THE ROLES OF THE IG-ALPHA AND IG-BETA CYTOPLASMIC DOMAINS IN B CELL ANTIGEN RECEPTOR (BCR) INTERNALIZATION AND TRAFFICKING

by

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ABSTRACT

The B cell antigen receptor (BCR) is expressed on the surface of B lymphocytes where it can bind antigen then transmit signals which regulate activation, growth, and differentiation. These signals can induce a number of cytoskeletal rearrangements leading to changes in adherence, spreading, polarity, immune synapse formation, migration and internalization. In the latter situation, BCR internalization results in the uptake of antigen which can then be processed and presented to T cells on MHC II. The relative importance of regions within the Igα and Igβ cytoplasmic domains have been studied in terms of signaling but their roles in BCR internalization and trafficking are less clear. We hypothesize that the structure of Igα and Igβ cytoplasmic domains is important for normal BCR internalization and trafficking. An Igα and Igβ deficient lymphoid cell line has been used to express mIgM along with a panel of Igα and Igβ mutants in order to compare their internalization and subcellular localization in both a qualitative and quantitative manner. Using this system it was shown that the Igα and Igβ cytoplasmic domains are each sufficient for internalization and that the internalization signal is contained in a region past the first tyrosine residue on either chain, Y176 and Y195 respectively. It was also determined that the Igα cytoplasmic domain makes a larger contribution to internalization than the Igβ tail and that a 4 amino acid motif normally contained within the Igβ ITAM is sufficient to allow internalization. In terms of receptor trafficking it was shown that each cytoplasmic domain is sufficient for trafficking to lysosomal compartments but that a normal rate of trafficking likely requires the tandem effects of both Igα and Igβ. It was also shown that BCR-induced signaling is generally reduced in mutants with an Igα truncation or an Igβ duplication, but that the Igα truncation results in an increased and sustained level of ERK phosphorylation. Further studies with the panel of mutants will determine the role of Igα and Igβ structure in other processes involving cytoskeletal rearrangements.
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<tr>
<td>3D</td>
<td>3-dimensional</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>Abp1</td>
<td>Actin binding protein1</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin-related protein-2/3</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>BLNK</td>
<td>B cell linker protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CL</td>
<td>Cytoplasmic loop</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CT</td>
<td>Cytoplasmic tail</td>
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<tr>
<td>Cx43</td>
<td>Connexin 43</td>
</tr>
<tr>
<td>DAG</td>
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</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>Deoxyribonucleic acid</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
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<td>Early endosome</td>
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<tr>
<td>EEA1</td>
<td>Early endosome antigen-1</td>
</tr>
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<td>EL</td>
<td>Extracellular loop</td>
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<tr>
<td>EPS15</td>
<td>Epidermal-growth-factor-receptor-pathway substrate 15</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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</table>
FITC  Fluorescein isothiocyanate
Gab1  Grb2-associated binding protein 1
Gab2  Grb2-associated binding protein 2
GDP  Guanosine diphosphate
GJ  Gap junction
Grb-2  Growth factor receptor-bound protein 2
GTP  Guanosine triphosphate
HIP1R  Huntingtin-interacting protein-1 related
HRP  Horseradish peroxidise
hrs  Hours
HS1  Hematopoietic lineage cell-specific protein 1
Ig  Immunoglobulin
Ii  Invariant chain
IL-2  Interleukin-2
IP3  Inositol tris phosphate
ITAM  Immunoreceptor tyrosine-based activation motif
ITIM  Immunoreceptor tyrosine-based inhibition motif
IκB  Inhibitor of NF-κB
KCl  Potassium chloride
kDa  Kilodalton
L  Lysosome
LAMP-1  Lysosome associated membrane protein-1
LB  Luria Bertani
LDL  Low density lipoprotein
LE  Late endosome
MAPK  Mitogen-activated protein kinase
MFI  Mean fluorescent intensity
MgSO4  Magnesium sulphate
Min  Minutes
MHC  Major histocompatibility complex
N-WASP  Neuronal Wiscott-Aldrich syndrome protein
mIg  Antigen binding subunit/membrane Ig
MIIC  MHC II-containing compartments
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MKP</td>
<td>Mitogen activated kinase phosphatase</td>
</tr>
<tr>
<td>mSOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>Sodium phosphate</td>
</tr>
<tr>
<td>Na$_3$VO$_4$</td>
<td>Sodium pervanadate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDK</td>
<td>Phosphoinositide-dependent kinase</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI(3,4)P$_2$</td>
<td>Phosphatidylinositol-3,4-bisphosphate</td>
</tr>
<tr>
<td>PI(3,4,5)P$_3$</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
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<tr>
<td>PI(4)P</td>
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<tr>
<td>PI(4,5)P$_2$</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol-3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC$_\gamma$</td>
<td>Phospholipase C $\gamma$</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>RasGRP</td>
<td>Ras guanyl nucleotide releasing protein</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homologous and collagen protein</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2 domain-containing inositol phosphate 5'-phosphatase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
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<tr>
<td>SHP-1</td>
<td>Src homology phosphatase-1</td>
</tr>
<tr>
<td>SHP-2</td>
<td>Src homology phosphatase-2</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS + 0.1% Tween-20</td>
</tr>
<tr>
<td>TfnR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>WEHI</td>
<td>Walter and Eliza Hall Institute</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
</tbody>
</table>
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1.1 The immune system – an overview

No organism exists in complete isolation. All are influenced in some way by the environment in which they are found, by both living and non-living factors. We are constantly exposed to pathogens which are themselves adept at colonizing hosts, so the fact that most of us lead healthy lives is a tribute to our immune systems. The vertebrate immune system can be loosely divided into two parts, though they are not completely independent of each other: the innate immune system and the adaptive immune system.

The innate immune system is the first line of defense against a pathogen and is immediately present in all normal individuals, providing a rapid and non-specific response. This system relies on the recognition of broad classes of pathogens sharing invariant patterns which are recognized by pattern recognition receptors such as Toll-like receptors (TLRs) (Akira et al., 2001; Kobayashi et al., 2002). The acellular component, includes proteolytic cascades such as complement and the cellular component, includes phagocytic cells such as macrophages, neutrophils and dendritic cells. These components cooperate to mount a response which functions both to clear the pathogen and to prime the cells of the adaptive immune system (Akira, 2006; Barton and Medzhitov, 2002).

The adaptive immune response is mediated by B and T lymphocytes and comes into play in the event that the innate immune component fails to clear an immunological insult. As a population, these cells have a large and diverse repertoire of antigen (Ag) specificity due to their randomly
generated receptors (Tonegawa, 1983), and during an immune challenge, the correct cells will proliferate and differentiate into effector cells in order to mount a highly specific response to clear the pathogen from the host system (Moretta, 2005). Though slower, the adaptive immune response is efficient, highly specific, and generates immunological memory which protects against re-infection. B cells act as professional Ag presenting cells (APCs) to help activate T cells and also secrete antibody (Ab) (Chapter 1.2). T cells are classified in different categories: cytotoxic, helper, or regulatory, all of which cooperate to achieve the eradication of pathogens.

1.2 B lymphocytes – an overview

Like all immune cells, B lymphocytes trace their lineage back to pluripotent hematopoietic stem cells which then commit to the lymphoid lineage, becoming common lymphoid progenitors. B cells develop in the bone marrow, interacting with stromal cells prior to migrating to secondary lymphoid tissues such as lymph nodes and the spleen where they encounter Ag becoming activated. Once terminally differentiated into Ab-secreting plasma cells, they migrate back to the bone marrow. It should also be noted that sub-categories of B cells exist however this discussion is limited to the classical B-2 cells.

Ag is recognized via B cell antigen receptors (BCRs) on the cell surface and its binding to BCRs causes signaling and activation which includes uptake by receptor-mediated endocytosis, and ultimately results in proliferation and differentiation. However BCR engagement on cells in different developmental stages can also result in receptor editing, deletion, or anergy (Rolink et al., 2001).
In the case of endocytosis, the Ag is processed into peptides and presented on the surface of the cell on a major histocompatibility complex (MHC) II (Guagliardi et al., 1990). In this way, B cells can act as Ag presenting cells (APCs) for naïve T cells (Lanzavecchia, 1985; Reth and Wienands, 1997). This activates the T cells causing a sequence of events eventually resulting in the secretion of cytokines which, in turn, activates B cells causing them to proliferate and differentiate, the end result of which is a large population of terminally differentiated, Ab-secreting plasma cells.

