mIgM only. Coligation of the μ YS/VV BCR with the endogenous BCR induced tyrosine phosphorylation of a spectrum of proteins similar to those seen in wt μ -expressing cells (Fig. 2-4). But phosphorylated proteins around 70 kDa were much less in the coligated cells than those seen in cells treated with anti- γ _{2a} antibody (Fig. 2-4). Thus, the μ YS/VV BCR interferes with the signaling function of the wt BCR when they are coligated.

The μ YS/VV BCR fails to facilitate antigen processing and does not target to late endosomes

The ability of the wt μ and μ YS/VV BCRs to facilitate antigen processing and presentation was tested using either myoglobin (Myo), taken up by fluid phase pinocytosis, or anti-mouse μ antibody-conjugated Myo (anti- μ -Myo), taken up by the BCR. Cells were incubated with graded concentrations of Myo or anti- μ -Myo and Myo-specific T cell hybrids for 24 h at 37°C, and the culture supernatants were assayed for IL-2 as a measure of T cell activation. Cells expressing wt μ required much less anti- μ -Myo than Myo to activate T cells (Fig. 2-5). Comparing to the wt μ BCR, the ability of μ YS/VV BCR to present the μ -specific antigen, anti- μ -Myo, was significantly decreased (Fig. 2-5). Thus, the mutation YS to VV inhibits the ability of the BCR to facilitate antigen processing.

Figure 2-5. The mutation YS to VV reduces BCR-mediated antigen presentation.

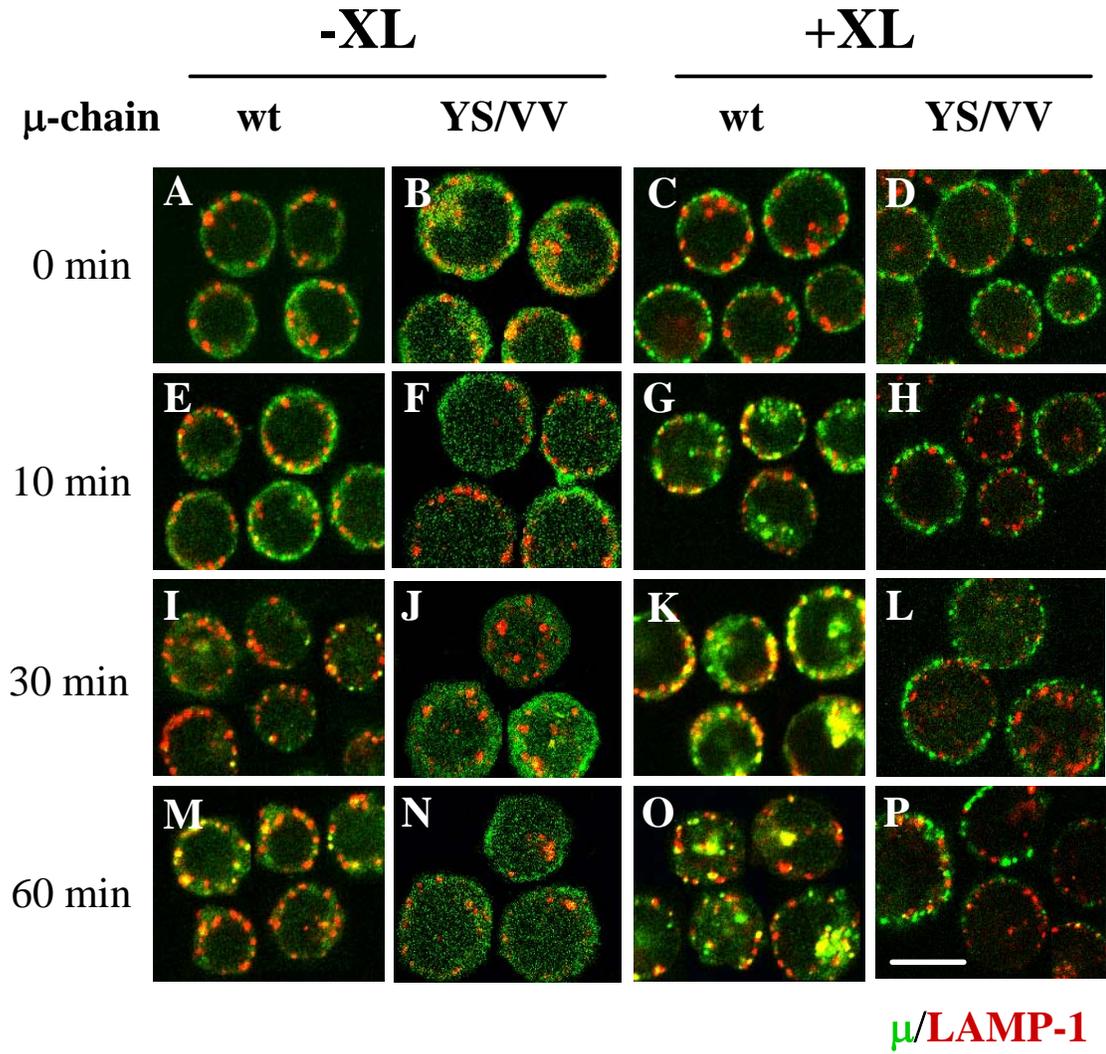
Transfectant A20 cells (5×10^4) were cultured with myoglobin-specific T cell clones (5×10^4) in the presence of graded concentrations of myoglobin (Myo) or myoglobin conjugated to anti- μ antibody (anti- μ -Myo) for 24 h. Culture supernatants were assayed for the presence of IL-2 as a measure of T cell activation. The concentration of IL-2 in the supernatants was determined by its ability to maintain the growth of the IL-2-dependent T cell line, CTLL. The proliferation of CTLL was measured by incorporation of [3 H]thymidine. Assays were done in triplicates. Shown are the means (\pm S.D.) of one of three independent experiments.



The decreased ability of the μ YS/VV BCR to facilitate antigen processing suggests that it may not correctly target antigens to the processing compartment. To investigate this possibility, the intracellular trafficking of the BCR was followed using immunofluorescence microscopy. The BCR on the cell surface was labeled with monovalent FITC-Fab-anti- μ antibody at 4°C, and a polyclonal anti- μ antibody was added to cross-link the BCR. Previous studies showed that the FITC-Fab-anti- μ does not compete with the polyclonal anti- μ for binding to the BCR [98]. The cells were washed and chased at 37°C for varying lengths of time. Late endosomes/lysosomes were labeled with LAMP-1-specific mAb. The intracellular distribution of BCR was analyzed using confocal fluorescence microscopy. Before the chase, FITC-Fab-anti- μ was primarily on the surfaces of the cells in all cases (Fig. 2-6A-D). In the absence of cross-linking, after 60 min of chase the wt μ BCR showed punctate staining at the cell periphery where it was partially colocalized with LAMP-1-staining (Fig. 2-6M). After only 10 min of cross-linking, the punctate staining of Fab-anti- μ moved to the center of cells and was colocalized with LAMP-1 (Fig. 2-6G). By 60 min, most of Fab-anti- μ and a significant portion of LAMP-1-stainings clustered in the perinuclear location where they were colocalized with each other (Fig. 2-6O). The colocalization of Fab-anti- μ and LAMP-1 in the cross-linked cells was far more extensive than that seen in the untreated cells (Fig. 2-6M and 6O). The intracellular distributions of the wt μ BCR in the presence or absence of cross-linking antibodies were similar to those of the endogenous BCR, indicating that transfected wt μ BCR behaves like the normal BCR.

Figure 2-6. The mutation YS to VV blocks the movement of the BCR from the plasma membrane to the LAMP-1⁺-compartment.

Cells were incubated with monovalent FITC-Fab-anti- μ in the presence (+XL) or absence (-XL) of anti- μ antibody (20 μ g/ml) to cross-link the BCR at 4°C for 60 min. After washing, the cells were chased at 37°C for times as indicated. Then the cells were fixed, permeabilized and incubated with LAMP-1-specific mAb, 1D4B, and TRITC-conjugated secondary antibodies to label late endosomes and lysosomes. The FITC and TRITC emissions were captured using a scanning laser confocal microscope. Single optical sections were taken from the middle of the cells. Shown are the representative images from three independent experiments. Bar, 10 μ m.



In contrast, in cells expressing μ YS/VV, FITC-Fab-anti- μ -staining remained on the cell surface up to 60 min of chase at 37°C (Fig. 2-6N). Treating the cells with the cross-linking antibody induced neither the clustering of Fab-anti- μ and LAMP-1 nor the colocalization of Fab-anti- μ and LAMP-1 (Fig. 2-6H, 6L, and 6P). Thus, the mutation YS to VV blocks the constitutive and cross-linking-stimulated movement of BCR to late endosomes/lysosomes.

To quantify the level of internalization of the wt μ and μ YS/VV BCR, cells were incubated with [¹²⁵I]Fab-anti- μ in the presence and absence of anti- μ antibody at 4°C. The cells were washed and incubated at 37°C for varying lengths of time to allow internalization. At the end of each time point, the radioactivity released from the cells, on the cell surface, and inside the cells was measured. At the end of each time period, the incubation media were collected and counted as radioactivity released. [¹²⁵I]Fab-anti- μ on the surface of the cells was stripped with an acid solution, collected and counted as radioactivity on the cell surface. [¹²⁵I]Fab-anti- μ associated with the cells after stripping was counted as internalized. These values were added and the data are presented as a percentage of the total radioactivity associated with cells before warming. Without cross-linking, wt μ internalized [¹²⁵I]Fab-anti- μ at a low rate, reaching 24% of the ligand initially bound to the cell surface by 10 min (Fig. 2-7A). Cross-linking the μ -chain dramatically increased the internalization rate, reaching 57% during the same length of time (Fig. 2-7A). This is consistent with our previously published results [97] that the IgM-based BCR in CH27 cells constitutively internalizes at a low rate, and cross-linking BCR stimulates BCR internalization. Without cross-linking, μ YS/VV internalized

Figure 2-7. Internalization of the transfected mIgM under different stimulation conditions.

Cells were incubated with [¹²⁵I]Fab-anti- μ in the presence or absence of different cross-linking or coligation antibodies at 4°C for 60 min. The cells were treated as follows: *A*, medium alone or 20 μ g/ml anti- μ antibody and/or 100 μ M PP2, *B*, medium alone or 20 μ g/ml anti- γ_{2a} antibody, and *C*, 20 μ g/ml anti- μ or 20 μ g/ml anti-IgG+M antibody. After extensive washing at 4°C, the cells were allowed to internalize the ligand at 37°C for the times indicated. At the end of each time period, the incubation media were collected and counted. [¹²⁵I]Fab-anti- μ on the surface of the cells was stripped with an acid solution. [¹²⁵I]Fab-anti- μ associated with the cells after stripping was counted as internalized. Internalized fractions are expressed as percentages of the total radioactivity initially associated with the cells. The results are the averages (\pm S.D.) of four independent experiments. (A) * indicates a significant difference between wt μ and μ YS/VV ($p < 0.01$).

Fig 2-7

[¹²⁵I]Fab-anti- μ at a rate lower than wt μ , only reaching 15% during 10 min of incubation (Fig. 2-7A). Cross-linking the μ -chain only slightly increased the internalization to 26% by 10 min. The cross-linking-induced increase in the internalization rate of μ YS/VV was significant and sensitive to the tyrosine kinase inhibitor, PP2 (Fig. 2-7A). However, this increase fell far short compared to wt μ . Since the surface expression levels of wt μ and μ YS/VV are similar (Fig. 2-1B), the expression levels cannot be accounted for the difference in their internalization rates. Thus, the mutation YS to VV inhibits both the constitutive and accelerated internalization of BCR.

Cross-linking the endogenous BCR independently and coligating the μ YS/VV BCR with the endogenous BCR cannot restore the internalization of the μ YS/VV BCR

To determine if signaling from the endogenous BCR stimulates the internalization of the transfected μ -chains, the wt μ or μ YS/VV BCR was labeled with [¹²⁵I]Fab-anti- μ and the endogenous BCR cross-linked with the γ_{2a} -specific antibody (Fig. 2-3B). The internalization of [¹²⁵I]Fab-anti- μ was analyzed as described above. Cross-linking the endogenous BCR did not significantly increase the internalization of either wt μ or μ YS/VV BCR (Fig. 2-7B). This is despite the fact that the cross-linking induced high levels of tyrosine phosphorylation in the wt μ or μ YS/VV expressing cells (Fig. 2-4). Thus, the signaling initiated by the endogenous BCR does not stimulate the internalization of the μ -chains.

To test whether the coligation of the endogenous BCR and the μ YS/VV BCR influences the internalization of the μ YS/VV BCR, the μ YS/VV and endogenous BCRs on the cell surface were cross-linked using the anti-IgG+M antibody (Fig. 2-3C). The internalization of μ YS/VV in cells treated with anti-IgG+M was analyzed using [125 I]Fab-anti- μ . Compared to cells treated with anti- μ alone, the internalization of μ YS/VV in cells treated with the coligation antibody was largely unchanged (Fig. 2-7C). Thus, coligation of the μ YS/VV BCR and the endogenous BCR cannot restore the internalization of the μ YS/VV BCR to the level seen with the wt μ BCR.

Coligation of the μ YS/VV BCR with the endogenous BCR promotes internalized μ YS/VV BCR to enter the LAMP-1⁺-compartment

Next, we asked whether the activation of the endogenous BCR or the coligation of the μ YS/VV BCR with the endogenous BCR affects the targeting of μ YS/VV to the LAMP-1⁺-late endosomes. The transfectants were incubated with FITC-Fab-anti- μ and anti- γ_{2a} (Fig. 2-3B) or anti-IgG+M antibody (Fig. 2-3C) sequentially at 4°C, washed, and chased at 37°C for 60 min. The cells were labeled with LAMP-1-specific mAb to mark the late endosomes/lysosomes. The colocalization of the μ -chain and LAMP-1 indicates the entry of the BCR to late endosomes/lysosomes. As shown earlier, most of the μ YS/VV BCR remained on the cell surface over a course of 60 min following cross-linking, and was not colocalized with LAMP-1 (Fig. 2-8A). Cross-linking the endogenous BCR resulted in the LAMP-1⁺-compartment moving from the cell periphery to center of the cells. However, the μ YS/VV BCR remained on the outer rim of cells, and no significant colocalization of μ YS/VV and LAMP-1 was detected (Fig. 2-8B). Thus, signaling

Figure 2-8. Movement of the transfected μ -chain and endogenous BCR to late endosomes/lysosomes.

To label the transfected μ -chain, cells were incubated with monovalent FITC-Fab-anti- μ antibody (*A-D*) in the presence of different cross-linking and coligation antibodies at 4°C for 60 min. To label the endogenous BCR, cells were incubated with TRITC-anti- γ_{2a} antibody (*E-F*). *A*, μ YS/VV transfectants were treated with 20 μ g/ml anti- μ antibody; *B*, μ YS/VV transfectants were treated with 20 μ g/ml anti- γ_{2a} antibody; *C* and *E*, μ YS/VV transfectants were treated with 20 μ g/ml anti-IgG+M; *D* and *F*, wt μ transfectants were treated with 20 μ g/ml anti-IgG+M antibody. After washing, the cells were chased at 37°C for 60 min. Then the cells were fixed, permeabilized and incubated with LAMP-1-specific mAb, 1D4B, and TRITC- (*A-D*) or FITC- (*E-F*) conjugated secondary antibodies to label late endosomes/lysosomes. The FITC and TRITC emissions were captured using a scanning laser confocal microscope. Single optical sections were taken from the middle of the cells. Shown are the representative images from three independent experiments. Bar, 10 μ m.

Fig 2-8

transduced by the endogenous BCR independently of the transfected μ -chain does not induce the targeting of μ YS/VV BCR to late endosomes/lysosomes.

Coligation of the endogenous BCR and μ YS/VV BCR resulted in colocalization of a small but significant portion of μ YS/VV and LAMP-1 in the center of cells (Fig. 2-8C). The majority of μ YS/VV and LAMP-1-stainings remained on the cell surface and at the cell periphery. Thus, compared to cross-linking the μ YS/VV BCR alone, the coligation of the endogenous BCR and μ YS/VV BCR significantly increased the colocalization of μ YS/VV and LAMP-1, but to a far less extent than that seen for wt μ BCR (Fig. 2-8C and 8D). Since the coligation does not increase the internalization of μ YS/VV, the small amount of μ YS/VV entering the LAMP-1⁺-compartments is probably derived from the small pool of internalized μ YS/VV.