1.3 The BCR

1.3.1 BCR structure and assembly

The BCR, first identified in the 1970's (Raff et al., 1970), is an oligomeric protein complex found on the surface of B cells as shown in Figure 1.1 (DeFranco, 1993; Reth, 1994). It is composed of an Ag binding subunit (mIg) and a signaling subunit. The Ag binding subunit is itself composed of 2 immunoglobulin (Ig) heavy chains and 2 Ig light chains, all held together by disulfide bonds. The signaling subunit is made up of an Igα-Igβ heterodimer, again, disulfide linked. These two subunits are non-covalently associated with each other in the endoplasmic reticulum (ER) and traffic together in a 1:1 ratio through the secretory pathway to the cell surface (Schamel and Reth, 2000). In the event that a BCR component is missing during assembly, the remaining proteins are trapped within the ER and are eventually degraded, surface expression of the BCR requires all 4 chains (Matsuuchi et al., 1992; Venkitaraman et al., 1991).

The mIg can be one of 5 isotypes: mIgM, mIgD, mIgA, mIgG, mIgE. This discussion is limited to the mIgM isotype. The murine Igμ heavy chain is a glycoprotein with a molecular weight (MW)
Figure 1.1. Schematic representation of the BCR with an in depth look at the Igα and Igβ cytoplasmic domains.
Cytoplasmic domain of Igα spans amino acids 160-220, cytoplasmic domain of Igβ spans amino acids 181-228. Letters indicate amino acids in single letter amino acid code.
ranging from 67-78 kilodaltons (kDa) (Vitetta et al., 1971) due to differential glycosylation at 5 sites (Finley et al., 1990). The heavy chain is anchored to the plasma membrane by a 25 amino acid transmembrane domain and a 3 charged amino acid lysine-valine-lysine cytoplasmic tail (Reth, 1992). Along with 2 heavy chains, 2 light chains make up the Ag binding subunit. The light chain has a MW of 25-28 kDa, and each chain is disulfide linked to a heavy chain. The light chain can be a lambda (λ) or a kappa (κ) chain, though in either case random germline rearrangements occur between V and J segments to generate unique receptor specificity, as with the heavy chain.

The signaling subunit is composed of one Igα chain and one Igβ chain which are disulfide linked and form a heterodimer (Hombach et al., 1988; Hombach et al., 1990). This heterodimer is sufficient for proper trafficking and surface expression of the mIgM form of the BCR (Matsuuchi et al., 1992; Venkitaraman et al., 1991), and it has been shown that the heterodimer displaces ER retention proteins associated with the heavy and light chains, allowing for their release from the ER (Melnick et al., 1992; Melnick et al., 1994). Neither Igα nor Igβ have specific tertiary structures reported for their cytoplasmic domains.

The Igα chain (or CD79a) is a 34 kDa transmembrane glycoprotein (Hombach et al., 1988) encoded by the mb-I gene (Kashiwamura et al., 1990; Sakaguchi et al., 1988). The cytoplasmic portion contains an immunoreceptor tyrosine-based activation motif (ITAM) signaling motif D/EX7D/EXXYXXI/LX7YXXI/L, single amino acid code where X represents any amino acid. Two tyrosine residues (Y) are separated by 11 amino acids with the third residue following each tyrosine being a leucine or an isoleucine (Cambier, 1995; Reth, 1989). Igα also contains an internalization signal which allows the BCR and any bound antigen to be taken up by receptor-mediated endocytosis (Lankar et al., 1998), but the precise signal has yet to be identified. There is,
however, there is a general consensus that the signal is contained within the region between the non-ITAM tyrosines Y176 and Y204, based on existing studies within the field.

The Igβ chain (or CD79b) is encoded by the B29 gene and has a molecular weight of 39 kDa (Hermanson et al., 1988). It too has a glycosylated extracellular domain, and a transmembrane and cytoplasmic domain with the cytoplasmic domain containing an ITAM signaling motif (Reth, 1989), and an internalization signal (Lankar et al., 1998), but again the precise signal has not been identified, though existing studies have focused on the ITAM. Igα and Igβ must heterodimerize in the extracellular and transmembrane regions for proper assembly and surface expression of the 4 chain BCR (Dylke et al., 2007).

1.3.2 BCR signaling

Ligation of the BCR by its cognate ligand can lead to activation and growth, or to apoptosis in different situations (Koncz et al., 2002). Two events initiated by this are differentiation and cytoskeletal rearrangements which lead to events such as receptor internalization, cell spreading, cell migration, and polarization. Signaling from the BCR is necessary for the proper activation and function of B cells and a basal level of signaling is required for survival (Casola et al., 2004). Signaling through the pro-, pre-, and complete BCR is a necessary component of development and cells deficient in signaling through the BCR in any of its forms do not progress through to the subsequent developmental stages, and are arrested or deleted. Signal transduction involves an ordered interaction and alteration of effector proteins generally resulting in the transcription of genes vital for B cell survival, proliferation, and development (Figure 1.2).
Figure 1.2. General overview of BCR-mediated signaling pathway activation.
A simplified schematic representation of the membrane-proximal events occurring upon BCR cross-linking and the main pathways activated: the PLCγ pathway, the Ras/MAPK pathway and the PI3K pathway. Igα is shown in orange, Igβ is shown in green with phosphorylated tyrosine residues on both chains represented by yellow circles. Kinases are represented by blue ovals, adaptor proteins by rectangles, dashed arrows indicate that intermediate effector proteins are involved. Schematic does not include negative regulation. For details see Chapter 1.3.2.
While signaling pathways in B cells can be discussed at length, it is useful to bear in mind that the cytoplasmic tails of Igα and Igβ have an essential role in the first steps of signal initiation and so this discussion is mostly limited to those events. Signaling is initiated upon ligand-induced aggregation of BCRs that may reside within sphingosine-rich, cholesterol-rich membrane microdomains called lipid rafts, though BCR signaling may also occur outside lipid rafts as well (Cherukuri et al., 2001; Dykstra et al., 2003; Gupta and DeFranco, 2003; Langlet et al., 2000). This receptor clustering results in the phosphorylation of tyrosine residues found within and outside of the ITAMs on the cytoplasmic tails of Igα and Igβ (Campbell et al., 1991; Gold et al., 1991), presumably due to the receptor being in close proximity to Src family kinases. This signaling event is a common first step of all pathways activated from the BCR. It is generally agreed upon that the phosphorylation is mediated by the Src family kinases that are expressed by B lymphocytes, Lyn, Fyn, Blk, and Lck depending on the B cell type (DeFranco, 1997; Kurosaki, 1997, 1999; Kurosaki et al., 2000; Reth and Wienands, 1997). In particular, active Lyn is known to be localized within lipid rafts in B cells (Cheng et al., 1999; Pierce, 2002; Resh, 1999; Xu et al., 2005) and can associate with the non-phosphorylated Igα and Igβ (Pleiman et al., 1994), bringing them close to the receptor complex. The phosphorylation of ITAM tyrosines facilitates the recruitment and activation of more Src family kinases as well as the nonreceptor tyrosine kinase Syk via its tandem Src-homology 2 (SH2) domains binding to a pair of phosphorylated tyrosine residues. Syk is necessary for normal downstream signaling but not for ITAM phosphorylation or Src family kinase activation (Dal Porto et al., 2004; Takata et al., 1994). The Src family kinases and Syk also function to recruit and phosphorylate Bruton’s tyrosine kinase (Btk) resulting in its activation (Kurosaki and Tsukada, 2000; Rawlings and Witte, 1995). This assortment of phosphorylated and activated effectors then continue the signal transduction cascade through three main pathways mediated by the signaling enzymes.
phosphoinositol-3 kinase (PI3K), phospholipase C (PLC) γ, and Ras/mitogen-activated protein kinase (MAPK), which are themselves interconnected.

The PI3K pathway is necessary for B cell development as well as for activation and survival (Clayton et al., 2002; Fruman, 2004; Fruman et al., 1999; Gold, 2002b; Gold and Aebersold, 1994). Following the membrane proximal events initiated by BCR clustering, PI3K is recruited to the membrane through SH2 domains binding to phosphorylated tyrosine residues in the receptors themselves or on co-receptors such as CD19 (Tuveson et al., 1993). This positions PI3K near its substrates, phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂), which it phosphorylates to create phosphtidylinositol-3,4-bisphosphate (PI(3,4)P₂) and phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P₃).