To analyze the effect of the μ YS/VV BCR on the intracellular movement of the endogenous BCR, cells were incubated at 4°C with TRITC-anti- γ_{2a} antibody to label the endogenous BCR prior to the coligation. The cells were washed at 4°C and chased at 37°C for 60 min. In wt μ -expressing cells, the endogenous BCR and LAMP-1 moved to the perinuclear area where they were extensively colocalized with each other (Fig. 2-8F). In contrast, the movement of the endogenous BCR from the cell surface to the perinuclear area and its colocalization with LAMP-1 were significantly decreased by the coligation with μ YS/VV BCR (Fig. 2-8E). This suggests that the coligation of the μ YS/VV BCR with the endogenous BCR inhibits the movement of the endogenous BCR

to late endosomes/lysosomes. The effects of the different crosslinking or coligation conditions on the BCRs is summarized in Figure 2-9.

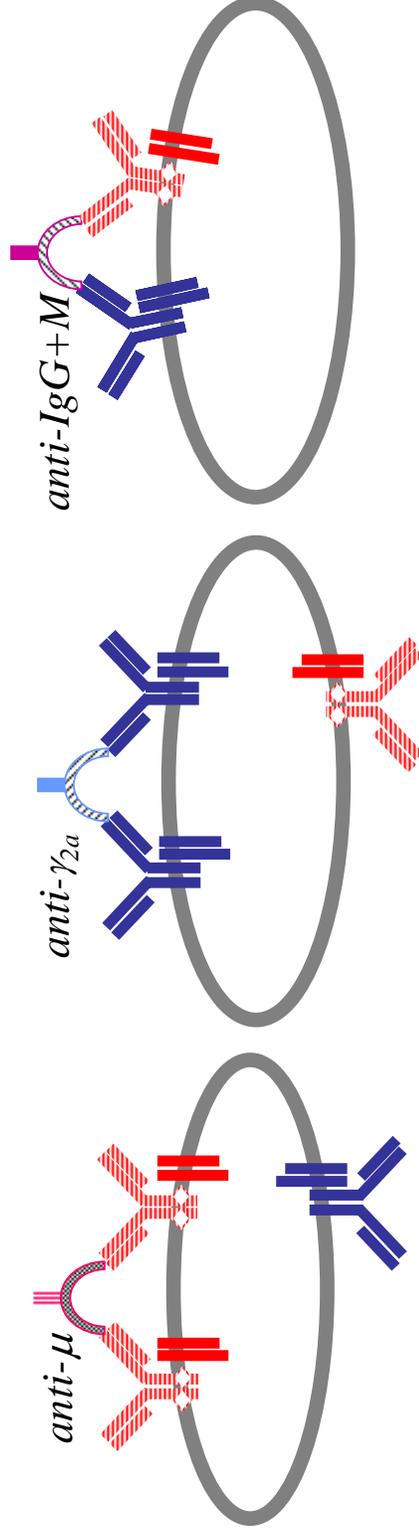
2.5 Discussion

The BCR plays a central role in B cell activation. The BCR senses the presence of antigens, initiates signaling cascades, and internalizes antigens for processing and presentation that acquires T cells' help. The binding avidities of antigens to the BCR modulate the stability of BCR and BCR-antigen complexes [132] and regulate the outcome of BCR signaling and the efficiency of antigen presentation [130, 144, 145]. To understand how the stability of BCR influences their functions, we have examined the effect of mutation YS to VV in the transmembrane domain of the μ -chain on BCR-induced protein tyrosine phosphorylation and BCR-mediated antigen targeting. Previous work with this mutant BCR first showed that there was a decrease in signaling and antigen presentation [8]. Later work indicated that this decrease in signaling was due to a lack of association of mIgM with $Ig\alpha/Ig\beta$ [9, 11]. Another study confirmed that when there is a lack of association of mIgM with $Ig\alpha/Ig\beta$, there is a decrease in signaling and a decrease in antigen presentation [10]. In this study, we wanted to examine the effect of this mutant unstable BCR on the internalization and intracellular trafficking of the BCR, which had not been observed yet, as well as signaling and antigen presentation. We also wanted to see if the destabilized BCR affected the endogenous BCR and vice versa, which also had not been determined. We found that the mutation YS to VV in the transmembrane domain of the μ -chain weakens the association of mIgM with $Ig\alpha/Ig\beta$, reduces the protein tyrosine phosphorylation induced by BCR cross-linking, inhibits both the

Figure 2-9. Summary of the effects of the different crosslinking or coligation conditions on the BCRs.

Crosslinking the mutated μ -chain independently shows defects in signal transduction, antigen presentation, intracellular trafficking and internalization of the BCR.

Crosslinking the endogenous BCR independently does not improve defects seen when crosslinking the mutated μ -chain independently. Coligation of the mutant μ and endogenous BCR improves the transport but not internalization of the mutant μ BCR.



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constitutive and signaling-stimulated internalization of the BCR, and blocks the movement of the BCR to late endosomes/lysosomes. These results indicate that the stability of BCR is essential for both the signaling and antigen-targeting functions of the BCR.

Modulating the stability of BCR appears to be one of the major regulatory mechanisms for BCR signaling. Vilen et al. [132] reported that the binding of multivalent, low affinity antigens desensitizes the BCR by weakening the interaction between mIg and Ig α /Ig β , indicating that BCR destabilization plays a role in generating the unresponsiveness of antigen-desensitized and perhaps anergic B cells. More recently they showed that destabilization does lead to a physical separation of mIg and Ig α /Ig β , with each component being found in different microdomains on the cell surface [146]. Here we show that destabilizing the BCR by mutations not only inhibits BCR signaling, but also blocks the internalization and targeting of the BCR to late endosomes/lysosomes, consequently decreasing the antigen-presenting efficiency of the B cells. Blocking BCR-mediated antigen transport probably is an additional mechanism by which the unresponsive and anergic stages of B cells are generated and maintained.

In vivo, concentrations of self-antigens are unlikely to be high enough to saturate the cell surface BCR and destabilize every receptor on the cell surface. How the destabilized BCR influences the functions of the rest of stable BCRs to generate the unresponsiveness of B cells is unclear. The mutation YS to VV destabilizes the BCR, resulting in an antigen-unresponsive BCR. Using B cells coexpressing the μ YS/VV BCR and

endogenous wt IgG_{2a} BCR, we analyzed the effect of the destabilized BCR on the functions of the stable BCR. Cross-linking the endogenous BCR independently of the μ YS/VV BCR induces protein tyrosine phosphorylation at a level similar to that seen in wt μ -expressing cells. However, the signaling from the endogenous BCR does not restore the internalization rate and intracellular movement of the μ YS/VV BCR. These suggest that two populations of BCRs, mIgM- and mIgG_{2a}-based, function independently of each other. Activation of one population of the BCR by cross-linking does not automatically bring the other population into the activating complex. Coligation of the μ YS/VV BCR with the endogenous BCR does not induce a full spectrum of phosphorylated proteins as seen in the wt μ -expressing cells, and inhibits the movement of the endogenous BCR to the LAMP-1⁺-compartment. Thus, even though the endogenous BCR remains competent for signaling and antigen targeting, coligation with the destabilized BCR inhibits both of its signaling and antigen-targeting functions. This could be due to the fact that the colligating antibody is crosslinking more of the μ YS/VV BCR and fewer of the endogenous BCR, thus not allowing the endogenous BCR to be stimulated at the same level as when it is crosslinked individually. This data could also suggest that destabilized BCR can interfere with the signaling and antigen-targeting capabilities of the stable BCR, blocking antigen-specific response of B cells. The minimum percentage of destabilized BCR required for generation of unresponsiveness of B cells has not been determined. The expression level of the μ YS/VV BCR is similar to that of the endogenous BCR, suggesting that the unresponsiveness of B cells can be achieved when 50% of the surface BCRs are destabilized. More recently Vilen et al showed that early, but not later, steps in endogenous BCR signaling are impaired coaggregation with

destabilized receptors, with the destabilized receptors making up as few as 15% of the total [147].

Ig α /Ig β and mIg, the two components of the BCR, are held together through non-covalent interaction. Through this non-covalent interaction, the binding of antigen to mIg is translated into activation of the receptor-associated Src-kinases and phosphorylation of the cytoplasmic tails of Ig α /Ig β . The transmembrane domain of mIgM consists of nine hydrophilic residues that are distributed around the putative α -helix potentially formed. One side of this putative α -helix, where the sequence is conserved between all classes of Ig, has been demonstrated to be important for mIgM to interact with Ig α /Ig β . The opposite side, where the sequence is class-specific but evolutionarily conserved, has been shown to be involved in the formation of the class-specific oligomeric BCR complex [13, 148]. Early studies have shown that mutating tyrosine and serine in the N-terminal end of the transmembrane domain to valines disrupts the interaction between mIgM and Ig α /Ig β [8, 11]. Using surface biotinylation and mild detergent, digitonin, the association of the Ig α /Ig β with wt μ and μ YS/VV during their intracellular movement was analyzed. We found that wt μ remained associated with Ig α /Ig β for up to 2 h when a significant amount of the surface-labeled BCR was transported to the LAMP-1⁺-late endosomes/lysosomes, which is consistent with our earlier published report [120]. To our surprise, in spite of a weak association shown earlier in whole-cell lysate analysis [11], the association between the biotinylated Ig α /Ig β and μ YS/VV was still prominent over the first 30 min of the chase, but rapidly decreased as the chase time increased. This result suggests that μ YS/VV forms a short-lived complex with Ig α /Ig β on the cell

surface. This transient association of μ YS/VV with Ig α /Ig β provides an explanation for its ability to initiate a low level of protein tyrosine phosphorylation and Ca²⁺-influx [11]. A question raised by this observation is why μ YS/VV can interact with Ig α /Ig β during the first 30 min of the chase, but not retain the interaction. There are at least two possible explanations. The BCR undergoes constitutive internalization. Within 30 min at 37°C, the internalization and recycling of the BCR reach equilibrium. When the BCR enters the endocytic endosomes, the acidic environment of the endosomes may disrupt the weak interaction between μ YS/VV and Ig α /Ig β . In addition, μ YS/VV may compete with the endogenous mIgG_{2a} for Ig α /Ig β . The stronger interaction between mIgG_{2a} and Ig α /Ig β removes Ig α /Ig β from μ YS/VV when mIgG_{2a} internalizes with Ig α /Ig β , leaving μ YS/VV behind. Our results from the internalization analyses show that μ YS/VV does not internalize very well, supporting the second hypothesis.

In response to antigens, BCR signaling and trafficking pathways need to communicate with each other in order to steer B cells to a proper response. Cross-linking the BCR by bivalent or multivalent antigens induces signaling cascades, and also accelerates antigen targeting to enhance antigen processing [97, 99]. Blocking BCR internalization inhibits the attenuation of BCR signaling [98]. At present, BCR-mediated signaling and antigen transport pathways have been intensively studied, but the relationship between the signaling and antigen-targeting functions of the BCR remains to be understood. The mutation YS to VV generates a signaling-desensitized BCR. Using B cells expressing both signaling-desensitized (μ YS/VV) and competent BCRs (the endogenous), we analyzed the relationship between BCR-signaling and BCR-trafficking events. Cross-

linking the desensitized BCR does not restore the rate of BCR internalization and targeting to late endosomes/lysosomes, indicating that receptor aggregation itself in the absence of proper interaction between mIg and Ig α /Ig β and signaling is not sufficient to induce the internalization and intracellular movement of BCR. Cross-linking the endogenous BCR independently of transfected μ -chains induces a normal level of signaling, including increases in protein tyrosine phosphorylation and the clustering of late endosomes/lysosomes, but does not stimulate the internalization of either wt μ or μ YS/VV BCR, indicating that signals from neighbor receptors cannot stimulate BCR internalization. When the desensitized and competent BCRs are brought together through coligation, the internalization of μ YS/VV BCR remains low, indicating that the proper interaction between mIg and Ig α /Ig β is essential for BCR internalization. These results demonstrate that physical association of signaling events with the BCR is essential for stimulating BCR internalization. This association is achieved by the interaction between the antigen binding-domain of the BCR, mIg, with the signaling component of the BCR, Ig α /Ig β . The maintenance of this association is dependent on the stability of BCR complexes. Coligation of the μ YS/VV BCR with the endogenous BCR does not increase the internalization of the μ YS/VV BCR, but does induce a small amount of internalized μ YS/VV to enter late endosomes/lysosomes. This suggests that BCR signaling differentially regulates BCR internalization and entry of BCR to late endosomes/lysosomes.

Shaw et al. [8] reported earlier that introducing YS to VV mutations to a human-mouse chimeric μ -chain expressed in A20 cell inhibits its ability to facilitate antigen processing

and presentation, which is consistent with our results. Here we show that mouse μ YS/VV BCR fails to internalize and target antigens to the processing compartments, which explains its low antigen processing and presentation efficiency. However, unlike mouse μ YS/VV, human-mouse chimeric μ YS/VV internalizes at the same rate as the wt BCR [10]. This discrepancy may result from the differences in the structures of the μ -chains. Indeed, there is a single amino acid difference between the transmembrane domains of mouse and human membrane μ -chains (Fig. 2-1) [11] and additional differences in the extracellular domains [12]. The structural differences of mIgs from different species could influence their interaction with $Ig\alpha/Ig\beta$ and their functions.

The activation of B cells to respond to T cell-dependent antigens and to generate B cell memory requires both BCR signaling and T cells' help through antigen processing and presentation. The binding of antigens to mIg is transduced through the interaction between mIg and $Ig\alpha/Ig\beta$ into the phosphorylation of $Ig\alpha/Ig\beta$, activation of signaling cascades and fast internalization and intracellular movement of the receptor. The binding affinity and avidity of antigens modulate the stability of BCR to fine-tune the signaling and antigen-targeting functions of the BCR. The signaling and antigen-targeting pathways of the BCR regulate each other in order to mount an appropriate response. Further studies on the mechanisms for finely tuning BCR functions and for the cross talk between BCR signaling and trafficking pathways will greatly extend our understanding of B cell biology.

Chapter III: The tyrosines of the Ig α ITAM are required for efficient transport of the BCR to antigen processing compartments

3.1 Abstract

The B cell antigen receptor (BCR) has dual roles. Upon the binding of antigen, the BCR initiates signaling cascades which begin activation of the B cells. Subsequently the BCR internalizes antigen and transports it to the MHC class II-containing endosomal compartment for processing and presentation. Current evidence indicates that BCR-triggered signaling is important for rapid internalization and transport of the BCR to antigen loading compartments. Here we analyzed the role of BCR signaling in regulation of the BCR-mediated antigen targeting by mutating the key amino acids of the immunoreceptor tyrosine-based activation (ITAM) motif of the Ig α cytoplasmic tail. Mutation of the N-terminal ITAM tyrosine of Ig α (Y182A) had no significant effect on BCR-triggered signaling and BCR internalization and intracellular transport. Mutation of the C-terminal ITAM tyrosine of Ig α (Y193A) caused a decrease in BCR signaling and a defect in BCR internalization and intracellular transport. Mutating both tyrosines led to a greater decrease in BCR signaling and a greater defect in the internalization and transport of antigen to LAMP-1⁺ compartments than the Y193A mutation. Mutating each or both tyrosines and their associated bulky hydrophobic residue in the Ig α ITAM showed a decrease in signaling and intracellular transport of the BCR, but it was equivalent to mutating the respective tyrosines alone. Thus, the ITAM of the Ig α cytoplasmic tail, which is known to be necessary for signaling, is also important for accelerated

internalization of the BCR and efficient transport of BCR through the endocytic pathway to the antigen-processing compartments.

3.2 Introduction

A central event that leads to T cell dependent antibody responses is the presentation of antigen by B cells to helper T cells. The antigens are first recognized, internalized and processed by B cells before they are presented on the B cell surface. B cells express clonally specific antigen receptors which bind antigen. Binding of antigen initiates signaling cascades, which are the first signals needed for B cell activation [30, 133, 134]. After binding, the B cell antigen receptor (BCR) internalizes the antigen and transports it through the endocytic pathway [120]. The antigen is targeted to the processing compartments where it is proteolytically degraded into peptides. These peptides are loaded into major histocompatibility complex (MHC) class II molecules and the antigenic peptide:MHC class II complexes are transported to the surface [136, 137]. The recognition of an antigenic peptide:MHC class II complex by a T cell leads to the second signal that is needed for B cell activation.

The BCR is composed of the membrane Ig (mIg) and the $Ig\alpha/Ig\beta$ heterodimer ($Ig\alpha/Ig\beta$). The mIg and the $Ig\alpha/Ig\beta$ associate non-covalently to form the BCR complex [12]. The mIg is the antigen binding component, while the $Ig\alpha/Ig\beta$ is known to be the signaling component [9, 133, 134]. The cytoplasmic tails of the $Ig\alpha/Ig\beta$ contain conserved motifs known as immunoreceptor tyrosine-based activation motifs (ITAMs), which have been shown to be essential for signaling functions in the BCR [23]. The BCR also serves to

transport antigens. The BCR allows B cells to be very efficient antigen presenting cells. Antigens can enter cells through fluid phase pinocytosis, but it requires 1,000 to 10,000 times more antigen to reach a similar antigen presentation level than antigen internalized by the BCR. This allows B cells to present antigen even when the concentration of antigen is low [149]. The BCR can internalize monovalent antigen, which leads to the processing and presentation of the antigen, but divalent antigen, which crosslinks the BCRs and triggers the signaling cascade, can be present at $1/10^{\text{th}}$ the concentration, suggesting a regulatory role of BCR signaling in its antigen processing function [150].

Although the mIg is known to be the antigen binding component and the $Ig\alpha/Ig\beta$ is known to be the signaling component, it appears that signaling and antigen processing are not separate activities. Previous studies indicate that signaling is required for optimum antigen processing and presentation. Several studies, including my own, show that mutated mIgM that no longer associates with $Ig\alpha/Ig\beta$ not only fail to initiate signaling, but the antigen processing and presentation efficiency of these B cells is also greatly reduced, indicating that $Ig\alpha/Ig\beta$ is important for BCR-mediated antigen processing [8-11, 94, 151]. Cross-linking the BCR, which initiates the signaling cascade, increases the rate of internalization, accelerates the targeting of the BCR to the antigen processing compartments, and increases antigen presenting efficiency [97, 152]. Tyrosine kinase inhibitors that prohibit initiation of signaling also inhibit accelerated antigen transport and lower the antigen-presenting efficiency of B cells [125, 126]. The tyrosine phosphorylation sites on both the cytoplasmic tail of the $Ig\alpha$ and the tyrosine kinase Syk have been shown to be important for BCR-mediated antigen processing [95, 122, 124].

When a dominant negative Syk mutant is overexpressed, signaling and antigen presentation are inhibited [122]. Initiation of signaling in B cells has been shown to induce aggregation and fusion of the late endosomal compartments where antigens are loaded onto MHC class II molecules [127]. Thus, signaling mediated by the BCR is important not just as a first activation step, but is also necessary for optimal efficiency of antigen processing.

The ITAMs in the cytoplasmic tails of $Ig\alpha$ and $Ig\beta$ have distinct roles in initiation of signal transduction [153, 154]. Previous studies have shown that chimeras consisting of either the $Ig\alpha$ or the $Ig\beta$ cytoplasmic tail can internalize antigen almost as well as wt BCR and present antigen, but at a greatly reduced efficiency compared to wt BCR [121, 124]. It has also been shown that both $Ig\alpha$ and $Ig\beta$ are required to stimulate protein tyrosine phosphorylation, induce the aggregation of late endosomal compartments and present antigen as efficiently as wt BCR [121, 123, 124, 154]. Although $Ig\alpha$ or $Ig\beta$ alone can present antigen at a reduced efficiency, $Ig\beta$ alone transports antigen only to early endosomal compartments [121]. On the other hand, $Ig\alpha$ alone transports antigen to the MIIC where antigens can be efficiently processed [121, 122]. Therefore, $Ig\alpha$ appears to have a more important role in antigen transport and presentation. A chimera consisting of only the ITAM of $Ig\alpha$ still internalized constitutively, but a mutation of the first tyrosine of the ITAM abolished constitutive internalization [95]. Another chimera which consisted of a wt $Ig\beta$ cytoplasmic tail and an $Ig\alpha$ cytoplasmic tail with both ITAM tyrosines mutated failed to initiate signaling or to present antigen [124]. These results

suggest that in addition to signaling the ITAMs of the BCR also play an important role in BCR-mediated antigen processing.

How the ITAM of the BCR is involved in antigen processing and presentation remains to be elucidated. The signaling cascade initiated by the ITAM of the BCR could regulate the endocytic machinery, which is required for BCR-mediated antigen uptake and processing. In addition, the ITAM could provide a binding site for the clathrin adaptor protein (AP-2), which is important for BCR-antigen internalization. The sequence YXX ϕ , where ϕ is a bulky hydrophobic amino acid, has been shown to be an internalization motif on the cytoplasmic tail of other receptors [155]. This sequence is found twice in the Ig α ITAM, as YXXL at the N-terminal tyrosine and YXXI at the C-terminal tyrosine.

In this study we investigated the role of the ITAM of the Ig α in the internalization and intracellular trafficking of the BCR. To accomplish this, we mutated each of the tyrosines individually and in combination. In addition to the tyrosines of the ITAM, we further mutated the leucine and isoleucine of the Ig α ITAM, as they may be part of internalization or targeting motifs. Thus we created Ig α s in which the tyrosine and leucine of one putative motif were mutated, the tyrosine and isoleucine of the other putative motif were mutated, or all four residues were mutated. HA-tagged, mutated Ig α s were expressed in B cell lymphoma A20 cells. The effect of mutations on signaling and antigen transport functions was examined.

3.3 Materials and Methods

Cell culture and antibodies

The B cell lymphoma, A20 IIA1.6, is an H-2^d, IgM⁻, IgG_{2a}⁺ and FcγRIIb1⁻ cell line. The A20 cells were cultured in DMEM supplemented as described [140], containing 10% FCS. The rat hybridoma 1D4B producing a LAMP-1-specific mAb was obtained from the Development Studies Hybridoma Bank (Iowa City, IA). Goat antibodies specific for rat IgG, mouse μ chain (anti-μ), F(ab')₂ fragment of goat anti- mouse IgG+M (F(ab')₂-anti-Ig), Fab fragment of goat anti-mouse μ chain antibody (Fab-anti-μ), and their FITC, TRITC or Cy3-conjugates were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Tyrosine phosphate-specific mAb, 4G10, was from Upstate Biotechnology (Waltham, MA).

Plasmid construction and transfection

Plasmid pEVmb-1 HAN neo containing a functionally rearranged genomic clone of the murine Igα gene with a hemagglutinin tag inserted at the N-terminal were kindly provided by Dr. Anthony L. DeFranco. This gene was cut from the plasmid using NdeI restriction enzyme and ligated it into the smaller plasmid pcDNA3 plasmid which contains G418 resistance gene *neo*. The mutations of tyrosine, leucine or isoleucine to alanine were engineered using complementary primers and PCR [156]. Mutant HA-Igαs were generated using oligonucleotides with the following sequences: Y182A, 5'-GACT ATGAAGATGAAAATCTCGCTGAGGGCCTGAACCTTG-3'; Y193A, 5'-GATGACT GTTCTATGGCTGAGGACATCTCCAG-3'; YALA, 5'GACTATGAAGATGAAAATC TCGCTGAGGGCGCGAACCTTGATGACTG-3'; YAIA, 5'GACTGTTCTATGGCTG

AGGACGCCTCCAGGGGACTC-3' (BioServe Biotechnologies, Laurel, MD).

Mutations were verified by DNA sequencing (Macrogen, Inc.). Double mutations (Y182A/Y193A and Y2LAIA) were generated by using one oligonucleotide to create a single mutation, verifying the sequence, then using a second oligonucleotide to create the second mutation. Plasmid was transfected into A20 cells by electroporation. Stable drug-resistant clones were selected in medium containing 0.75 mg/ml G418. Drug-resistant clones were screened for expression of HA-Ig α by flow cytometry.

Analysis of tyrosine phosphorylated proteins

Cells were incubated with anti-mouse IgG (20 μ g/ml) at 4°C for 10 min, warmed to 37°C for 2, 5, 10 and 20 min, and lysed in a buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM Na₃VO₄, and protease inhibitors) at 4°C. The cell lysate was analyzed by SDS-PAGE and Western blotting. The cell lysates generated from an equal number of cells were loaded onto each well of gels. Blots were probed with mAb 4G10.

Immunofluorescence microscopy

A20 cells were incubated with FITC-anti- μ for 40 min at 4°C on polylysine-coated slides (Sigma, St. Louis, MO). The cells were then washed at 4°C and chased for varying lengths of time at 37°C. After the chase, the cells were fixed by incubating them with 4% paraformaldehyde in PBS for 20 min. For LAMP-1 staining, after the chase, cells were fixed, and incubated with a permeabilization buffer (1% gelatin, 0.05% saponin, 10 mM glycine, 10 mM HEPES, pH 7.4) for 15 min. They were then incubated with 1D4B mAb

in the permeabilization buffer for 1 h. The cells were then washed and incubated for 30 min with TRITC-goat-anti-rat IgG. The cells were then washed, post-fixed and mounted with Gel/Mount (Biomedex, Foster City, CA). Double immunofluorescence analyses were carried out on a scanning laser confocal microscope (Zeiss LSM 510). Images were acquired using a 100X oil immersion objective, and cropped using Photoshop (Adobe, Mountain View, CA). Optical sections in the middle of cells were selected.

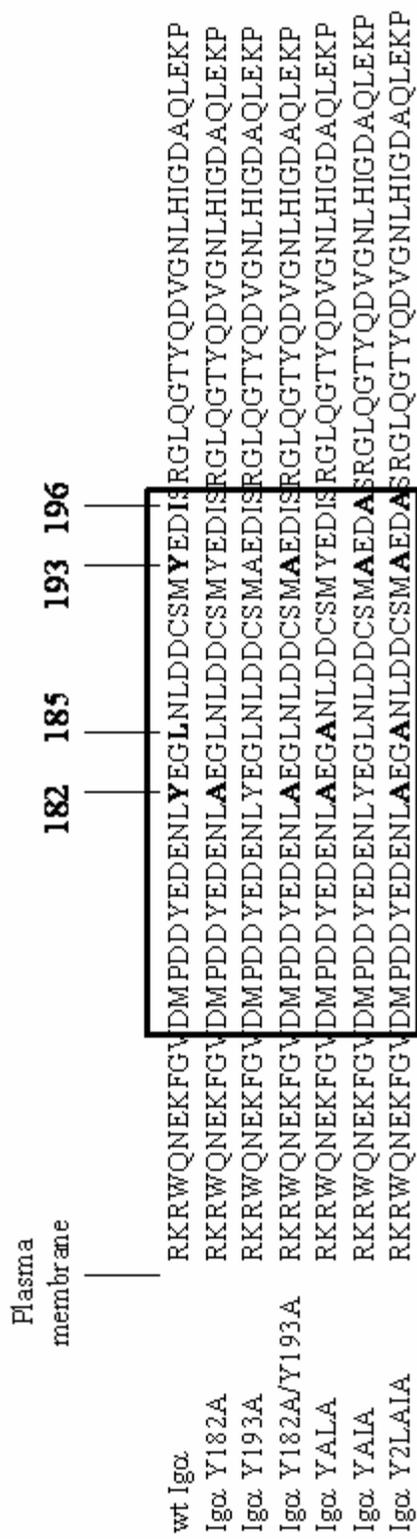
3.4 Results

The expression of wt and mutant HA-tagged Ig α

We expressed either a wild-type (wt) or a mutated Ig α in a murine B lymphoma A20 cell line which express an endogenous IgG_{2a}-based BCR. Both wt and mutated Ig α s have an extracellular hemagglutinin (HA) epitope tag to differentiate them from the endogenous wt Ig α . The mutated Ig α contain one or more point mutations of residues of the ITAM (Fig. 3-1). The mutant Ig α Y182A contains a point mutation of the first tyrosine in the ITAM to an alanine, while Ig α Y193A contains a point mutation of the second tyrosine in the ITAM to an alanine. The mutant Ig α Y182A/Y193A contains both of these point mutations. To study the putative internalization motifs YXXL/I, mutant Ig α were also created which contained mutations of both the tyrosine and its respective leucine or isoleucine. The mutant Ig α YALA contains two point mutations, the N-terminal ITAM tyrosine and the downstream leucine mutated to alanines. The mutant Ig α YAIA contains two point mutations, the C-terminal ITAM tyrosine and the downstream isoleucine mutated to alanines. The expression levels of HA-tagged Ig α (HA-Ig α) was

Figure 3-1: Ig α cytoplasmic tail sequence.

Protein sequence of the Ig α cytoplasmic tail for wt and mutated Ig α s, with the immunoreceptor tyrosine activation motif marked with a box. The introduced mutations are marked by bold letters. Numbering begins at the amino terminus.



analyzed by flow cytometry using anti-HA mAb. Clones expressing wt or mutant HA-Ig α at similar levels were chosen for further analysis (Fig. 3-2A-F).

BCR-triggered protein tyrosine phosphorylation in B cells expressing mutated HA-Ig α

To examine the effect of the transfected Ig α s on BCR-induced signaling, we analyzed protein tyrosine phosphorylation triggered by BCR cross-linking in transfected A20 cells. Cells were incubated with anti-mouse IgG (20 μ g/ml) at 4°C for 10 min, and then warmed to 37°C for 2, 5, 10, and 20 min to activate the BCR. The cells were immediately lysed at 4°C, and the cell lysates were analyzed using SDS-PAGE and Western blot, probing for tyrosine phosphorylated proteins. Crosslinking the BCR in A20 cells expressing wt HA-Ig α induced protein tyrosine phosphorylation at a level similar to that in untransfected A20 cells (Fig. 3-3A). The protein tyrosine phosphorylation peaked at 2 min, followed by a decrease occurring by 5 min and continuing through 20 min. The gross tyrosine phosphorylation level induced in A20 cells expressing HA-Ig α Y182A was equivalent to that in A20 cells expressing wt HA-Ig α , which suggests that expression of this mutated HA-Ig α does not affect the ability of BCR to transduce signal. The A20 cells expressing HA-Ig α Y193A showed a decreased ability to induce signaling. Tyrosine phosphorylation did increase after 2 min, but not to the level seen with A20 cells expressing wt or HA-Ig α Y182A. Thus, this mutation impairs BCR signal induction, but does not eliminate it. Induction of signaling was not seen in A20 cells expressing HA-Ig α Y182A/Y193A as there is no increase in protein tyrosine phosphorylation after BCR crosslinking. Therefore, this double mutation significantly reduced BCR signal induction.

Figure 3-2: Expression wt and mutant HA-tagged Ig α .

Cells were incubated with FITC-conjugated anti-HA antibodies at 4°C for 20 minutes, then washed and then fixed for flow cytometry and analyzed with FACScan. A-C, grey line is wt HA-Ig α expression, dotted line is mutant HA-Ig α expression. (A) HA-Ig α Y182A, (B) HA-Ig α Y193A, (C) HA-Ig α Y182A/Y193A. (D-F), black line is wt HA-Ig α expression, grey line is mutant HA-Ig α expression. (D) HA-Ig α YALA, (E) HA-Ig α YAIA, (F) HA-Ig α Y2LAIA.