Phosphoinositide-dependent kinase (PDK) and Akt are both recruited to the membrane via their Pleckstrin homology (PH) domains and their close proximity allows for the phosphorylation and activation of Akt by PDK (Alessi et al., 1997a; Alessi et al., 1997b; Anderson et al., 1998; Kandel and Hay, 1999; Stokoe et al., 1997; Wick et al., 2000). Because PI3K pathway activation results in Akt phosphorylation (Astoul et al., 1999; Gold et al., 1999) it is often used as an indicator of PI3K pathway activation. Akt activation eventually results in β-catenin and NF-κB translocation into the nucleus and the subsequent transcription of pro-survival genes (Datta et al., 1997; Kane et al., 1999; Monick et al., 2001; Ozes et al., 1999; Pogue et al., 2000, Romashkova and Marakov, 1999; Rubinfeld et al., 1996).

Signaling through the PLCγ pathway is necessary for both development and proliferation of B cells (Hashimoto et al., 2000; Wang et al., 2000). Following BCR engagement and subsequent initiation events, the adapter protein B cell linker protein (BLNK) is recruited, via its SH2 domain, to phosphorylated tyrosine residues on Iγ (Dal Porto et al., 2004; Engels et al., 2001;
Kabak et al., 2002). Btk is also recruited to the membrane. BLNK, now in close proximity with Syk, is phosphorylated, creating a binding platform for PLCγ and other signaling molecules through its SH2 domain (Fu et al., 1998; Ishiai et al., 1999a; Ishiai et al., 1999b; Kurosaki and Tsukada, 2000; Wienands et al., 1998). Once bound to this signaling complex, PLCγ is phosphorylated by Syk and Btk and activated, though it appears that the activation is not dictated as much by the phosphorylation status as it by the compartmentalization (T. Jackson, PhD thesis, UBC, 2005). Activated PLCγ hydrolyzes its substrate PI(4,5)P2 to produce the second messengers inositol 1,4,5-triphosphate (IP3) and diacetylglycerol (DAG) (Bijsterbosch et al., 1985; Dal Porto et al., 2004; Fahey and DeFranco, 1987; Gold, 2002a; Kurosaki et al., 2000; Rhee et al., 1989). IP3 causes calcium release from intracellular stores (Kurosaki et al., 2000; Miyakawa et al., 1999; Sugawara et al., 1997; Takata et al., 1995) which eventually leads to NFAT translocation to the nucleus (Dal Porto et al., 2004; Dolmetsch et al., 1997; Kurosaki and Tsukada, 2000; Timmerman et al., 1997). DAG is an activator of protein kinase C (PKC) which can phosphorylate IκB, allowing NF-κB nuclear translocation (Saijo et al., 2003). The second messenger DAG, in concert with calcium and PKC, can also play into the Ras/MAPK pathway resulting in extracellular signal-regulated kinase (ERK) phosphorylation (Dal Porto et al., 2004; Gold et al., 1992).

Ras/MAPK pathway activation is a necessary pathway for B cell development and proliferation (Iritani et al., 1997; Richards et al., 2001). DAG, produced as a result of PLCγ activation, can activate Ras guanyl nucleotide releasing protein (RasGRP) (Coughlin et al., 2005) which in turn causes a GDP for GTP switch, effectively activating Ras. Alternately, Ras is activated through the action of mSOS which is localized to the membrane via an interaction with Grb2 which is bound to Src homologous and collagen protein (Shc) or SHP-2 (Dal Porto et al., 2004; Gold, 2002a). Shc may be linked to the plasma membrane via Gab1 or Igα (Gold et al., 2000), whereas
SHP-2 interaction with the membrane is through Gab1 and Gab2 (Gold et al., 2000; Gu et al., 1998). Activated Ras allows signaling to continue by activating Raf-1 which in turn phosphorylates and activates mitogen-activated protein kinase kinase (MEK) 1 and MEK2. These kinases phosphorylate and activate ERK. Activated ERK translocates into the nucleus and activates transcription factors initiating transcription of the appropriate genes.

It should also be mentioned that the efficiency of B cell signaling requires a balanced state of signaling. This is achieved mainly through the phosphorylation of immunoreceptor tyrosine-based inhibition motifs (ITIMs) found on some membrane receptors and CD antigens (D'Ambrosio et al., 1996; Daeron, 1996) and dephosphorylation of activated components by protein tyrosine phosphatases, which may include CD45, Src homology phosphatase-1 (SHP-1) and Src homology phosphatase-2 (SHP-2).

1.3.3 BCR-mediated cytoskeletal rearrangements

In addition to BCR signaling leading to transcription factor activation, signaling through the BCR can also induce a number of cytoskeletal rearrangements leading to changes in cell shape, adherence, spreading, process extension, polarity, immune synapse formation, migration and internalization. These are all necessary and normal processes in the life of a B lymphocyte and would be required for migration to or from the bone marrow, or for extravasation through tissue layers, for example. It is becoming clear that there is a link between BCR-mediated signaling and actin cytoskeletal reorganization although the precise mechanism by which the two are connected remains unclear. One possible link which has been suggested is that Src-family kinases and the tyrosine kinase Syk regulate the cytoskeleton through modulation of a cortactin homolog, hematopoietic lineage cell-specific protein 1 (HS1) (Gomez et al., 2006). It is known
that upon initiation of BCR signaling there is a rapid wave of actin depolymerization (Hao and August, 2005), and that stabilization of existing actin filaments or inhibition of actin polymerization affects many of these BCR mediated processes (Brown and Song, 2001; Melamed et al., 1991; Stoddart et al., 2005). This thesis focuses on one of these processes: receptor internalization (Figure 1.3).

1.4 Focus on BCR internalization

1.4.1 BCR internalization

An important function of the BCR upon Ag binding is the mediation of Ag internalization leading to processing and presentation of that Ag to helper T cells in the context of MHC II (Lanzavecchia, 1985; Reth and Wienands, 1997). While the end result of BCR internalization is clear, there is still some debate as to precisely how this comes about and what elements are involved. BCR clustering leads to receptor internalization in what is generally agreed to be a clathrin-dependent manner (Guagliardi et al., 1990; Salisbury et al., 1980) although there has been a report of clathrin-independent BCR internalization as well (Stoddart et al., 2005). The involvement of lipid rafts in this process has been contested as well (Caballero et al., 2006; Cheng et al., 1999; Putnam et al., 2003; Stoddart et al., 2002; Stoddart et al., 2005).

Clathrin-mediated endocytosis in general has been very well characterized in the past with the major models being the low density lipoprotein (LDL) receptor and the transferrin receptor. Clathrin accumulates at the plasma membrane via adaptor proteins to form clathrin-coated pits which then bud and pinch off in a dynamin-dependent manner to form clathrin-coated vesicles. The vesicles uncoat and are targeted to early endosomes where sorting occurs. From there the
Figure 1.3. Simplified schematic of the BCR internalization, trafficking and Ag presentation.

(1) Ag binding causes clustering of BCRs at the cell surface, clathrin and adaptor proteins accumulate and the BCR-Ag complexes are internalized in an actin-dependent manner. (2) BCR-Ag complexes are found within early endocytic compartments (EE) which (3) mature into later endocytic compartments (LE) which (4) fuse with the MIIC where degradation of the Ag occurs by resident proteases (yellow). (5) Newly synthesized MHC II (purple) is trafficked from the Golgi apparatus to the MIIC where it is (6) loaded with the newly degraded peptide then the peptide-MHC II complex traffics to the cell surface. For details see Chapter 1.4. N represents the nucleus, GA represents the Golgi Apparatus.
unligated receptors are recycled and return to the plasma membrane and those bound to antigen proceed as the endocytic pathway matures to the late endosomes and lysosomes where degradation occurs.

The structure of the BCR, in particular of the Igα/Igβ heterodimer, is important for the process of internalization and for the subsequent targeting of vesicles. Studies have shown that the Igα/Igβ heterodimer is sufficient for internalization and that the BCR is constitutively internalized through clathrin-coated pits (Patel and Neuberger, 1993). A closer look revealed that Igα and Igβ each contain an internalization signal (Lankar et al., 1998) but the identities of these signals are not well defined though the focus has been on the region between the non-ITAM tyrosines in Igα and within the ITAM for Igβ. The cytoplasmic domain of Igα, specifically the first tyrosine in the ITAM has been reported to be both necessary and sufficient for constitutive internalization of the BCR (Cassard et al., 1998), and it has also been shown that both ITAM and non-ITAM tyrosines contribute to BCR internalization, however mutation of these residues does not abrogate the process entirely (Hou et al., 2006). The ITAM tyrosines of the Igβ cytoplasmic domain have also been reported to be necessary for ligand-induced receptor internalization (Gazumyan et al., 2006). It has also been reported that the 3 amino acid cytoplasmic domain of Igμ can be ubiquitynated (Drake et al., 2006), which may also affect receptor internalization or trafficking. Generally speaking, the relative importance of various regions of the cytoplasmic domains has been the subject of conflicting reports for many years.

Although BCR signaling and BCR internalization are discussed as separate entities it is useful to remember that each affects the other. Inhibitors of signaling proteins also affect internalization, trafficking, and growth control. Inhibitor and knock out studies have shown that several proteins involved in BCR signaling, such as Src-family kinases, appear to be involved in receptor
internalization (Ma et al., 2001; Stoddart et al., 2002) and that the inhibition of BCR internalization may result in the prolonged activation of signaling pathways (Hao and August, 2005; Stoddart et al., 2005) although it has recently been suggested that receptor signaling and internalization are mutually exclusive events for any given BCR and that only one or the other will occur (Hou et al., 2006). The involvement of other proteins has also been reported, for example, the ubiquitin ligase Cbl has been reported to be necessary for BCR internalization as well as linking the process to the actin cytoskeleton (Jacob et al., 2008).

1.4.2 BCR trafficking to intracellular compartments and antigen presentation

Though the BCR is internalized by clathrin-mediated endocytosis, it proceeds through the endocytic pathway with a few amendments. When internalization has been induced by ligand binding, BCR-ligand complexes are trafficked to MHC II-containing (MIIC) compartments where normal cargo would be degraded (Amigorena et al., 1994; Cheng et al., 1999; Drake et al., 1999; Lankar et al., 2002; Neefjes et al., 1990; Siemasko et al., 1998). MIICs have many of the same markers as both late endosomes and lysosomes and indeed were initially described as late endosome-like, lysosome-like compartments. They are most commonly described as lysosomes containing a unique protein, MHC II. They are acidic compartments resembling late endosomes with a complex multivesicular and multilamellar structure, lysosome associated membrane protein-1 (LAMP-1) positive, and contain newly synthesized MHC II (Harding and Geuze, 1993; Kleijmeer et al., 1997; Qiu et al., 1994; Rudensky et al., 1994). MHC II molecules are synthesized in the ER where they also associate with the invariant chain (Ii) which prevents their premature association with endogenous peptide as well as targeting them to endosomal compartments (Bakke and Dobberstein, 1990; Lamb et al., 1991; Lotteau et al., 1990; Miller, 1994). The proteolytic action of cathepsin S exposes the peptide-binding groove which is filled
by the ligand-derived peptide, and the newly-formed peptide-MHC II complexes are transported to the cell surface for presentation to helper T cells.

The importance of the cytoplasmic domains of Igα and Igβ have been mostly examined with respect to Ag presentation, as measured by the presence of a given peptide on surface MHC II or by the T cell response elicited. Less is known regarding the ability of either cytoplasmic domain to direct BCR-ligand complexes to the correct intracellular compartment so that the aforementioned process can occur. The contributions of the cytoplasmic domains to intracellular targeting remain unclear because of conflicting reports (Bonnerot et al., 1995; Cassard et al., 1996; Granboulan et al., 2003; Hou et al., 2006; Lankar et al., 1998). The cytoplasmic domains of the Igα-Igβ heterodimer are necessary and sufficient for BCR trafficking. Individually, the cytoplasmic domain of Igα targets the BCR to compartments containing newly synthesized MHC II whereas the cytoplasmic domain of Igβ targets the BCR to a recycling pool of MHC II (Bonnerot et al., 1995). It has been reported that the phosphorylation of the non-ITAM tyrosine on Igα targets the BCR to the late endosome (Siemasko et al., 1999), but also that it is the ubiquitynation of Igβ which allows for receptor trafficking to late endosomes (Zhang et al., 2007). Further evidence for the unique roles of Igα and Igβ was gathered from experiments showing that Igα mediates presentation of all tested Ags but that Igβ allowed presentation of only one of the tested Ags (Lankar et al., 1998), which may have resulted from the differential targeting to MHC II-containing compartments. But while that report states that Igα is required for efficient Ag presentation, another report states that PI3K activation is required for this and that it is linked to Igβ (Granboulan et al., 2003). These reports illustrate the fact that although there are clearly differences between Igα and Igβ in terms of their contributions to trafficking and Ag presentation, the details remain unclear. Because of the BCRs dual role in signaling and Ag presentation, BCR-mediated signaling and trafficking may also be linked. Inhibitor and
knockout studies have shown that several proteins involved in BCR signaling such as Syk and BLNK, while not directly involved in internalization, may be involved in targeting (Cassard et al., 1996; Siemasko et al., 2002).

1.5 Purpose of thesis study

BCR internalization and intracellular trafficking are important precursors to antigen presentation, which is in turn important for the adaptive immune response. There is an ongoing effort in this field of study to convincingly identify the elements involved, such as lipid rafts and clathrin, and to link the processes involved in Ag presentation to BCR-mediated signaling and cytoskeletal reorganization. However, the contributions of the cytoplasmic domains of Igα and Igβ to the processes remain enigmatic. Despite several groups having published reports to this end, they are often contradictory and so this field of study continues to lack consensus. The contrary reports may stem from the fact that many of the past reports have utilized a chimeric receptor system. In these systems, the cytoplasmic domain of either Igα or Igβ is fused to the transmembrane and extracellular portions of either the platelet-derived growth factor (PDGF) receptor or the Fc receptor which are then clustered using antibodies to mimic Ag binding. This is useful in that it facilitates the study of the effects of mutations in these domains, but may be misleading since it is likely not reflective of what happens with a full, 4 chain BCR. Clustering of these chimeric receptor results in homotypic clustering, whereas BCR clustering results in the cytoplasmic clustering of multiple Igμ, Igα, and Igβ cytoplasmic domains.

This thesis project tests the hypothesis that the structure of the Igα and Igβ cytoplasmic domains is important for normal BCR function with respect to receptor internalization and intracellular
trafficking. The aim is to clarify the relative contributions made by regions within the cytoplasmic domains of Igα and Igβ in the context of a full 4 chain mIgM-containing BCR. J558 murine plasmacytoma tissue culture cell lines were utilized as an experimental system because of their deficiency in multiple BCR components (Carson and Weigert, 1973; Cesari and Weigert, 1973). Plasma cells do not normally express the BCR on the cell surface, however we are in possession of several variants which have been stably transfected with varying combinations of BCR components. In this way, we were able to study the effects of mutations to the Igα and Igβ chain, while maintaining a normal B lymphocyte cytoplasmic microenvironment, since this system allows for stable and transient transfection of missing BCR components. It should also be noted that plasma cells no longer express MHC II and therefore no longer interact with T cells.

1.6 Summary of findings

In order to better understand the contributions of the cytoplasmic domains, we compared a panel of BCRs with mutations in the Igα and Igβ cytoplasmic domains with WT BCRs, with respect to receptor uptake and subsequent subcellular localization in both a qualitative and quantitative manner. Pre-existing Igα and Igβ constructs with cytoplasmic swaps with the opposite chain were employed (Dylke et al., 2007) and Igα and Igβ constructs with cytoplasmic truncations as well as mutations within the ITAMs were created. The constructs were expressed in J558 recipient cells in varying combinations and the function of the cells was assessed.

To determine the contribution made by the complete cytoplasmic domains or regions within the cytoplasmic domains of Igα and Igβ to BCR internalization, a panel of 7 mutants was compared to WT for their ability to internalize BCR following crosslinking by flow cytometry. The data
shows that the Igα cytoplasmic domain makes a larger relative contribution to BCR internalization than the Igβ cytoplasmic domain and that a 4 amino acid motif from Igα is sufficient to restore BCR internalization to normal levels in defective mutants (Chapter 3.1).

To determine the role of the Igα and Igβ cytoplasmic domains in trafficking to intracellular compartments, stimulated cells were co-immunostained for BCR and intracellular compartments and assessed for co-localization by confocal microscopy. The data shows that both the Igα and the Igβ cytoplasmic domains are sufficient for trafficking to lysosomes, but that both may be required for a normal rate of trafficking (Chapter 3.2).