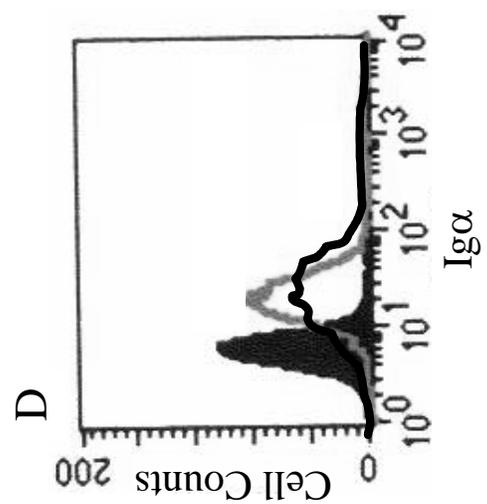
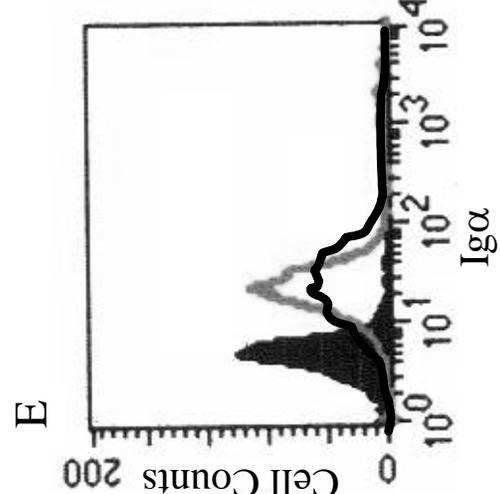
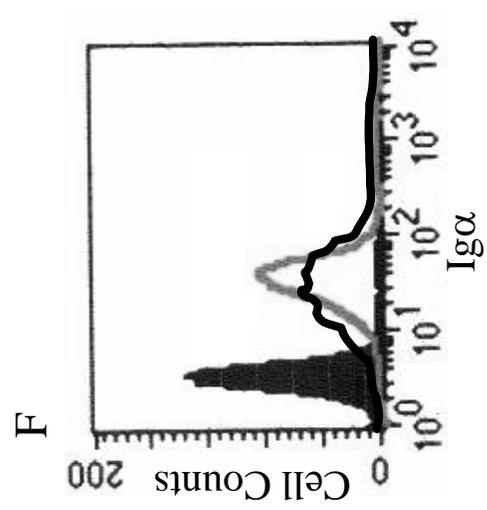
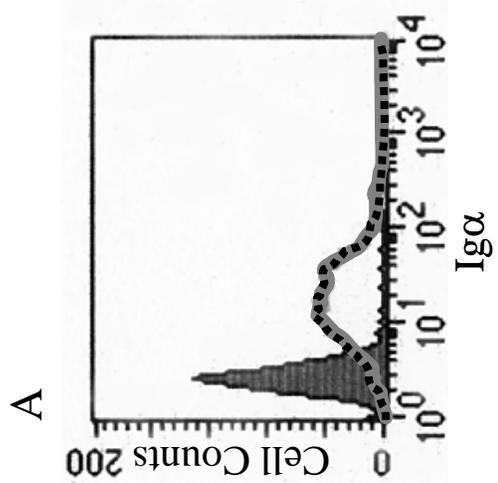
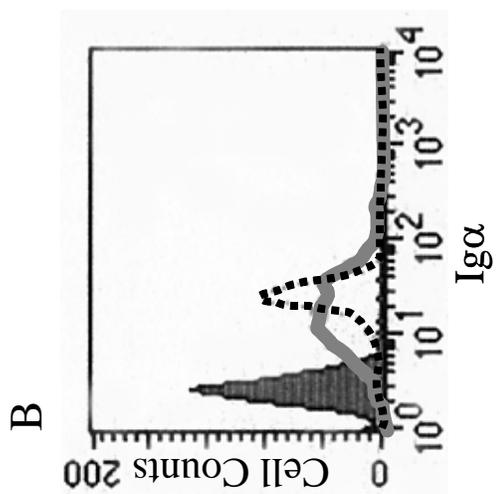
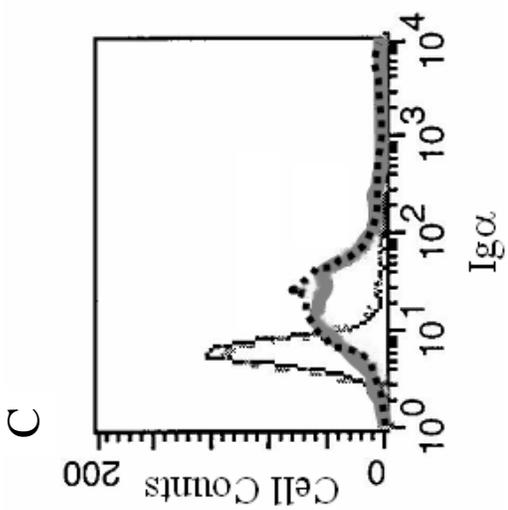
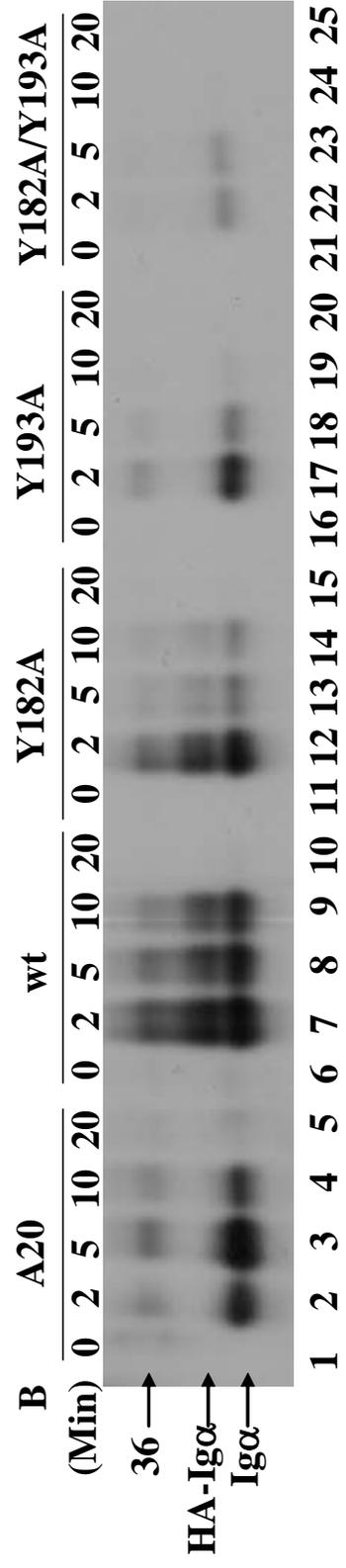
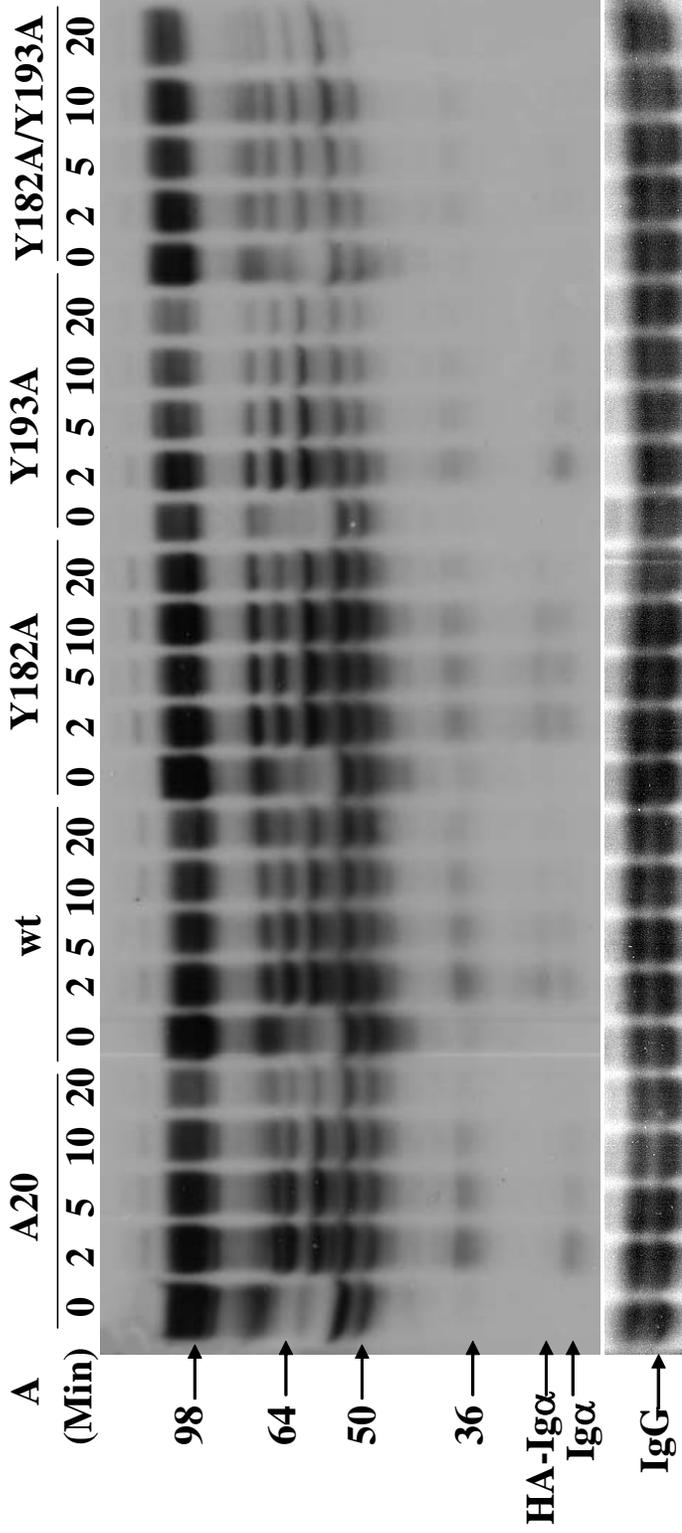


Figure 3-3: Protein tyrosine phosphorylation in untransfected A20 and A20 cells expressing HA-Ig α .

(A) Cells were incubated with anti-mouse IgG (20 μ g/ml) at 4°C for 10 min, and then warmed to 37°C for 2, 5, 10 and 20 min to activate the BCR. The cells were immediately lysed at 4°C. Equal amount of proteins from the cell lysate was loaded onto gel and analyzed by SDS-PAGE and Western blot, probing with mAb 4G10 to detect tyrosine phosphorylated proteins. The blots were stripped and reblotted with anti-mouse IgG antibody for loading control. Shown is a representative blot of 4 independent experiments. (B) Cells were treated as in (A), the cell lysates with equal amount of proteins were subjected to immunoprecipitation using a polyclonal anti-Ig α antibody. The immunoprecipitation was then analyzed using SDS-PAGE and Western blot, probing with mAb 4G10 to detect tyrosine phosphorylated proteins. The band at 36 kDa may be Ig β , but this was not verified.



Next we further studied the tyrosine phosphorylation of endogenously expressed Ig α and transfected HA-Ig α . Ig α was isolated from the cell lysate by immunoprecipitation using a polyclonal anti- Ig α antibody. The immunoprecipitation was analyzed using SDS-PAGE and Western blot, probing for tyrosine phosphorylated proteins. In untransfected A20 cells, two major tyrosine phosphorylated proteins, with molecular weights corresponding to Ig α and Ig β , become visible after BCR crosslinking (Fig. 3-3B), showing the tyrosine phosphorylation of endogenous Ig α and Ig β . By 20 min, their phosphorylation level returned to the basal level. In cells expressing wt HA-Ig α , the phosphorylation levels of endogenous Ig α and Ig β were similar to what was seen in untransfected A20 cells. The tyrosine phosphorylation of wt HA-Ig α was only detected in A20 cells expressing wt HA-Ig α , but not in untransfected A20 cells (Fig. 3-3B). The phosphorylation level of wt HA-Ig α was similar to that of the endogenous one. In A20 cells expressing HA-Ig α Y182A, the phosphorylation levels of both endogenous Ig α and HA-Ig α were reduced (Fig. 3-3B). The phosphorylation levels of endogenous Ig α and HA-Ig α were further reduced in A20 cells expressing HA-Ig α Y193A, and became almost undetectable in A20 cells expressing HA-Ig α Y182A/Y193A (Fig. 3-3B).

Taken together, these results suggest that Ig α mutants, when coexpressed with the endogenous wt Ig α , can interfere with BCR signaling function. While both tyrosines of the Ig α ITAM are important for BCR signaling function, Y193A appears to have a more essential role. This could mean that the 193 tyrosine has a more dominant role in

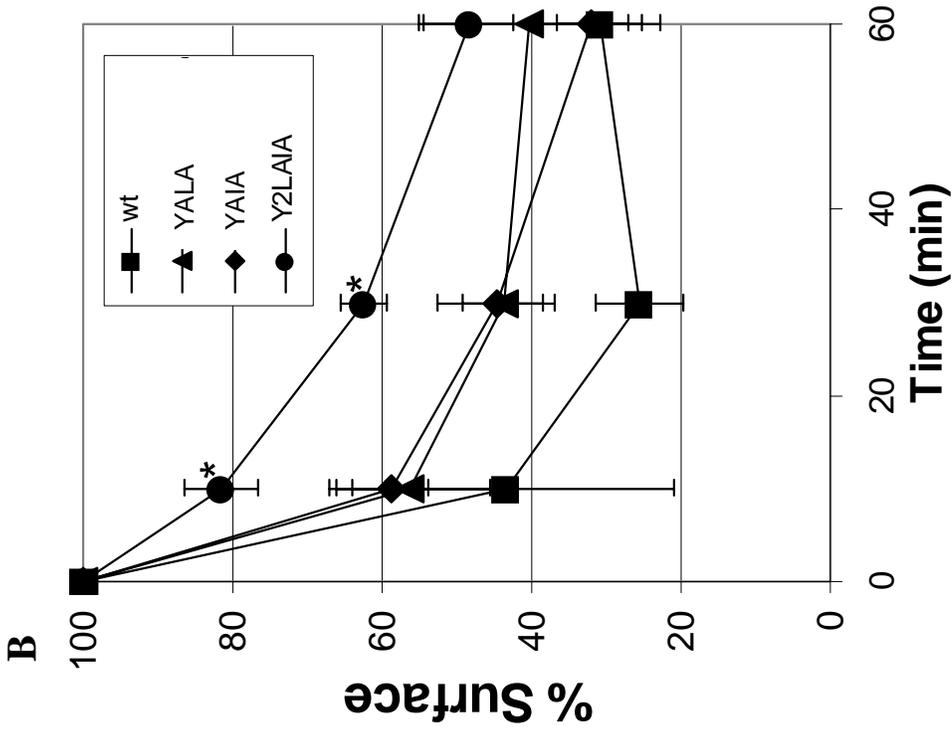
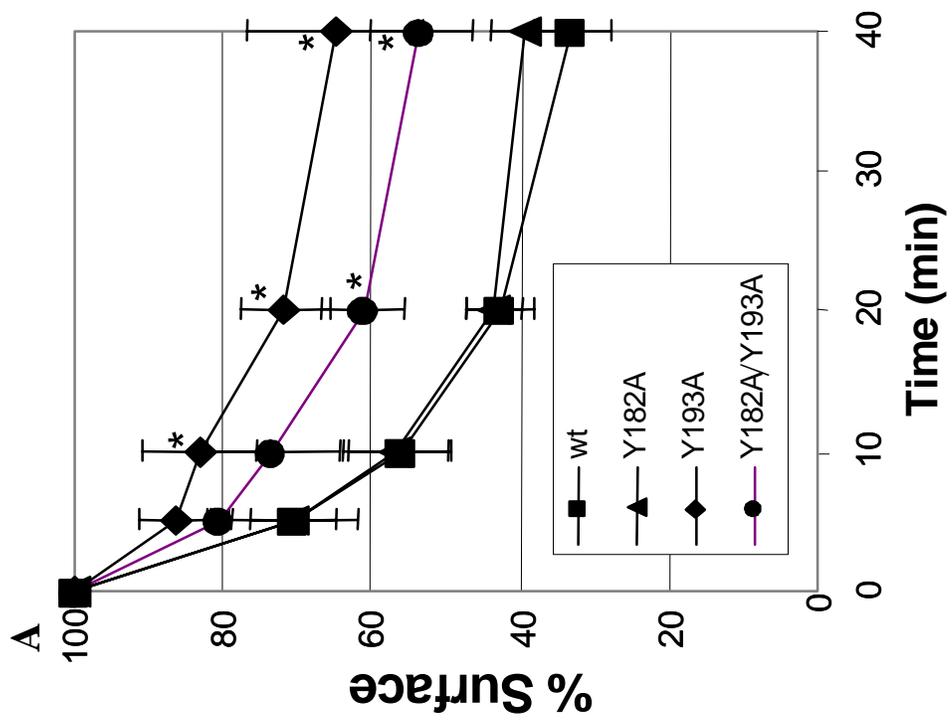
signaling, or it may be that it plays a role in the phosphorylation of the 182 tyrosine, which is why a decrease in signaling is seen.

Effects of Ig α ITAM tyrosine mutations on its antigen internalization and transport functions

Previous studies have indicated a role for Ig α /Ig β cytoplasmic tails in the kinetics of antigen uptake and specificity of antigen targeting [121-124]. To determine whether and how the Ig α ITAM is involved in BCR antigen transport, we analyzed the internalization and intracellular trafficking of the Ig α mutants. To determine the amount of HA-Ig α internalized, cells were incubated with biotin-conjugated anti-HA antibodies (10 μ g/ml) at 4°C and chased at 37°C for varying lengths of time. At the end of each time point, cells were washed and incubated with TRITC-conjugated avidin (10 μ g/ml) at 4°C. Cells were washed again at 4°C and fixed for flow cytometry. Using the biotin-avidin system ensured that only HA-Ig α that was originally on the surface at zero time, and therefore bound by the biotin-conjugated antibody, would be labeled by the TRITC-conjugated avidin and therefore measured as being on the surface at the end of each time point. As seen in Figure 3-4A, prelabeled surface wt HA-Ig α reduced with time, with 70% of wt HA-Ig α remaining at the cell surface at 5 min and 35% remaining at 40 minutes. The internalization kinetics of HA-Ig α Y182A was similar to that of wt HA-Ig α (Fig. 3-4A). The mutation of Y193A and the double mutation Y182A/Y193A, specifically the latter, significantly reduced internalization of Ig α (Fig. 3-4A).

Figure 3-4: Effect of the ITAM mutations on the internalization of Ig α .

A20 cells that express wt or mutated HA-Ig α were incubated with anti-HA mAb at 4°C, washed, and chased at 37°C for varying lengths of time. At the end of each time point, cells were washed and incubated with FITC-conjugated secondary antibody to label the HA-Ig α remaining on the surface after the chase. Cells were then fixed and analyzed by flow cytometry. The amount of HA-Ig α remaining on the surface was plotted as a percentage of the amount of HA-Ig α at time 0. Shown is the average (\pm S.D.) of three or four independent experiments. * indicates a significant difference between mutated HA-Ig α and wt HA-Ig α ($p < 0.01$).