To determine the contribution made by the cytoplasmic domains to BCR-mediated pathway activation, stimulated cells were stained for total tyrosine phosphorylation, phosphorylated Akt and phosphorylated ERK. The data shows that the presence of both cytoplasmic domains is necessary for a normal rate and magnitude of phosphorylation and that the Igα cytoplasmic domain may play a role in negative regulation of some signaling pathways (Chapter 3.3).
CHAPTER 2
MATERIALS AND METHODS

2.1 Reagents

2.1.1 Antibodies

The polyclonal goat anti-mouse IgM (μ chain specific) antibody (Ab) used for stimulations and the polyclonal rabbit anti-mouse IgM (μ chain specific) Ab used for immunoblotting were both purchased from Jackson ImmunoResearch Labs, Inc. (West Grove, Pennsylvania). The polyclonal rabbit anti-mouse Igλ light chain Ab was purchased from Bethyl Laboratories (Montgomery, Texas). The polyclonal rabbit anti-mouse Igα cytoplasmic domain Ab was produced against a 34 amino acid peptide from the Igα cytoplasmic tail (187-220) was developed by the Matsuuchi Lab (University of British Columbia, Vancouver BC) and has been previously described (Gold et al., 1991). The polyclonal rabbit anti-mouse Igα extracellular Ab produced against a 29 amino acid peptide from the Igα extracellular domain, and the polyclonal rabbit anti-mouse Igβ extracellular Ab produced against a 29 amino acid peptide from the Igβ extracellular domain, were both gifts from Abeome (Athens, Georgia) and Dr. Richard Meagher and Elizabeth McKinney (University of Georgia, Athens, Georgia).

The mouse monoclonal anti-phospho-tyrosine Ab (4G10) used for immunoblotting was purchased from Upstate Biotechnology (Charlottesville, Virginia) and the polyclonal rabbit anti-mouse phosphotyrosine Ab used for intracellular staining was purchased from BD Biosciences. The polyclonal rabbit anti-mouse phospho-Akt (Ser473) Ab and the mouse monoclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) Ab, both used for intracellular staining, were
purchased from Cell Signaling Technology (Danvers, Massachusetts). The monoclonal mouse anti-human transferrin receptor Ab was purchased from Invitrogen Life Technologies and the rat anti-mouse LAMP-1 hybridoma supernatant (1D4B) was purchased from the Developmental Studies Hybridoma Bank (DSHB) (University of Iowa, Iowa City, Iowa). The polyclonal rabbit anti-human Connexin 43 Ab was purchased from Sigma Aldrich. The monoclonal mouse anti-β-actin Ab was purchased from MP Biomedical (Solon, Ohio).

The horseradish peroxidase (HRP)-conjugated Protein A secondary reagent was purchased from Amersham (Baie d'Urfe, Quebec), and the goat anti-mouse IgG conjugated HRP and goat anti-rabbit IgG conjugated HRP secondary reagents were purchased from Invitrogen Life Technologies and Jackson ImmunoResearch Labs, Inc. respectively.

The fluorescein isothiocyanate (FITC)-conjugated affinipure goat anti-mouse IgG Ab (Fc fragment specific) was purchased from Jackson ImmunoResearch Lab, Inc. The Alexa Fluor 647-conjugated donkey anti-goat IgG (H+L) Ab and the Alexa Fluor 488 F(ab)_2 fragment of rabbit anti-goat IgG (H+L) Ab were both purchased from Invitrogen Molecular Probes. The Alexa Fluor 633-conjugated goat anti-mouse IgM Ab, the Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) Ab, the Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) Ab, the Alexa Fluor 647-conjugated goat anti-mouse IgG and the Alexa Fluor 568-conjugated goat anti-rat IgG (H+L) Ab, all used for intracellular staining, were purchased from Invitrogen Molecular Probes.

2.1.2 Plasmids

The plasmid pWZL-Blast3 was a gift from Dr. Stephen Robbins (University of Calgary, Alberta). The pBCMGSneo-μM2 plasmid encoding Igμ was created by Dr. Linda Matsuuchi and
has been previously described (Matsuuchi et al., 1992). The pCMVmb1 plasmid encoding WT Igα was a gift from Dr. Rudolf Grosschedl (Max Planck Institute, Freiburg, Germany). The pMIGR1-Igβ plasmid encoding WT Igβ was a gift from Dr. Marcus Clark and was previously described (Wang et al., 2004). The pWZL-Blast1-Cα and pWZL-Blast3-Cβ plasmids encoding Igα and Igβ each with a cytoplasmic swap for the opposite chain were made by Janis Dylke and have been previously described (Dylke et al., 2007).

2.1.3 Plasmids Created

2.1.3.1 Construction of pWZL-blast3-Igatrunc

The truncated Igα construct (Igα trunc) (Table 2.1) was made using polymerase chain reaction (PCR) using pCMVmb1 as a template. The primers shown in Table 2.2 were designed to amplify the coding region of Igα from the start codon up to and including the 3 nucleotides encoding the amino acid immediately preceding the most N-terminal tyrosine residue (Y176). A flanking BamHI restriction enzyme site was also added N-terminal to the start codon and a flanking EcoRI restriction enzyme site was added C-terminal to the coding region bound by the reverse primer. Following PCR, the resulting fragments were concentrated then inserted into pWZL-blast3 between the EcoRI and BamHI restriction digest sites and successful ligations were selected through transformation into competent bacteria (Chapter 2.2.4). The truncation was confirmed by sequencing.
Table 2.1. **Summary of Igα and Igβ cytoplasmic mutant constructs.** Schematic representations and descriptions of the mutant constructs used in this study. Normal Igα sequences are depicted in orange and normal Igβ sequences are depicted in green. Only cytoplasmic domains are shown, extracellular and transmembrane domains are wild type. Black lines represent the plasma membrane. Schematic diagrams are not drawn to scale.

<table>
<thead>
<tr>
<th>Construct Name</th>
<th>Schematic Representation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Igα</td>
<td><img src="image" alt="WT Igα Schematic" /></td>
<td>Wild type Igα sequence</td>
</tr>
<tr>
<td>WT Igβ</td>
<td><img src="image" alt="WT Igβ Schematic" /></td>
<td>Wild type Igβ sequence</td>
</tr>
<tr>
<td>Igα trunc</td>
<td><img src="image" alt="Igα trunc Schematic" /></td>
<td>Igα truncation immediately preceding the first cytoplasmic tyrosine residue (Y176)</td>
</tr>
<tr>
<td>Igβ trunc</td>
<td><img src="image" alt="Igβ trunc Schematic" /></td>
<td>Igβ truncation immediately preceding the first cytoplasmic tyrosine residue (Y195)</td>
</tr>
<tr>
<td>Cα</td>
<td><img src="image" alt="Cα Schematic" /></td>
<td>Swap of the normal Igβ cytoplasmic domain for the Igα cytoplasmic domain</td>
</tr>
<tr>
<td>Cβ</td>
<td><img src="image" alt="Cβ Schematic" /></td>
<td>Swap of the normal Igα cytoplasmic domain for the Igβ cytoplasmic domain</td>
</tr>
<tr>
<td>Igα QTAT</td>
<td><img src="image" alt="Igα QTAT Schematic" /></td>
<td>Mutation of the DCSM amino acids normally found within the Igα ITAM to the QTAT amino acids normally found within the Igβ ITAM</td>
</tr>
<tr>
<td>Igβ DCSM</td>
<td><img src="image" alt="Igβ DCSM Schematic" /></td>
<td>Mutation of the QTAT amino acids normally found within the Igβ ITAM to the DCSM amino acids normally found within the Igα ITAM</td>
</tr>
</tbody>
</table>
Table 2.2. Oligonucleotide primer sequences used for PCR and site-directed mutagenesis reactions.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’-3’)</th>
<th>Description</th>
<th>DNA construct created</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iga Bam start forward</td>
<td>CGG GAT CCC GAT GCC AGG GGG TCT A</td>
<td>Binds non-coding strand of Iga starting 2 amino acids N-terminal to the start codon. Creates a Bam HI restriction enzyme site.</td>
<td>Iga trunc</td>
</tr>
<tr>
<td>Iga trunc Eco reverse</td>
<td>GGA ATT CCC TAG TCA TCT GGC ATG TC</td>
<td>Binds coding strand of Iga and creates a stop codon and an EcoRI restriction enzyme site.</td>
<td>Iga trunc</td>
</tr>
<tr>
<td>Igβ Bam start forward</td>
<td>CGG GAT CCC GAT GGC CAC ACT GGT G</td>
<td>Binds non-coding strand of Igβ starting 2 amino acids N-terminal to the start codon. Creates a Bam HI restriction enzyme site.</td>
<td>Igβ trunc</td>
</tr>
<tr>
<td>Igβ trunc Eco reverse</td>
<td>GGA ATT CCC TAG GTG TGA TCT TCC TC</td>
<td>Binds coding strand of Igβ and creates a stop codon and an EcoRI restriction enzyme site.</td>
<td>Igβ trunc</td>
</tr>
<tr>
<td>Iga no DCSM forward</td>
<td>GGC CTG AAC CTT GAT TAT GAG GAC ATC TCC</td>
<td>Binds non-coding strand of Iga excluding the 12 nucleotides encoding DCSM amino acids.</td>
<td>Iga no DCSM</td>
</tr>
<tr>
<td>Iga no DCSM reverse</td>
<td>GGA GAT GTC CTC ATA ATC AAG GTT CAG GCC</td>
<td>Binds coding strand of Iga excluding the 12 nucleotides encoding DCSM amino acids.</td>
<td>Iga no DCSM</td>
</tr>
<tr>
<td>Iga QTAT forward</td>
<td>GAA CCT TGA TCA GAC AGC CAC CTA TGA GGA CAT C</td>
<td>Binds non-coding strand of Iga and includes a 12 nucleotide insertion encoding the amino acids QTAT.</td>
<td>Iga QTAT</td>
</tr>
<tr>
<td>Iga QTAT reverse</td>
<td>GAT GTC CTC ATA GGT GGC TGT CTG ATC AAG GTT C</td>
<td>Binds coding strand of Iga and includes a 12 nucleotide insertion encoding the amino acids QTAT.</td>
<td>Iga QTAT</td>
</tr>
</tbody>
</table>
2.1.3.2 Construction of pWZL-blast3-Igβtrunc