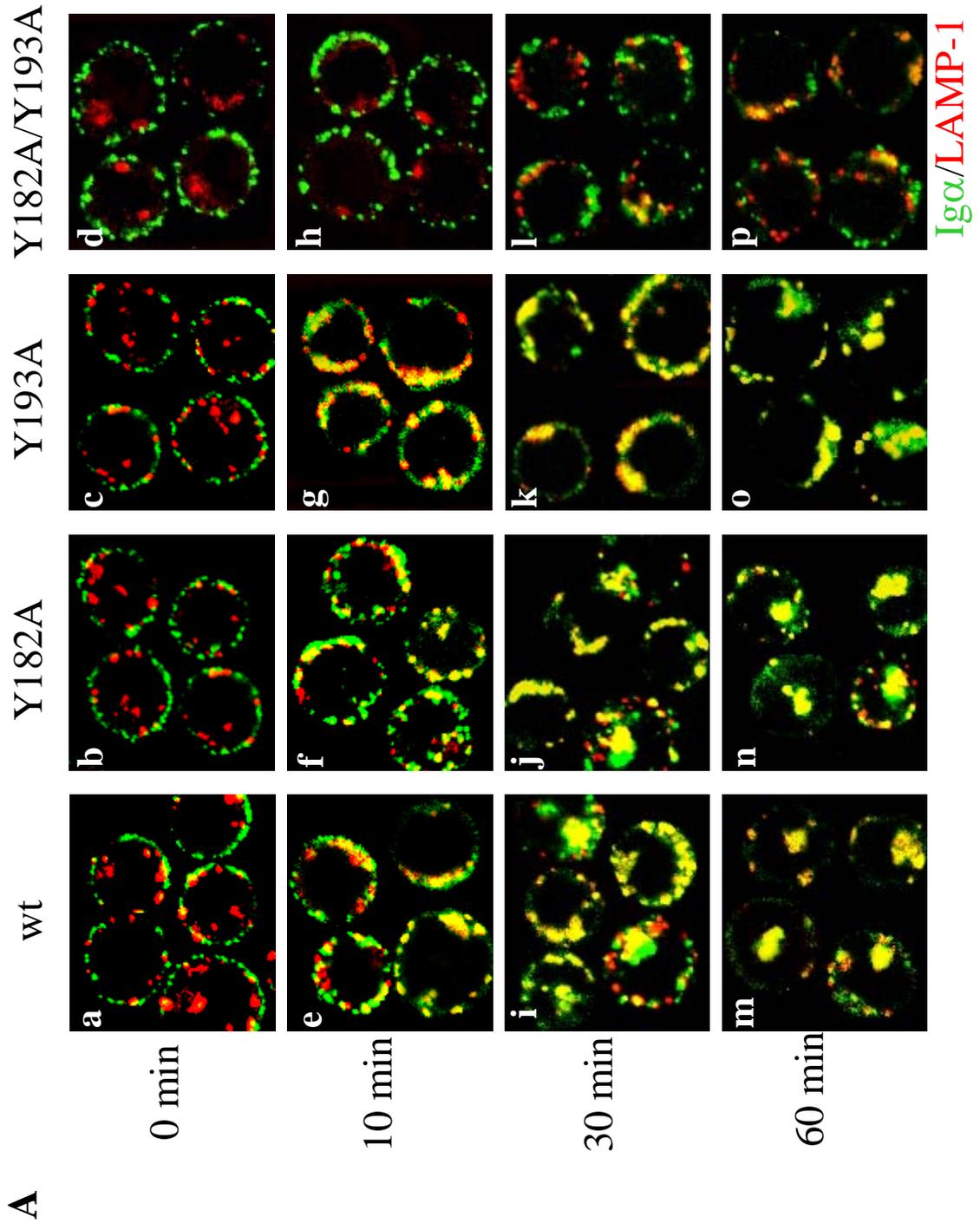


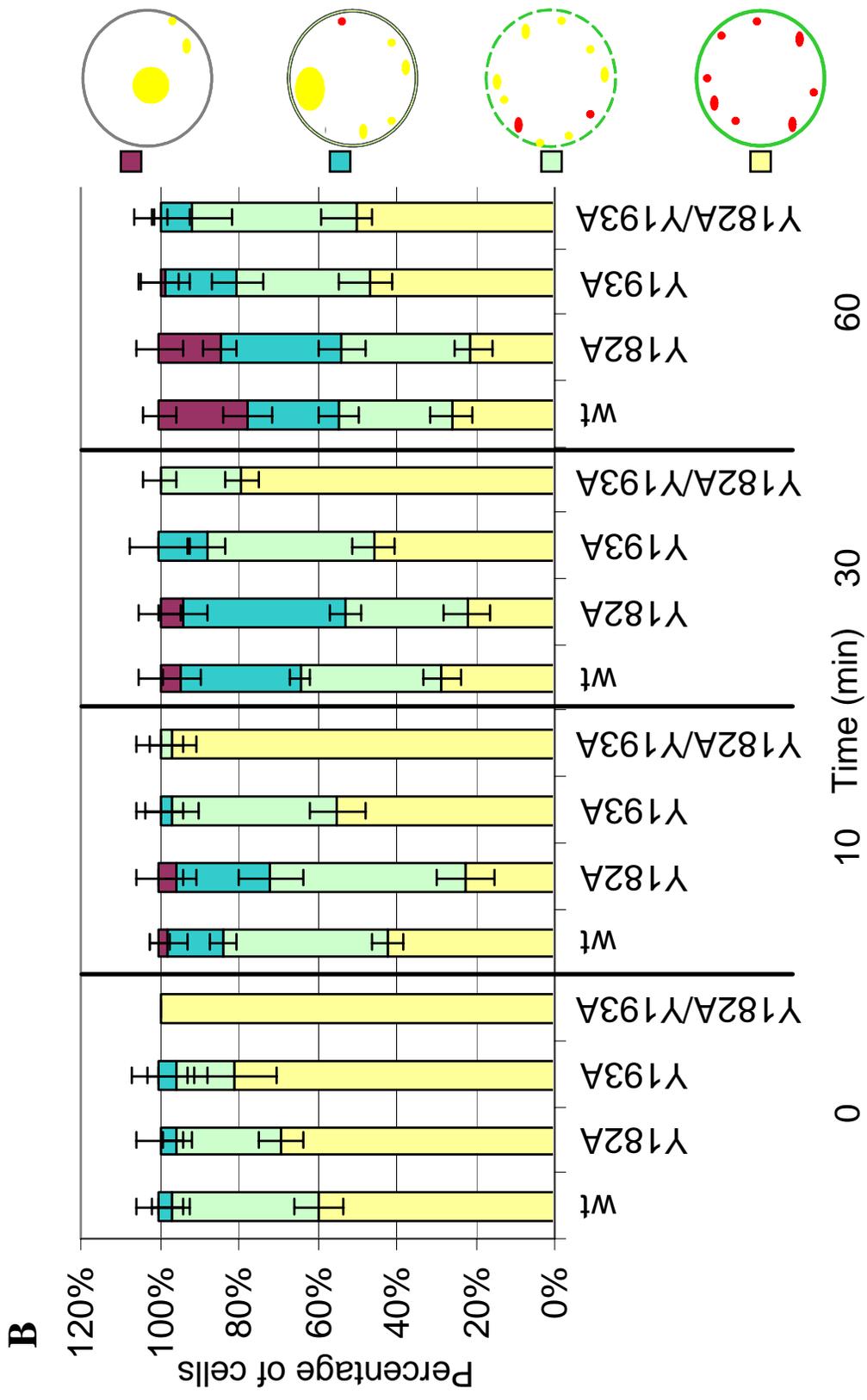
To follow the intracellular trafficking of the HA-Ig α , the cells were incubated with FITC-conjugated anti-HA antibodies at 4°C, washed and chased at 37°C for varying lengths of time. Late endosomes were labeled with TRITC-conjugated LAMP-1-specific mAb. At 0 min, the wt HA-Ig α shows staining at the surface while the LAMP-1 shows punctuate staining in the cell periphery (Fig. 3-5A-a). At 10 min, the wt HA-Ig α staining is punctate on or near the surface, with some colocalization with LAMP-1 (Fig. 3-5A-e). At 30 min the colocalization of wt HA-Ig α with LAMP-1 increased, appearing either punctuate or clustering near the surface (Fig. 3-5A-i). At 60 min, most cells showed the colocalization of wt HA-Ig α and LAMP-1, with both HA-Ig α and LAMP-1 clustering in a perinuclear location (Fig. 3-5A-m).

In order to phenotypically analyze of the immunofluorescence images, the cells in the images were placed into one of four categories based on the cellular distribution of HA-Ig α and LAMP-1. The first phenotype is HA-Ig α staining at the surface and punctuate LAMP-1 staining near the surface. The second phenotype is punctate HA-Ig α staining on or near the surface, with some colocalization with punctate LAMP-1. The third phenotype is more colocalization of HA-Ig α with LAMP-1 clustering near the surface. The fourth phenotype is colocalization of HA-Ig α and LAMP-1 with perinuclear clustering. At 0 min, most of the cells expressing wt HA-Ig α show the first phenotype (60%), and the percentage of cells in the first phenotype decreases with time (Fig. 3-5B). By 10 and 30 min, the number of cells moved to the second and third phenotypes increased. At 30 min, cells showing the fourth phenotype became detectable and significantly increased by 60 min (Fig. 3-5B).

Figure 3-5: Effect of tyrosine mutations of Ig α ITAM on the intracellular trafficking of Ig α .

(A) Cells were incubated with FITC-conjugated anti-HA antibodies at 4°C, washed, and chased at 37°C for varying lengths of time. Late endosomes were labeled with LAMP-1-specific mAb and a TRITC-conjugated secondary antibody after fixation and permeabilization. Cells were analyzed using a confocal fluorescence microscope. (B) Cells in the immunofluorescence images were categorized into four different phenotypes based on the cellular distribution of HA-Ig α and LAMP-1. A range of 50-150 cells were taken from each mutant and each time point. The number of cells showing each of the four phenotypes were counted and plotted as a percentage of the total number of cells in the images. Shown is the average (\pm S.D.) of three or four independent experiments.





The HA-Ig α Y182A showed similar staining patterns as wt HA-Ig α at each time point (Fig. 3-5A-b, f, j, n), indicating that the mutation did not interfere with BCR-triggered LAMP-1⁺ compartment aggregation or the trafficking of the HA-Ig α Y182A. Compared to wt HA-Ig α , the Ig α Y182A followed the same cellular distribution pattern with time. This indicates that the Y182A mutation did not impair the ability to properly traffic to late endosomes (Fig. 3-5B).

Compared to wt HA-Ig α , at each time point there were more cells with HA-Ig α Y193A remaining on its surface (Fig. 3-5A-c, g, k and o), indicating that the HA-Ig α Y193A BCR does not internalize as well. At 10 min the HA-Ig α Y193A staining is similar to wt HA-Ig α (Fig 3-5A-e and g). At 30 min, more colocalization of HA-Ig α Y193A and LAMP-1 is seen, and some clustering of the LAMP-1⁺ compartments is seen in the cell periphery (Fig 3-5A-k), although not as much compared to wt HA-Ig α (Fig. 3-5A-i). At 60 min the cells look similar to those at 30 minutes (Fig. 3-5A-o), with few showing LAMP-1⁺ compartment clustering at a perinuclear location as is seen with wt HA-Ig α (Fig. 3-5A-m). This indicates that although the HA-Ig α Y193A is trafficked to the LAMP-1⁺ compartments and induces clustering, it does not cause the compact perinuclear clustering as seen with wt HA-Ig α . This trend is also seen when looking at the distribution of phenotypes (Fig 3-5B). At 0 min, cells expressing HA-Ig α Y193A are similar to cells expressing wt HA-Ig α . At 10 min, cells expressing HA-Ig α Y193A have more cells of the first phenotype compared to wt HA-Ig α , but about the same number of the second phenotype. At 30 min, cells expressing HA-Ig α Y193A have many of the first and second phenotypes (87%) compared to wt HA-Ig α (64%). There is an increase in the

number of the third phenotype, but fewer than wt HA-Ig α (13% vs. 31%), and there are none of the fourth phenotype, though at this time wt HA-Ig α has some cells in this group (5%). At 60 min, cells expressing HA-Ig α Y193A have mostly cells of the first two phenotypes (81%). The percent of cells of phenotype three (17%) and four (2%) are far fewer than wt HA-Ig α (25% and 22%, respectively). Therefore, HA-Ig α Y193A has reduced kinetics of movement to the LAMP-1⁺ compartments, a lower level of LAMP-1⁺ compartment clustering, and BCR-triggered translocation of LAMP-1⁺ compartments from the cell periphery to a perinuclear location.

Compared to wt HA-Ig α , at each time point there were more cells with HA-Ig α Y182A/Y193A remaining on its surface (Fig. 3-5A-d, h, l and p), indicating that the HA-Ig α Y193A BCR does not internalize as well. At 10 min, most of the HA-Ig α Y182A/Y193A BCR staining is still at or near the surface, with little to no colocalization with LAMP-1⁺ compartments as seen with wt HA-Ig α at this time (Fig. 3-5A-e, h). At 30 min, much of the HA-Ig α Y182A/Y193A staining is punctate at or near the surface, with some colocalization of HA-Ig α Y182A/Y193A with punctate LAMP-1 (Fig. 3-5A-l). This is less colocalization than seen with wt HA-Ig α , and there is no clustering of LAMP-1⁺ compartments as is seen with wt HA-Ig α at this time (Fig. 3-5A-i). At 60 min, many cells expressing Ig α Y182A/Y193A show staining at or near the surface, with some colocalization of HA-Ig α Y182A/Y193A with LAMP-1 and a few are showing clustering of the LAMP-1⁺ compartments at the cell periphery (Fig. 3-5A-p). At this time point wt HA-Ig α shows more LAMP-1 clustering and movement to a perinuclear location (Fig. 3-5A-m). This suggests that the HA-Ig α Y182A/Y193A has greatly

reduced internalization kinetics and impaired ability to cluster the LAMP-1⁺ compartments compared to wt HA-Igα. The phenotypic analysis confirms the difference between HA-Igα Y182A/Y193A and wt HA-Igα (Fig 3-5B). At 10 min, the vast majority of the cells expressing HA-Igα Y182A/Y193A are of the first phenotype (96%), with just a few of the second phenotype (4%). At 30 min, all of the cells expressing HA-Igα Y182A/Y193A are still of the first or second phenotype. In comparison, wt HA-Igα at this time has many cells of the third phenotype (31%) and some of the fourth phenotype (5%). At 60 min, most of the cells expressing HA-Igα Y182A/Y193A are still first or second phenotype (90%), with a few of the third (8%) and fourth phenotype (1%). At this time point, wt HA-Igα has fewer of the first and second phenotype and more of the third (25%) and fourth phenotype (22%). Thus, the HA-Igα Y182A/Y193A is impaired in trafficking and has a greatly reduced ability to cluster the LAMP-1⁺ compartments and induce their movement to a perinuclear location in comparison to the wt HA-Igα.

Effects of Igα ITAM tyrosine mutations on LAMP-1⁺ compartment clustering

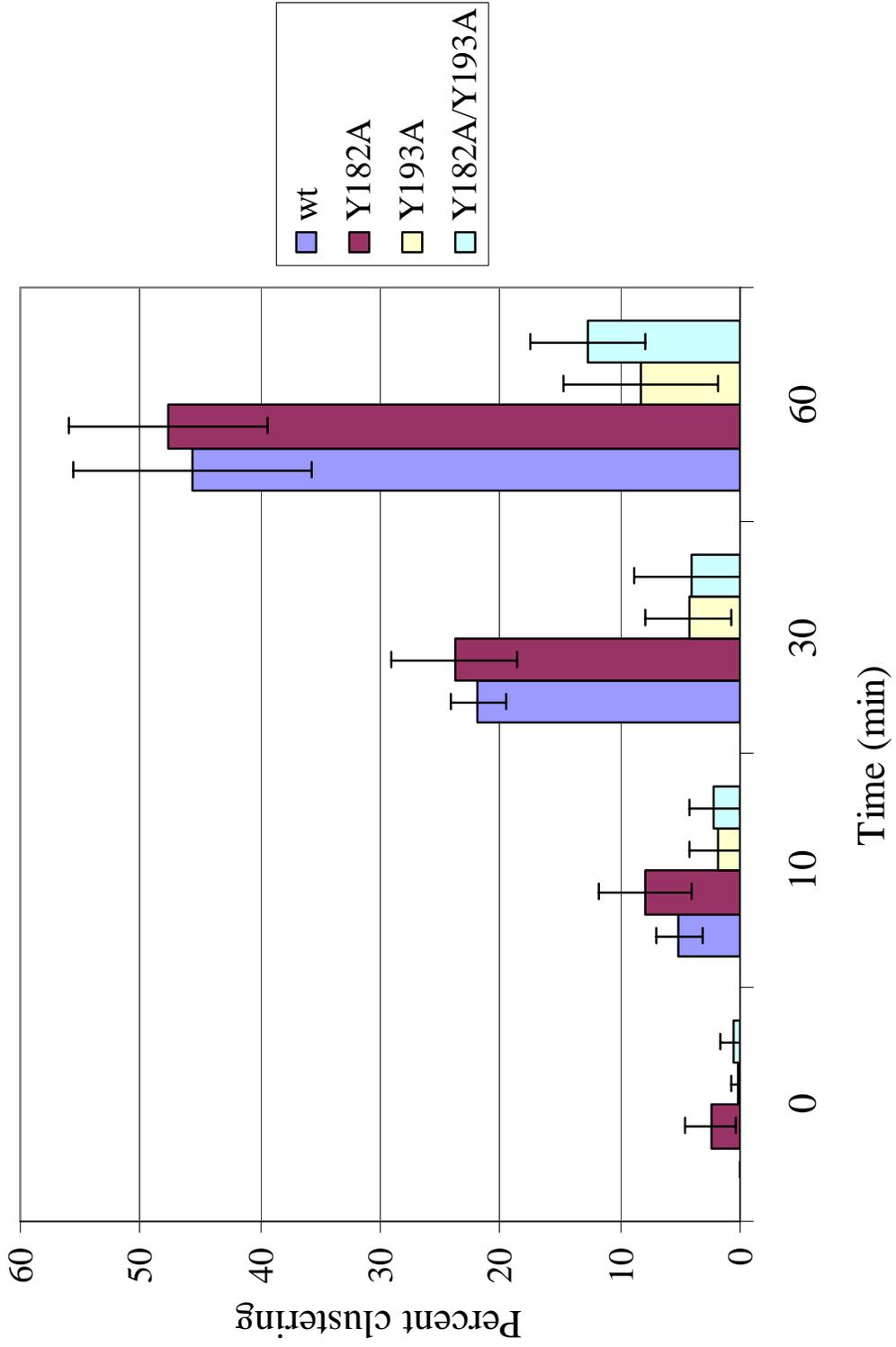
When the cell images were analyzed to put the cells into either LAMP-1⁺ compartment clustering or no clustering, we see that cells expressing HA-Igα Y182A have the same number of cells with LAMP-1 clustering as wt HA-Igα at all time points (Fig. 3-6). At all time points cell expressing HA-Igα Y193A have far fewer cells with clustering of LAMP-1⁺ compartments compared to wt HA-Igα (Fig. 3-6). The number of cells expressing HA-Igα Y182A/Y193A with LAMP-1 clustering is greatly reduced compared

Figure 3-6: Effect of mutations of Ig α ITAM on signaling triggered redistribution of LAMP-1⁺-compartment.

Cells with LAMP-1 staining dispersed at the cell periphery or clustered at the perinuclear location were counted and plotted as a percentage of the total number of each images.

Shown are the average (\pm S.D.) results from three or four independent experiments.

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to wt HA-Ig α at all time points, but is equal to the amount clustering induced by HA-Ig α Y193A (Fig. 3-6).

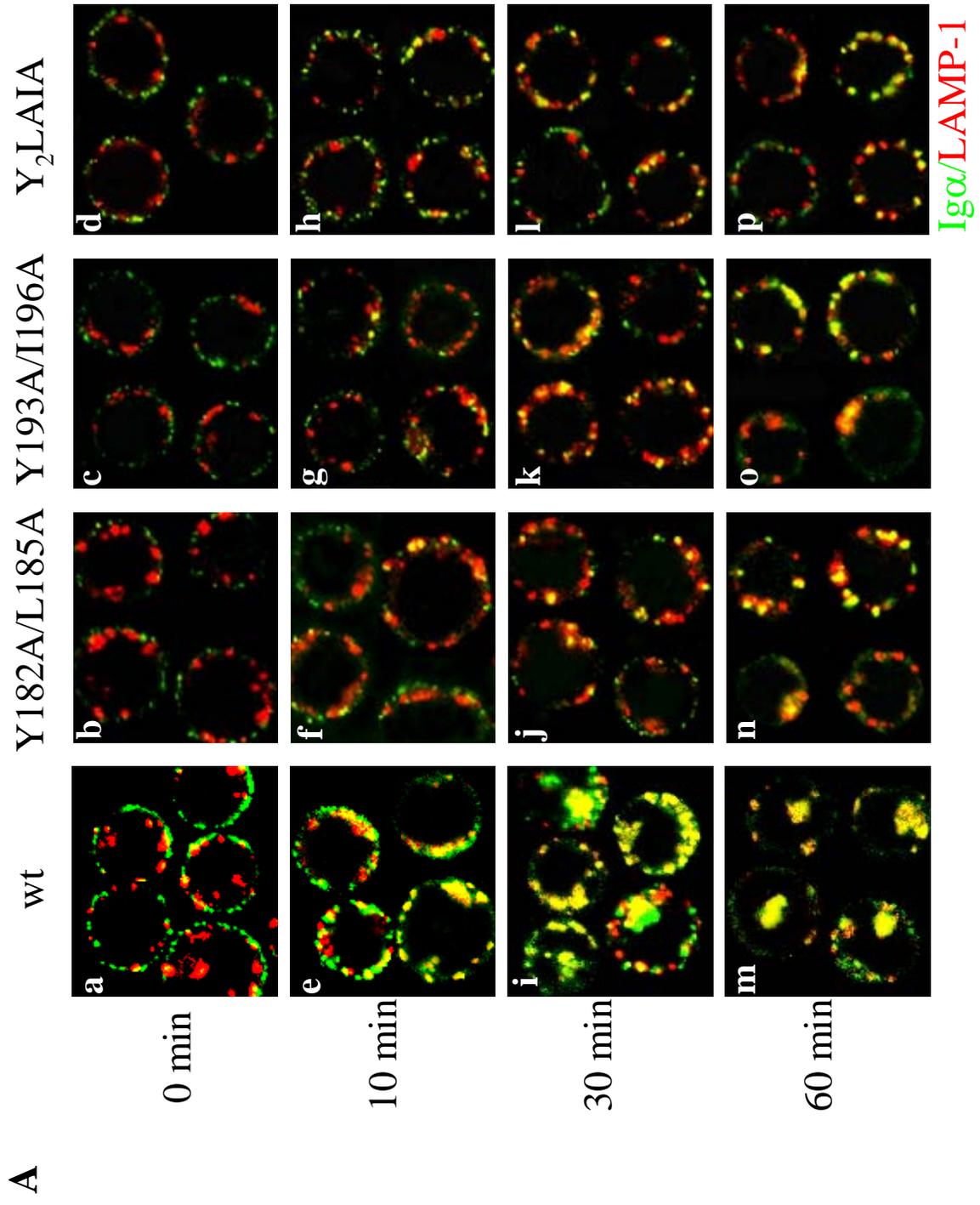
Effects of mutations of Ig α ITAM putative internalization motifs on its antigen internalization and transport functions

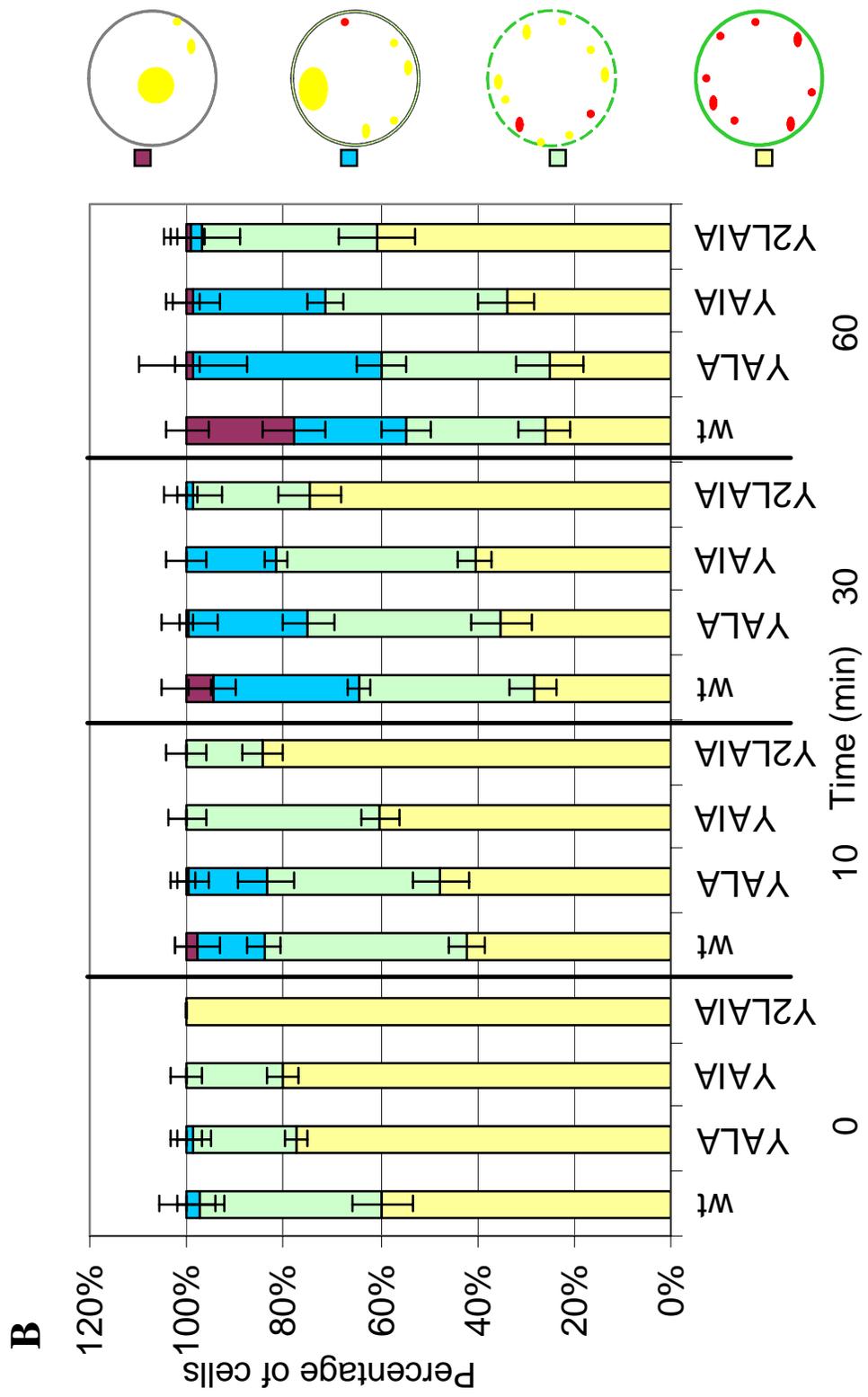
Figure 3-4B shows that the double mutation of Y182A/L185A does not affect the internalization capability of HA-Ig α YALA. The amount of HA-Ig α YALA internalized after 60 minutes is equivalent to that internalized by the wt HA-Ig α (Fig. 3-4B), and HA-Ig α Y182A (Fig. 3-4A). The amount of HA-Ig α YAIA internalized after 60 minutes is equivalent to that internalized by the wt HA-Ig α (Fig. 3-4B), although it appears to have slower kinetics. HA-Ig α Y2LAIA has decreased internalization compared to the wt HA-Ig α . Its internalization appears to be somewhat slower compared to the HA-Ig α Y182A/Y193A, but by 40 minutes their amount of internalization is equal (Fig 3-4A). Thus, the additional leucine or isoleucine mutations did not cause a greater defect in internalization.

The immunofluorescence data indicates that although HA-Ig α YALA is being internalized, it does not traffic the same as wt HA-Ig α . At 10 min HA-Ig α YALA staining is similar to wt HA-Ig α and HA-Ig α Y182A (Figs. 3-7A-e, f and 3-5A-f). At 30 min most HA-Ig α YALA staining is still at or near the surface, with a few cells showing colocalization of HA-Ig α YALA and clustered LAMP-1⁺ compartments at the cell periphery (Fig. 3-7A-j). In comparison, many cells expressing wt HA-Ig α have LAMP-1⁺ compartment clustering and show movement of the clustered compartments to a

Figure 3-7: Effect of tyrosine, leucine, and isoleucine mutations on the intracellular trafficking of Ig α .

The experiments were carried out and data were analyzed as described in Figure 5.