The truncated Igβ construct (Igβ trunc) (Table 2.1) was made using polymerase chain reaction (PCR) using pMIGR1-Igβ as a template. The primers shown in Table 2.2 were designed to amplify the coding region of Igβ from the start codon up to and including the 3 nucleotides encoding the amino acid immediately preceding the most N-terminal tyrosine residue (Y195). A flanking BamHI restriction enzyme site was also added N-terminal to the start codon and a flanking EcoRI restriction enzyme site was added C-terminal to the coding region bound by the reverse primer. Following PCR, the resulting fragments were concentrated, then inserted into pWZL-blast3 between the EcoRI and BamHI restriction digest sites and successful ligations were selected through transformation into competent bacteria (Chapter 2.2.4). The truncation was confirmed by sequencing.

2.1.3.3 Construction of pCMVmb1-αQTAT

The Igα construct with the DCSM residues mutated to QTAT (αQTAT) (Table 2.1) was made by performing successive rounds of site-directed mutagenesis using pCMVmb1 as a template. The primers shown in Table 2.2 were used to first delete the 12 nucleotides encoding DCSM and successful reactions were selected through transformation into XL-1 Blue supercompetent bacteria. The DNA isolated from these bacterial clones was amplified and used as a template for the second round of site-directed mutagenesis. The primers shown in Table 2.2 were used to insert the nucleotides encoding the amino acid residues QTAT into the site of the deletion from the previous round. Again, successful reactions were selected through transformation into XL-1 Blue supercompetent and the mutation was confirmed by sequencing.
**2.1.3.4 Construction of pMIGR1-IgβDCSM**

The Igβ construct with the QTAT residues mutated to DCSM (βDCSM) (Table 2.1) was made by May Dang-Lawson through successive rounds of site-directed mutagenesis using pMIGR1-Igβ as a template. The primers shown in Table 2.2 were used to first delete the 12 nucleotides encoding QTAT and successful reactions were selected through transformation into XL-1 Blue supercompetent bacteria. The DNA isolated from these bacterial clones was amplified and used as a template for the second round of site-directed mutagenesis. The primers shown in Table 2.2 were used to insert the nucleotides encoding the amino acid residues DCSM into the site of the deletion from the previous round. Again, successful reactions were selected through transformation into XL-1 Blue supercompetent bacteria and the mutation was confirmed by sequencing.

**2.1.4 Cell Lines**

The J558 tissue culture B cell line was derived from a murine plasmacytoma (Carson and Weigert, 1973; Cesari and Weigert, 1973) of which there are several variants. The J558μm3 cell line which contains a full mIgM BCR, as well as the J558 15-25 cell line which is deficient in the Igα chain, were gifts from Dr. Louis Justement (University of Alabama, Birmingham, Alabama). The J558L cell line, which is deficient in both the Igμ and Igα chains, was a gift from Dr. Marcus Clark (University of Chicago, Chicago, Illinois) and the subcloning of this cell line to isolate a clone also deficient in the Igβ chain was performed by S. Machtaler and C. Jang (unpublished results).
2.2 Molecular Biology Techniques

2.2.1 Restriction endonuclease digestion

Restriction enzymes purchased from Invitrogen Life Technologies (Burlington, Ontario) or New England Biolabs (Pickering, Ontario) were added to PCR-amplified DNA fragments or plasmids according to the manufacturer’s instructions and digested for 1-2 hours (hrs) at 37°C. The resultant digested DNA was then be used for ligation following purification.

2.2.2 Purification and concentration of DNA

In order to isolate DNA from solutions with the purpose of performing further molecular biology techniques, 100 µl of 7.5 M ammonium acetate (Fisher Scientific, Ottawa, Ontario) was added to 200 µl of DNA mix, followed by 760 µl of ice cold 95% ethanol. The solution was incubated at -20°C for 30 minutes (min) prior to centrifugation at 15000 revolutions per min (rpm) for 20 min. The resulting pellet was washed once with 95% ethanol then resuspended in 50 µl of distilled water for use in further procedures.

2.2.3 DNA ligation

DNA ligation of digested PCR-amplified fragments and vectors was performed using T4 DNA ligase (Invitrogen Life Technologies) used according to the manufacturer’s instructions. The PCR-amplified fragments and vector were mixed in a 4:1 ratio of mole ends. Ligations were performed at 37°C for 1 hr then were immediately used to transform competent bacteria (Chapter 2.2.4).
2.2.4 Bacterial transformation

The competent strains of *Escheria coli* (*E. coli*), XL-1 Blue Supercompetent Cells (Stratagene, La Jolla, California), were transformed according to the manufacturer’s instructions. The competent *E. coli* strain DH5α (Invitrogen Life Technologies) was used at 50 µl per reaction and transformed with 100-500 ng of the appropriate DNA in distilled water. The mixture was incubated on ice for 30 min then heat shocked for 45 seconds (sec) at 42°C. The reaction was incubated on ice for 2 min prior to the addition of 900 µl of warm Luria Bertani (LB) broth (10 g/L sodium chloride (NaCl) (Fisher Scientific), 5 g/L Bacto Yeast Extract and 10 g/L Bacto Tryptone (both BD Biosciences, Palo Alto, California)) and incubation at 37°C, shaking at 225 rpm for 1 hr. Finally, 200 µl of the mixture was plated on LB agar plates (LB agar, 15 g/L Bacto Agar (BD Biosciences) with 100 µg/ml ampicillin (Sigma Aldrich, St. Louis, Missouri)) and incubated at 37°C overnight.

2.2.5 DNA preparation

A single bacterial colony was used to inoculate 4 ml, 50 ml, or 150 ml of LB broth with 100 µg/ml ampicillin and was incubated at 37°C overnight while shaking at 225 rpm. The resultant bacterial culture was then used to purify small, intermediate, or large volumes of plasmid DNA using the GenElute Plasmid Miniprep Kit (Sigma Aldrich), the PureLink HiPure Plasmid Midiprep Kit (Invitrogen Life Technologies), or the GenElute HP Plasmid Maxiprep Kit (Sigma Aldrich), respectively according to the manufacturer’s instructions.
2.2.6 Polymerase chain reaction (PCR)

PCR was performed using PuRe Taq Ready-To-Go PCR beads (GE Healthcare, Piscataway, New Jersey) according to the manufacturer’s instructions with 10 pmol of each primer and 5 ng of template DNA. The reactions were placed in a DNA Thermal Cycler (Perkin Elmer Cetus, Woodbridge, Ontario) and run at 95°C for 45 sec, 55°C for 2 min, and 72°C for 2 min, for 35 cycles.