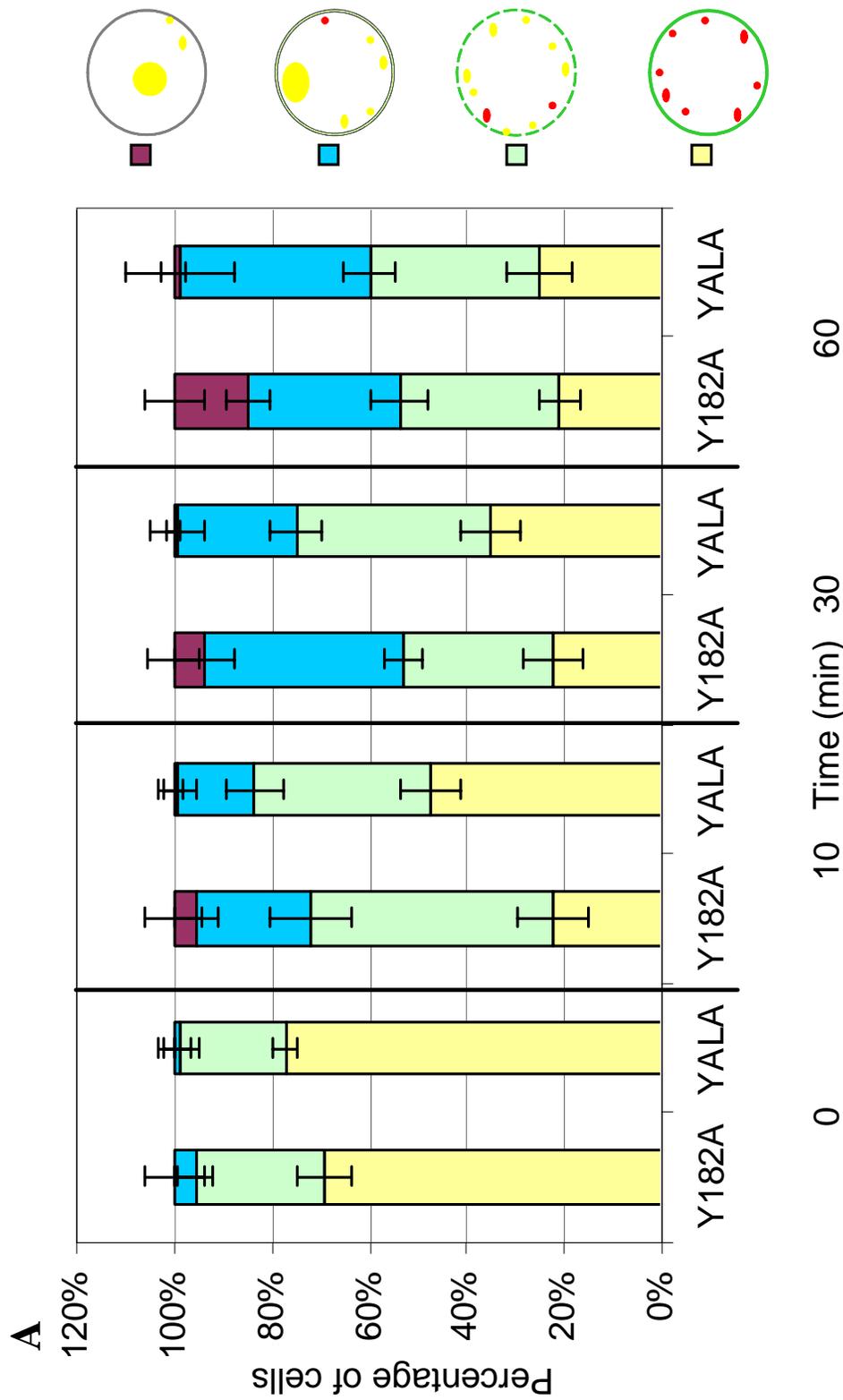


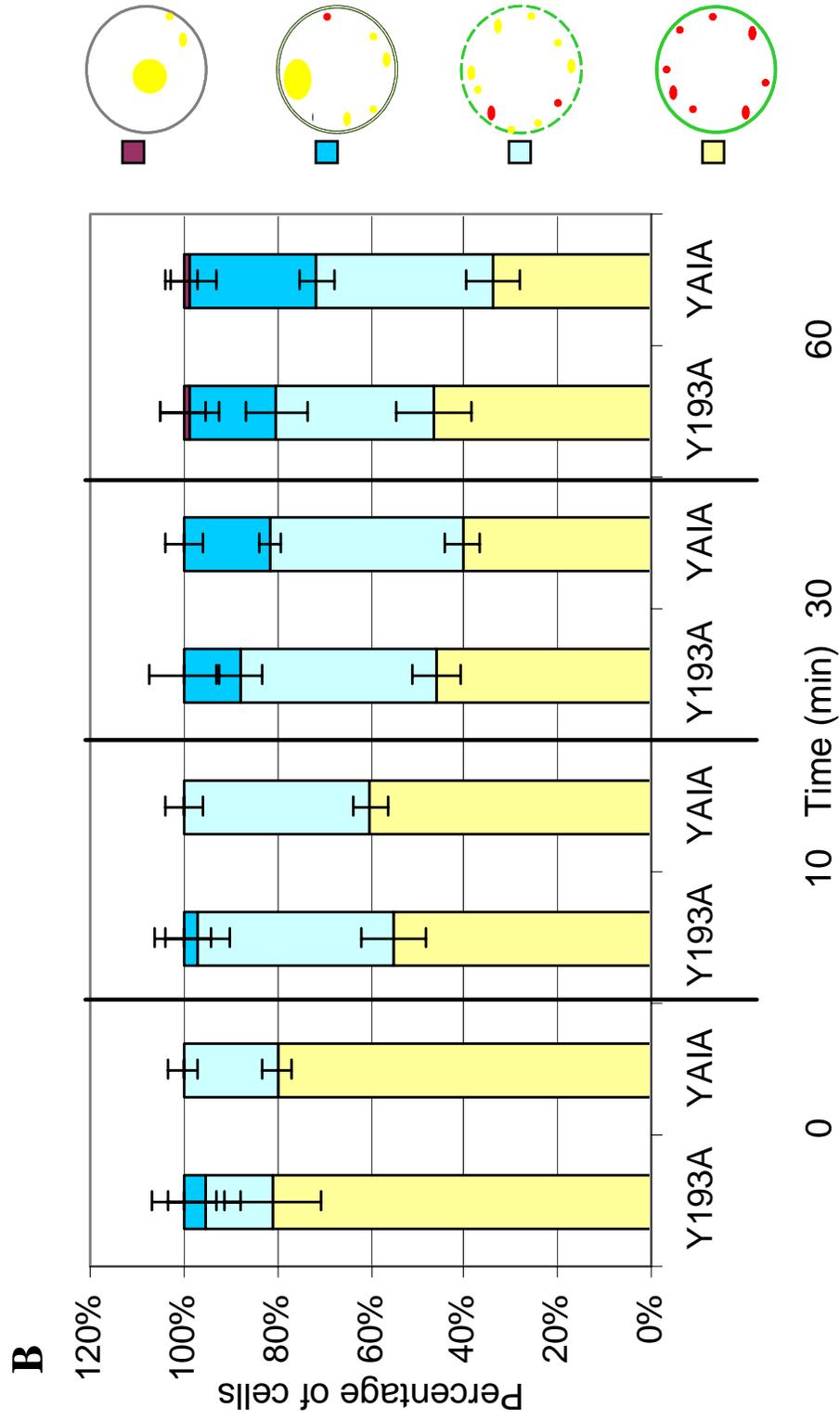
perinuclear location. At 60 min, many cells expressing HA-Ig α YALA still show colocalization of HA-Ig α YALA and punctate or periphery-clustered LAMP-1⁺ compartments, with little to no perinuclear clustering (Fig. 3-7A-n). Cells expressing wt HA-Ig α have a significant amount of perinuclear clustered LAMP-1⁺ compartments at this time (Fig. 3-7A-m). The phenotypic analysis confirms this. In Figure 3-7B it can be seen that at all time points, HA-Ig α YALA has fewer of the later phenotypes than wt HA-Ig α . HA-Ig α YALA has a similar phenotypic pattern to HA-Ig α Y182A, with the exception of HA-Ig α YALA having little to no cells of phenotype four (Fig. 3-8A). This data indicates that the double mutation of Y182A/L185A does not affect trafficking of the HA-Ig α YALA to LAMP-1⁺ compartments and does not prohibit it from inducing LAMP-1 clustering, but the movement of these clustered compartments from the surface to the perinuclear region is affected.

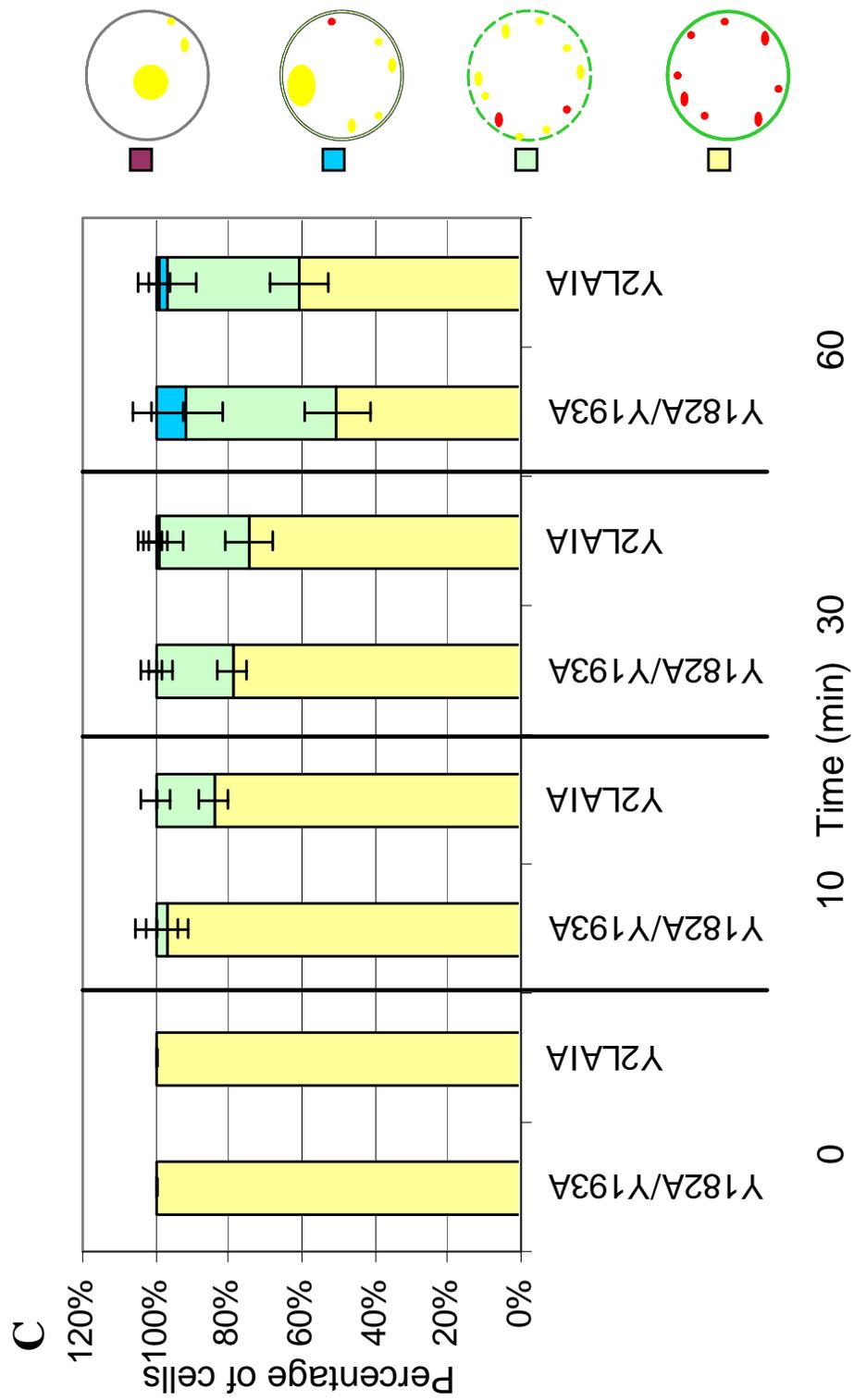
The immunofluorescence pictures show that at 10 min, HA-Ig α YAIA staining is on or near the surface with some colocalization of HA-Ig α YAIA with LAMP-1 (Fig. 3-7A-g). At 30 min, cells expressing HA-Ig α YAIA show more colocalization with punctate LAMP-1⁺ compartments and some clustering of LAMP-1⁺ compartments at the cell periphery, but many of the cells are still showing no colocalization (Fig. 3-7A-k). At this time cells expressing wt HA-Ig α have more cells with clustering of LAMP-1⁺ compartments and some movement of these clustered compartments to a perinuclear location (Fig. 3-7A-i). At 60 min, the HA-Ig α YAIA shows less surface staining and the cells have more surface clustering of colocalized LAMP-1 (Fig. 3-7A-o). This is similar to the HA-Ig α Y193A at this time, but the cells expressing wt HA-Ig α have much more

Figure 3-8: Comparison of phenotypes of HA-Ig α with tyrosine mutations and tyrosine/leucine double mutations.

Data from Figure 5B and Figure 7B were regrouped to compare the intracellular trafficking of HA-Ig α with tyrosine mutations only and HA-Ig α with both tyrosine and leucine or isoleucine mutations. (A) Comparison of phenotypes of cells transfected with HA-Ig α Y182A and HA-Ig α YALA. (B) Comparison of phenotypes of cells transfected with HA-Ig α Y193A and HA-Ig α YAIA. (C) Comparison of phenotypes of cells transfected with HA-Ig α Y182A/Y193A and HA-Ig α Y2LAIA.







perinuclear clustering of LAMP-1⁺ compartments. The trafficking of HA-Igα YAIA is impaired compared to wt HA-Igα but similar to HA-Igα Y193A. The phenotypic analysis shows that cells expressing HA-Igα YAIA lag behind wt HA-Igα in expressing the later phenotypes (Fig. 3-7B), but that its phenotypic pattern is similar to HA-Igα Y193A (Fig. 3-8B). This data indicates that the HA-Igα YAIA has a similar defect in trafficking, clustering LAMP-1⁺ compartments and inducing movement of clustered LAMP-1⁺ compartments to perinuclear clustering as HA-Igα Y193A.

As seen in Figure 3-7A, HA-Igα Y2LAIA staining is mostly at or near the surface with little to no colocalization of HA-Igα Y2LAIA with punctate LAMP-1 at all time points after 0 min (Fig. 3-7A-h, l and p). This is obviously a defect compared to wt HA-Igα (Fig. 3-7A-e, i and m), but is similar to what is seen with HA-Igα Y182A/Y193A (Fig 3-5A-h, l and p). Cells expressing HA-Igα Y2LAIA have a very different phenotypic pattern compared to cells expressing wt HA-Igα, in that most cells are of the first and second phenotype for all time points (Fig.3-7B), but this a similar phenotypic pattern as is seen for HA-Igα Y182A/Y193A (Fig. 3-8C). Thus, the quadruple mutation causes a greater impairment of trafficking than any of the other mutations. HA-Igα Y2LAIA takes longer to colocalize with punctate LAMP-1⁺ compartments, induces little clustering of colocalized LAMP-1⁺ compartments and does not induce movement of the clustered compartment to a perinuclear location.

3.5 Discussion

The BCR is a dual function receptor. Upon being bound by antigen, it initiates signaling cascades, and subsequently internalizes antigen for processing and presentation. The BCR initiated signaling has been shown to upregulate BCR-mediated antigen processing and presentation by accelerating antigen internalization, specifically targeting antigen to the processing compartment, and increasing expression of MHC class II and CD86 [95, 97, 122, 124, 125, 157]. It is not clear how BCR signaling regulates the internalization and trafficking of antigen, an initiation step of antigen processing. It has been demonstrated that the Ig α /Ig β heterodimer is essential for this regulation, as mutated IgMs that lack association with Ig α /Ig β or chimeras of Ig α or Ig β alone do not transport and present antigen as efficiently as wt BCR [8-11, 94, 151]. In this study, we examined the role of the Ig α cytoplasmic tail in the regulation of intracellular trafficking of the BCR, specifically how its ITAM, which has a key signaling role and contains putative internalization motifs as well, influences the internalization and movement of BCR through the endocytic pathway.

Most previously published studies used chimeric proteins that contain the cytoplasmic tail of either Ig α or Ig β [95, 121-124, 154]. These chimeras do not form disulfide-links with each other or with endogenous Ig α or Ig β and do not interact with mIg, the antigen-binding component of BCR. In this study, we express wt and mutated HA-Ig α in a B cell lymphoma line that express mIgG_{2a}-based BCR. Previously unpublished results from another member of Dr. Song's laboratory showed that transfected HA-Ig α formed a

disulfide-linked heterodimer with endogenous Ig β and non-covalently associated with mIg in A20 cells. Such a model system allowed us to study the function of Ig α in the BCR complex.

A previous study showed that when a chimera complex that contained a wt Ig β cytoplasmic tail and an Ig α cytoplasmic tail with both ITAM tyrosines mutated to alanines failed to initiate signaling and to present antigen [124]. Here, we examined the effect of mutating each tyrosine in Ig α 's ITAM individually as well as in combination on the internalization and intracellular trafficking of the BCR. The point mutation of the N-terminal tyrosine of Ig α ITAM, Y182A, appeared to have little effect on BCR-triggered protein tyrosine phosphorylation, the internalization of the HA-Ig α , and trafficking of the HA-Ig α to the LAMP-1⁺ compartments, a step known to be important for efficient antigen presentation [127]. The Y182A mutation of Ig α did reduce BCR-triggered tyrosine phosphorylation of both transected HA-Ig α and endogenously expressed wt Ig α . It may seem a contradiction that the Y182A mutation had little effect on the overall BCR-triggered protein tyrosine phosphorylation but had a definite effect on the phosphorylation of both transected HA-Ig α and endogenously expressed wt Ig α . This could be due to the fact that Ig β , which also has an ITAM, is inducing signaling that compensates for the lack of tyrosine phosphorylation of the Ig α s. In contrast, the point mutation of the C-terminal tyrosine of Ig α ITAM, Y193A, decreased the level of BCR-triggered protein tyrosine phosphorylation, the level of tyrosine phosphorylation of HA-Ig α and endogenous Ig α , and reduced the internalization kinetics of HA-Ig α and

movement of HA-Ig α to LAMP-1⁺ compartments. This mutation causes defects in both BCR signaling and antigen transport functions. In cells expressing HA-Ig α with both tyrosines of Ig α mutated, HA-Ig α Y182A/Y193A BCR-triggered protein tyrosine phosphorylation and Ig α tyrosine phosphorylation was dramatically reduced, and the tyrosine phosphorylation of HA-Ig α Y182A/Y193A was completely abrogated. The internalization of HA-Ig α Y182A/Y193A was greatly reduced and its movement to LAMP-1⁺ compartments was impaired compared to wt BCR. In cells expressing HA-Ig α Y182A/Y193A, BCR-triggered the translocation of LAMP-1⁺ compartments from the cell periphery to perinuclear location was almost undetectable. This defect in LAMP-1⁺ compartment translocation was even more pronounced in cells expressing HA-Ig α Y182A/Y193A than in cells expressing the Ig α Y193A. Thus, this double tyrosine mutation severely impaired BCR signaling and antigen transport function. Our results are consistent with a previous study using Ig α chimeric protein, where it was shown that an Ig α chimera with both ITAM tyrosines mutated is defective in both signaling and antigen presentation [127]. Taken together, the results presented in this study demonstrate that the tyrosines of Ig α ITAM are important for both the signaling and antigen transport functions. Comparing the two tyrosines of the ITAM, the C-terminal tyrosine appears to have a more essential role. This could mean that the 193 tyrosine has a more dominant role in signaling, or it may be that it plays a role in the phosphorylation of the 182 tyrosine, which is why a decrease in signaling is seen when the 193 tyrosine is mutated.

The sequence YXXΦ (Φ is a bulky, hydrophobic amino acid) has been found to be an internalization motif for clathrin-mediated internalization. Each ITAM contains two YXXΦ sequences. In this study, we further tested whether the leucine and isoleucine in the ITAM of the Igα cytoplasmic tail play a role in BCR-mediated antigen transport function. HA-Igα YALA internalized with similar kinetics as wt HA-Igα and HA-Igα Y182A BCR. HA-Igα YAIA also appeared to internalize well, but its trafficking was greatly affected, comparable to HA-Igα Y193A, with little LAMP-1⁺ compartment clustering. HA-Igα Y2LAIA had reduced internalization and a great defect in trafficking, equivalent to the HA-Igα Y182A/Y193A. Thus, mutating the associated leucine or isoleucine along with its associated tyrosine did not appear to cause a greater trafficking defect.

Although it is not clear how BCR internalization and trafficking is regulated, previous studies showed that BCR-mediated signaling is involved. B cells in which BCR signaling function is inhibited do not show accelerated BCR-mediated antigen transport and have greatly reduced antigen presentation. Here, we show that the tyrosine mutations of Igα inhibited both BCR signaling and antigen transport function. Interestingly, there is a correlation between the levels of signaling defects and intracellular trafficking defects of Igα mutants. HA-Igα Y182A still initiates signaling and has internalization and trafficking kinetics similar to wt BCR. On the other hand, HA-Igα Y193A has reduced signaling and reduced internalization and trafficking kinetics. Such a correlation suggests that the signaling and antigen functions of the BCR are interrelated, and the Igα ITAM

plays an important role in linking these two functions of the BCR. The signaling function of the Ig α ITAM has been well studied [9, 11, 61]. However, its role in BCR-mediated antigen transport is unclear. The Ig α ITAM could regulate the intracellular trafficking of the BCR indirectly by initiating signaling cascades. BCR-initiated signaling has been shown to regulate BCR internalization by phosphorylating clathrin heavy chain in lipid rafts [128]. In addition, BCR-triggered signaling has been shown to induce the redistribution of LAMP-1⁺ compartments from the cell periphery to a perinuclear location and coalescing the processing compartments. Here we found a correlation between the signaling defects of Ig α ITAM mutations with their inability to induce the translocation of LAMP-1⁺ compartments. This further supports the notion that the Ig α ITAM is involved in the intracellular trafficking of BCR through its signaling function.

Another possibility is that the Ig α ITAM has a direct function in BCR internalization and targeting to the processing compartment. The YXX Φ sequences in the Ig α ITAM could serve as internalization motifs and provide binding sites for clathrin adaptor protein upon dephosphorylation by phosphatase. It is known that the BCR is internalized by clathrin-mediated endocytosis. What is known from other systems is that the first step in the formation of the clathrin coats is the recruitment of adaptor protein (AP) to the receptor [158]. AP-2 is the adaptor protein involved in endocytosis. It is not known exactly how adaptor proteins find their way to the correct receptor, but they have been shown to interact with the internalization motifs carried by the cytoplasmic tails of receptors [155]. Previous studies have shown that upon crosslinking, the BCR moves into lipid rafts,

where initiation of signaling occurs [128, 159]. The phosphorylation of clathrin heavy chain in lipid rafts is required for BCR internalization [128]. It is possible that the ITAM is not only important for signaling, but it is also important to create a docking site for AP-2, which then associates with the phosphorylated clathrin and leads to internalization. In addition, the phosphorylation of the Ig α ITAM could change the conformation of the cytoplasmic tails of the BCR, which exposes the internalization motif outside the ITAM. Since the Ig α ITAM mutations inhibited both the signaling and internalization functions of the BCR and the additional mutation of isoleucine or leucine in the ITAM did not enhance the internalization defect of the BCR, our data could not confirm or exclude the possibility of the Ig α ITAM serving as binding sites for the endocytosis machinery or regulating the binding of the endocytosis machinery with the BCR. Further studies are required to answer these questions.

One interesting find is that expression of mutated Ig α reduced not only the tyrosine phosphorylation of transfected Ig α , but also the gross levels of cell protein tyrosine phosphorylation and the tyrosine phosphorylation level of endogenous Ig α triggered by cross-linking mIg. This indicates that transfected Ig α mutants interfere with the function of endogenous wt Ig α . This further confirms that transfected Ig α can form BCR complex with endogenous mIg and Ig β . Upon cross-linking mIg, transfected Ig α that associated with mIg is able to aggregate with endogenous wt Ig α , and through the BCR cross-linking aggregates mutated Ig α can interfere with the function of endogenous wt Ig α . The effect of the coexpressed mutated Ig α on the phosphorylation of endogenous

wt Ig α suggests that in the cross-linking BCR aggregates, each BCR complex does not function independently.

The important observations made in this study are that the tyrosines of the Ig α cytoplasmic ITAM are not only important for BCR signaling, but also critical for proper internalization and trafficking of the BCR. Signaling through the Ig α ITAM is not only important as the first step in activating the BCR, it also appears to be essential for the rapid internalization and proper trafficking of antigen to the MHC compartments to allow efficient presentation of antigen to T cells.

Chapter IV. General Discussion

B lymphocytes are among the most efficient cells of the immune system in capturing, processing, and presenting antigen to T cells, a crucial event in the humoral immune response. B cells differ from other antigen presenting cells in that they are clonal specific and usually only capture and process antigens recognized by the BCR. This mechanism is much more efficient than other ways by which antigen might gain access to the antigen processing compartments, such as pinocytosis [149]. Multivalent antigen, which is specifically binds the BCR, is transported to the antigen processing compartments much more rapidly than monovalent antigens, which do not crosslink the BCR [152].

Crosslinking the BCR initiates both the signaling cascade and the rapid internalization of the BCR, which is one of many indications that BCR signaling and antigen transport functions are linked. How they are interrelated and how BCR signaling regulates BCR transport have not been elucidated. The main goal of this work was to delineate the role of the $Ig\alpha/Ig\beta$, the known signaling component of the BCR, in BCR-mediated antigen processing and transport.

4.1 General conclusions

First, I examined the effects of dissociating mIgM, the antigen binding component, from $Ig\alpha/Ig\beta$ heterodimer, the signaling component of the BCR, on the internalization and intracellular transport of the mIgM. Previous studies have shown that cells with mIgM that no longer associate with $Ig\alpha/Ig\beta$ not only fail to initiate signaling, but have greatly reduced antigen processing and presentation [8-11, 94, 151]. The goal of this study was to determine if the reduced antigen presentation efficiency was due to reduced

internalization, improper transport of BCR-antigen complexes in the endocytic pathway, and/or lack of entry of antigen into the antigen processing compartments. To test this hypothesis, I expressed either a wild type (wt) of murine IgM or a murine mIgM containing two point mutations of the transmembrane domain (YS/VV) that disrupt the association of mIgM with Ig α /Ig β in B cell lymphoma A20 cells. I found that while both wt μ and μ YS/VV were expressed on the cell surface at a similar level, μ YS/VV had reduced abilities to initiate protein tyrosine phosphorylation and to facilitate antigen processing and presentation (Fig. 2-4 and 2-5), which is consistent with previous studies [8-11, 94, 151]. I further examined the effect of the mutation on the internalization and intracellular trafficking of the BCR and found that the YS/VV mutation appeared to block both the constitutive and crosslinking stimulated internalization, as well as the movement of the BCR from the plasma membrane to the late endosomes (Figs. 2-6 and 2-7). Thus, Ig α /Ig β is essential for antigen processing because it allows for rapid internalization and transport of the BCR to the late endosomal compartments.

To test how BCR-triggered signaling regulates BCR-mediated antigen transport function, we examined how the signaling triggered by endogenous wt BCR influences the internalization and intracellular movement of mIgM YS/VV that failed to initiate signaling. It was found that cross-linking the endogenous wt BCR triggered a normal level of protein tyrosine phosphorylation in mIgM YS/VV-expressing cells (Fig. 2-4). However, endogenous BCR-triggered signaling did not restore the defects of mIgM YS/VV in internalization and movement to the antigen processing compartments (Figs 2-7 and 2-8). This supports the notion that the association of mIg with Ig α /Ig β is critical

for the efficient internalization and transport of antigens for processing and presentation, and the signaling from neighboring receptors cannot substitute the role of Ig α /Ig β directly associated with mIgM.

The mechanism by which the Ig α /Ig β functions molecularly in the internalization and intracellular trafficking of the BCR remains to be elucidated. There are two possibilities. Signaling mediated through Ig α /Ig β may regulate the endocytosis and endocytic trafficking machineries. Another possibility is the cytoplasmic tails of Ig α /Ig β provide the binding sites for the endocytosis and endocytic trafficking machineries to bind. Interestingly, the ITAM motifs in the cytoplasmic tails of Ig α /Ig β not only are the key element for BCR signaling functions, they also contain putative endocytosis motif YXX ϕ (ϕ is a bulky and hydrophobic amino acid).

To determine if the ITAM motifs of the Ig α /Ig β cytoplasmic tails are important for regulation of BCR internalization and intracellular transport, I studied the effect of various mutations of the Ig α ITAM on the movement of the BCR. I found that the point mutation of the N-terminal tyrosine of Ig α ITAM (HA-Ig α Y182A) appeared to have little effect on BCR-triggered protein tyrosine phosphorylation, the internalization of the HA-Ig α , and trafficking of the HA-Ig α to the LAMP-1⁺ compartments (Figs. 3-3A, 3-4, and 3-5). In contrast, the point mutation of the C-terminal tyrosine of Ig α ITAM (HA-Ig α Y193A) decreased the level of BCR-triggered protein tyrosine phosphorylation and reduced the internalization kinetics of HA-Ig α and movement of HA-Ig α to LAMP-1⁺ compartments (Figs. 3-3, 3-4 and 3-5). Therefore, this mutation causes defects in both

BCR signaling and antigen transport functions. In cells expressing HA-Ig α with both tyrosines of Ig α mutated, Ig α (HA-Ig α Y182A/Y193A) BCR-triggered protein tyrosine phosphorylation was dramatically reduced. The internalization of HA-Ig α Y182A/Y193A was greatly reduced and its movement to LAMP-1⁺ compartments was impaired compared to wt BCR. In cells expressing HA-Ig α Y182A/Y193A, BCR-triggered translocation of LAMP-1⁺ compartments from the cell periphery to a perinuclear location was almost undetectable. This defect in LAMP-1⁺-compartment translocation was even more pronounced in cells expressing HA-Ig α Y182A/Y193A than in cells expressing HA-Ig α Y193A. Thus, this double tyrosine mutation severely impaired BCR signaling and antigen transport function. I also studied the potential internalization motifs (YXX ϕ) by mutating both the tyrosine and its associated leucine or isoleucine, as well as mutating all four residues at once. These mutations did not enhance the internalization defect of the HA-Ig α , therefore it cannot be concluded from this data whether these are internalization motifs that are necessary in addition to the signaling that is initiated through the tyrosines of the ITAM. These experiments do show that the tyrosines of the Ig α cytoplasmic ITAM are not only important for BCR signaling, but also critical for proper internalization and trafficking of the BCR.

In conclusion, the association of the mIgM with the Ig α /Ig β is essential for rapid internalization and proper transport of BCR through the endocytic pathway to the antigen loading compartments. The tyrosines of the Ig α ITAM, particularly the C-terminal tyrosine at 193, are necessary for optimization of these processes. It appears that the signaling role of the Ig α /Ig β is closely associated with its role in antigen transport for

antigen processing, since when there is intact signaling function of the mutated Ig α , there is rapid internalization and efficient transport of the BCR, but when there is reduced signaling function of the mutated Ig α , a defect in BCR internalization and intracellular trafficking were observed. Therefore, Ig α /Ig β heterodimer of the BCR regulates the movement of the BCR from the plasma membrane and through the endocytic pathway to the antigen processing compartment through activating signaling cascades.

4.2 Future experiments

The study described here supports the idea that signaling function of the BCR is important in increasing the efficiencies in antigen uptake, transport and presentation. Future work is needed to further understand the molecular mechanism by which BCR-mediated signaling regulates its antigen transport function. Compared to the chimeric systems used in most previous studies, our experimental system, where mutated mIgM or Ig α were expressed in B cells expressing endogenous mIg and Ig α /Ig β , allows the transfected mIg and Ig α to interact with endogenous partner. However, such a system may hinder clearly determining the role of Ig α /Ig β in regulation of BCR transport. In the case of transfected HA-Ig α , the endogenous Ig α may associate with the transfected HA-Ig α s upon crosslinking and compensate the functional defects of mutated HA-Ig α . For instance, even when both ITAM tyrosines were mutated, we still saw some internalization and transport to LAMP-1⁺ compartments of the HA-Ig α Y182A/Y193A. This could be due to several reasons, such as the tyrosines being important but not essential for these processes, or it could be due to the endogenous Ig α s compensating for the mutated Ig α s. It would be interesting to repeat the experiments done with the

mutated Ig α s using the J558 μ m cell line. This cell line does express mIgM but does not express Ig α . Transfecting these cells with mutated Ig α would ensure that the effects seen are due to the mutations, and not due to the influence of endogenous Ig α . If the cells transfected with mutant Ig α show abrogation of internalization and transport of the BCR compared to the cells transfected with wt Ig α , this would be a stronger indication that signaling is required for optimum BCR transport. One issue with this cell line is that it is a myeloma cell line, not a lymphoma cell line, but many researchers have used this cell line to study the BCR [20, 160, 161].

The putative internalization motifs found in the Ig α ITAM have the sequence YXX ϕ , where ϕ is a bulky hydrophobic amino acid. In order to separate the role of signaling from the role of the putative internalization motifs in the regulation of transport of the BCR, it would be interesting to repeat the experiments done with the Ig α mutants using Ig α that has either or both tyrosines mutated to phenylalanines. Since phenylalanine has a similar structure to tyrosine but cannot be phosphorylated, this could be a way to not allow signaling to occur but possibly keep the same conformation of the internalization motif. Another idea would be to mutate only the leucine or isoleucine, or both, to an alanine. But by mutating the ϕ residue of the putative internalization motif we would be changing the ITAM as well, since the putative internalization motifs are imbedded in the ITAM. If these experiments were done, it would have to be verified that these mutations are not affecting phosphorylation or signaling at all. The, if there was still a defect in internalization and intracellular transport of the BCR in cells expressing these mutated

Ig α s, this would indicate that these are necessary targeting motifs, as well as sites of signaling.

Previous work in our lab showed that AP-2, clathrin, and dynamin all become rapidly recruited to the plasma membrane upon BCR crosslinking, but this recruitment is inhibited by a Src kinase inhibitor, indicating that signaling is important for this event [128]. Work on other receptors has shown that clathrin-mediated endocytosis relies on an internalization motif in the cytoplasmic tail of the receptor [155, 162]. Experiments should be undertaken to further examine the role of signaling and the potential internalization motifs in BCR clathrin-mediated endocytosis. Each of the ITAM tyrosines should be mutated individually and in combination. After transfection of the wt and mutant Ig α into A20 and J558 μ m cells, the intracellular distribution of AP-2, clathrin and dynamin should be observed using immunofluorescence microscopy. A defect in recruitment of these molecules to the plasma membrane after crosslinking of the BCR in cells expressing mutant Ig α compared to cells expressing wt Ig α would indicate that signaling or the potential internalization motifs are important for this action. These experiments should be repeated with Ig α in which the leucine or the isoleucine of each putative internalization motif are mutated individually or in combination. A defect in recruitment of these molecules to the plasma membrane after crosslinking of the BCR in cells expressing mutant Ig α compared to cells expressing wt Ig α would indicate that these are required internalization motifs. An absence of defects with these mutations but a presence of defects with the tyrosine mutations would indicate that signaling, not the internalization motifs, is essential for efficient movement of the BCR. All of these

experiments would give us more insight into how signaling may be regulating BCR movement and if the putative internalization motifs are necessary for efficient internalization and intracellular transport of the BCR.

4.3 Implications

Antigen presentation by B lymphocytes to T lymphocytes is required to mount high-affinity humoral responses to invading pathogens. When antigen binds and crosslinks the BCR, signaling is initiated, the first signal required to activate the B cell. The BCR is internalized and transported through the endocytic pathway to the antigen loading compartments. Antigen that has been loaded into MHC class II molecules is presented on the surface, and recognition of the antigen peptide:MHC class II complex by a T cell leads to the second activation signal for the B cell. The B cell then proceeds to produce and secrete antigen-specific antibody. These specific antibodies will opsonize pathogens, neutralize toxins, and activate the complement system. Obviously, without proper presentation of antigen by B cells, the human immune system is much less effective and the vaccines that we rely upon to help fight disease by giving our immune system a “first look” at foreign antigens would be useless. Therefore, understanding in detail how the process of antigen internalization and intracellular transport occurs and how it is regulated is very important. Although the research explained here is not clinical, it does contribute to the foundation of understanding of how antigen is efficiently presented, which could lead to therapeutic intervention in the future. For instance, in autoimmune diseases such as systemic lupus erythematosus and diabetes, B cell presentation of self antigens to T cells is necessary for propagation of disease. In systemic lupus

erythematosus, recognition of presented antigen by the T cell activates the B cell to produce high-affinity autoantibodies [163]. In diabetes, presentation of a particular antigen by B cells is necessary to eliminate T cell tolerance to islet beta cells [164]. Thus, understanding how B cells regulate efficient processing and presentation of antigen would be important for creating therapeutics to down-regulate these activities to treat such diseases.

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