2.2.7 Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and reactions were run on a DNA Thermal Cycler (Perkin Elmer Cetus) according to the manufacturer’s instructions. Primers were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa), and mutations were confirmed through DNA sequencing (Nucleic Acid Protein Service Unit, Vancouver, BC) of plasmid DNA purified from transformed XL1-Blue Supercompetent Cells (Stratagene).

2.2.8 Agarose gel electrophoresis

Whole plasmid DNA, restriction endonuclease digested DNA, and PCR-amplified DNA fragments were electrophoresed on a 1% agarose gel (Invitrogen Life Technologies) in Tris-buffered ethylene diamine tetraacetic acid (EDTA) (90 mM Tris-HCl pH 8.2, 90 mM boric acid (Fisher Scientific) 2 mM EDTA (Fisher Scientific)) with 0.1% SYBRsafe (Invitrogen Molecular Probes) at 100V for 40-50 min. Samples were mixed with an appropriate amount of DNA sample preparation buffer (0.04% bromophenol blue (Sigma Aldrich), 0.04% xylene cyanol FF,
10% sucrose (Fisher Scientific)) prior to loading into wells. The 1kb DNA ladder (Invitrogen Life Technologies) was used as a size standard and DNA bands were imaged with ultraviolet light in an Alpha Imager EC MultiImage Light Cabinet (Alpha Innotech, San Leandro, California) using Alpha Imager software (Alpha Innotech).

2.3 Tissue culture techniques

2.3.1 Culture of cells and drug selection

J558 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen Life Technologies) with the following supplements: 10% heat inactivated fetal bovine serum (FBS) (Invitrogen Life Technologies or CanSera, PAA Laboratories, Etobicoke, Ontario), 4.5 g/L glucose, 2 mM L-glutamine (Invitrogen Life Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies), 50 u/ml penicillin (Invitrogen Life Technologies), 50 μg/ml streptomycin (Invitrogen Life Technologies), 50 μM β-mercaptoethanol (2-βME) (Sigma Aldrich). All cells were maintained in an incubator (Thermo Forma, Marietta, Ohio) at 37°C in a 5% CO₂ atmosphere, and passaged every 2 or 3 days as needed. For long term storage, cells were frozen in liquid nitrogen in heat inactivated FBS with 10% dimethyl sulfoxide (DMSO) (Fisher Scientific). Selection for drug resistant cells was achieved by the transfer and growth of transfected cells in complete RPMI 1640 with either 0.5 mg/ml G418 (Invitrogen Life Technologies) or 4 μg/ml Blasticidin S (Invitrogen Life Technologies) depending on the vector. Cells were transferred 24 hrs post-transfection.
2.3.2 DNA transfection and enrichment

J558 cell lines were transfected with DNA constructs using Nucleofection®. Recipient cells were resuspended with 2 µg of the appropriate DNA construct and solutions from the Amaxa Nucleofection Kit T® (Amaxa Biosystems, Gaithersburg, Maryland) according to the manufacturer’s instructions. The electrical parameters from the G-016 program in the Amaxa Nucleofector Device® (Amaxa Biosystems) were used. Drug resistant populations were enriched for BCR-expressing cells using fluorescent activated cell sorting (FACS). Cells were resuspended in 0.5 ml PBS with 2% FBS and labeled with 2 µg Alexa Fluor 633-conjugated goat anti-mouse IgM (µ chain specific). Sorting was performed on a BD FACS Aria (BD Biosciences) by the Jeffrey Duenas, UBC FACS Facility (University of British Columbia, Vancouver, BC).

J558m3 cells and WEHI 231 cells transfected with Cx43-GFP by incubation of 0.5x10^6 cells with 1 ml retroviral supernatant from 293GPG cells transfected with NAP2-Cx43GFP (a gift from Christian Naus and John Bechberger, Department of Cellular and Physiological Sciences, UBC) and 1 ml complete RPMI 1640. Cells were resupended in fresh RPMI 1640 24 hrs later. Successful transfectants were isolated from the untransfected population by FACS by virtue of their GFP expression.

2.3.3 Cell stimulation

For samples used in western blotting, 0.5 x 10^6 cells per sample were resuspended in quinsaline (25 mM sodium Hepes pH 7.4 (Invitrogen Life Technologies), 125 mM NaCl, 5 mM KCl (Sigma Aldrich), 1 mM CaCl2 (Fisher Scientific), 1 mM Na2HPO4 (Fisher Scientific), 0.5 mM
MgSO₄ (BDH Chemicals, Toronto, Ontario), 1g/L glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-βME) at 37°C in a 5% CO₂ atmosphere. Cells were stimulated with 20 μg/ml of goat anti-mouse IgM for the appropriate amount of time. Reactions were stopped with 1 ml of cold phosphate buffered saline (PBS) (1.5 mM NaCl, 1.9 mM Na₂HPO₄ · H₂O, 8.4 mM Na₂HPO₄ pH 7.2) with 1 mM sodium pervanadate (Na₃VO₄) (Sigma Aldrich) and washed once with the same solution.

For samples used in flow cytometry or confocal microscopy assays, an appropriate number of cells were washed once with (PBS) then resuspended in 500 μl of ice cold PBS with 2% heat inactivated FBS. Goat anti-mouse IgM was added to the cells in a 20 μg/ml concentration and cells were incubated on ice for 30 min. Following one rinse in ice cold PBS to remove any unbound Ab, cells were resuspended in 100 μl of ice cold complete RPMI 1640. Cells were stimulated at 37°C for the appropriate amount of time then fixed with 500 μl of 8% paraformaldehyde (Sigma Aldrich or Electron Microscopy Sciences, Hatfield, Pennsylvania) then incubated on ice for 15 min. Cells were then washed once with PBS then resuspended in 100 μl of the same. If the experiment was assaying the signaling capabilities, cells were transferred to complete RPMI 1640 with low serum (1% FBS) at least 4 hrs prior to stimulation.

2.4 Biochemical Procedures

2.4.1 Cell lysis and preparation of samples

Cells were lysed on ice while shaking for 15 min with an appropriate amount of Triton X-100 lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% Triton X-100 (Fisher Scientific), 2 mM EDTA) with 1 mM Na₃VO₄, 1 mM Pepstatin A (Sigma Aldrich), 1mM phenylmethylsulfonyl
fluoride (PMSF) (Roche Diagnostics, Indianapolis, Indiana), 1 µg/ml Aprotinin (Roche Diagnostics), and 10 µg/ml Leupeptin (Roche Diagnostics). Samples were then centrifuged at 14000 rpm at 4°C for 10 min to remove cellular and the protein concentration of each sample was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnologies, Rockford, Illinois) according to the manufacturer’s instructions. Lysates were mixed with an appropriate amount of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 4% glycerol (Fisher Scientific), 2.5% SDS (Bio-Rad Laboratories, Hercules, California), 0.02% bromophenol blue, 100 mM dithiotheitol (Sigma Aldrich)) and boiled for 5 min or were stored at -20°C for later use.

2.4.2 SDS-PAGE and western immunoblotting

Samples containing total cell lysate and SDS-PAGE reducing sample buffer were heated in a boiling water bath for 5 minutes prior to loading into wells in a 1.5 mm, 10% or 12% polyacrylamide (Bio-Rad Laboratories) mini-gel along with 4 µl of BenchMark Prestained Protein Ladder (Invitrogen Life Technologies). The gels were prepared according to standard lab procedures (Dylke et al., 2007) and run in a dual vertical mini-gel apparatus (CBS Scientific, Del Mar, California) in running buffer (50 mM Tris, 0.4 M glycine (Fisher Scientific), 0.1% SDS) at a constant voltage of 150 V for 1-2 hrs as needed. Electrophoresed proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (Bio-Rad Laboratories) using a Transblotter Transfer Apparatus (Bio-Rad Laboratories) filled with transfer buffer (20 mM Tris-HCl, 150 mM glycine, 20% methanol), at a constant voltage of 100 V for 1.5-2 hrs (Maniatis, 1989). Successful transfer of protein was confirmed by the presence of the Benchmark Prestained Protein Ladder (Invitrogen Life Technologies) on the filter or by Ponceau S (Sigma Aldrich) staining.
Nitrocellulose filters were blocked for 1 hr in a solution of tris-buffered saline (TBS) (with 5% skim milk powder (Safeway Canada, Calgary, Alberta) or bovine serum albumin (BSA) (MP Biomedicals, Solon, Ohio) while shaking for 1 hr at 22°C. The blocked filters were then incubated overnight with the appropriate primary antibody (diluted 1:500-1:2000 in TBS with 5% skim milk powder or BSA) while shaking gently at 4°C. Removal of the primary Ab was followed by 4 successive 15 min washes of the filter with TBS with 0.1% Tween 20 (Fisher Scientific) (TBST) at 22°C. Filters were then incubated with the appropriate HRP-conjugated secondary Ab (diluted 1:10000 in TBST with 5% skim milk powder) for 1 hr while shaking gently at 22°C. This was followed by a second round of 4, 15 min washes with TBST. Filters were then incubated in 10 ml of enhanced chemiluminescence (ECL) reagent (Pierce Biotechnologies) and exposed to Kodak X-Omat Blue XB-1 autoradiography film (Mandel Scientific, Guelph, Ontario). Films were developed using a Kodak X-OMAT 1000A Processor (MedTec Marketing Group, Burnaby, BC).

If needed, filters were reprobed with a different primary Ab following a 20 min stripping incubation in TBS pH 2.0 while gently shaking at 22°C, followed by a 5 min incubation with TBS pH 8.0 prior to immunoblotting according to the protocol outlined above.

2.5 Flow cytometry

2.5.1 Sample preparation and staining

To assess the cell surface expression, cells were stained by resuspension in PBS with 2% FBS and incubation on ice with 2 μg of Alexa Fluor 633-conjugated goat anti-mouse IgM for 30 min.
Cell were then washed once in cold PBS with 2% FBS and resuspended in 500 µl of the same buffer.

To assess mutant BCR internalization rates, cells were first stimulated with goat-anti mouse IgM as previously described (Chapter 2.3.3). Following fixation and subsequent rinses, BCR complexes remaining on the cell surface were stained with a fluorescently labeled secondary Ab against the stimulating antibody in a 1:500 dilution. Cells were washed twice with incubation buffer (PBS with 1% BSA) prior to resuspension in 500 µl of FACS buffer (PBS, 2% FBS, 2 mM EDTA, 0.01% sodium azide).

To assess the signaling capabilities of cells with mutant BCRs, stimulation with Alexa Fluor 633-conjugated anti IgM was performed as previously described (Chapter 2.3.3). Following fixation and subsequent rinses, cells were permeabilized with 900 µl of ice cold methanol on ice while shaking for 15 min. Cells were washed twice with incubation buffer then stained with the appropriate primary antibody for 30 min, then 500 µl of incubation buffer was added and cells were incubated overnight at 4°C. The next day, another wash with incubation buffer was followed by a 30 min incubation with the appropriate fluorescently labeled secondary Ab. Cells were washed twice with incubation buffer and resuspended in 500 µl of FACS buffer (Figure 2.1).

2.5.2 Data collection and analysis

Samples were processed using a BD LSRII Flow Cytometer (BD Biosciences) and BD FACS Diva software (BD Biosciences) using the appropriate lasers according to the secondary antibodies used. Data was analyzed using FlowJo Flow Cytometry Analysis Software (Tree Star
Figure 2.1. Schematic representation of BCR internalization assay.
Cells were incubated with goat anti-mouse IgM on ice for 30 min. Following rinsing to wash away excess Ab, cells were either kept on ice (unstimulated) or incubated at 37°C to allow cross-linking and stimulation to occur. All reactions were stopped with paraformaldehyde and cells were incubated in a fluorescently-conjugated secondary Ab without permeabilization in order to detect BCRs remaining on the cell surface. Data was collected by flow cytometry and MFI of stimulated samples were normalized with the appropriate unstimulated control to determine the fraction of BCR remaining on the cell surface, an indirect measurement of BCR internalization.
Inc., Ashland, Oregon). For internalization experiments, cells with BCRs on the surface were gated on, and the change in mean fluorescent intensity (MFI) was determined by normalizing values to the MFI of the unstimulated sample. For signaling experiments, cells with BCRs on the cell surface were gated on, and that population was analyzed for a change in MFI of the appropriate fluorescently labeled phosphorylated proteins. MFI values of each timepoint were normalized to the MFI of the unstimulated sample.

2.6 Confocal Microscopy

2.6.1 Sample preparation and staining

An appropriate number of cells were stimulated in suspension with Alexa Fluor 633-conjugated goat anti-mouse IgM and fixed as previously described (Chapter 2.3.3). Incubation with a solution of PBS with 0.5% Triton X-100 for 15 min on ice was performed to permeabilize cells followed by 2 washes with a solution of PBS with 3% BSA. Cells were incubated in a blocking solution of PBS with 3% BSA for 45 min then with appropriate dilutions of primary Ab against intracellular compartments in PBS with 3% BSA for 1.5 hrs at 22°C or overnight at 4°C. Cells were washed 3 times with PBS with 3% BSA then incubated with the appropriate secondary Ab in a 1:500 dilution for 45 min. This was followed by 3 more washes prior to resuspension of samples in a solution of PBS with 5 mM EDTA in 35 mm glass bottom microwell dishes (MatTek, Ashland, Massachusetts). All staining was performed in suspension.
2.6.2 Image acquisition and analysis

Images were acquired using the 60x or 100x planApochromat objectives (NA 1.35) of the Olympus FV1000 confocal microscope. Colocalization between BCR and transferrin receptor, and between BCR and LAMP-1 was determined using Pearson’s product moment coefficients generated by the Fluoview 10-ASW 1.6 software program. This statistic is a measure of the correlation between 2 variables and assumes they are independent of each other. The correlation coefficient may have any value between -1.0 and +1.0, with -1.0 indicating that the 2 variables have a negative linear relationship, a value of 0 indicating that there is no correlation and a value of 1.0 indicating that there is a positive linear relationship. This means a positive value measures co-localization with a value closer to +1.0 indicating more co-localization.
3.1 The effect of Igα and Igβ cytoplasmic domain mutation on ligand induced BCR internalization

3.1.1 Rationale

The presentation of Ag by Ag presenting cells, such as B lymphocytes, to T lymphocytes is an important part of eliciting an immune response. Internalization of BCR-Ag complexes is the necessary first step to that end and it is important to understand what is both necessary and sufficient for this process to occur.

Through the use of chimeric receptors, having the extracellular and transmembrane domains of the PDGF receptor but the cytoplasmic domains of either Igα or Igβ, it is now known that both the Igα and the Igβ cytoplasmic domains contain internalization signals (Lankar et al., 1998) though the precise identities of these signals have yet to be elucidated. However, because of the chimeric receptor system, each cytoplasmic domain was examined separately. What is lacking in our understanding, is the relative contribution each cytoplasmic domain makes towards the internalization of a complete BCR (made of mIgM, Igα and Igβ), and the identification of any functionally important regions within both cytoplasmic domains. It also speaks to our understanding of the underlying mechanism of endocytosis and our understanding of how the structure of the BCR is involved in this process.
In order to address these issues, we established a cell system in which a panel of mutant BCRs, containing Igα and Igβ proteins with mutated cytoplasmic domains, were expressed in different combinations. Using soluble Ab to mimic Ag binding, the internalization of the mutant BCRs was assayed as described (Chapter 2.5.1 and Figure 2.1) and compared to the internalization of WT BCRs for changes or an abrogation of this process. If found, these changes would show either the necessity or sufficiency of certain regions within the cytoplasmic domains for receptor internalization and this is important because the precise signals on both cytoplasmic domains have yet to be identified.

3.1.2 Stable expression of mutant BCR constructs in a J558 cell line

The stable populations of cells expressing BCRs with mutated Igα/βs, an Igα cytoplasmic truncation (atrunc) or 2 cytoplasmic Igβ domains (Cβ) were created by transfecting the J558 15-25 cell line (mIgM, Igβ, missing Igα) with the appropriate Igα construct (Figure 3.1), followed by selection in Blasticidin S (Chapter 2.3.1). BCR surface expression was verified by flow cytometry (Chapter 2.5.1). Stable expression of the mutant Igα proteins, as well as all other BCR components, was verified by western immunoblots (Figure 3.2) and levels of BCR surface expression was monitored periodically by flow cytometry. This resulted in cell lines expressing BCRs on the cell surface with a single Igβ cytoplasmic domain (atrunc) and 2 Igβ cytoplasmic domains (Cβ), respectively.

3.1.3 Transient expression of mutant BCR constructs in a J558 cell line

Since obtaining stable populations of cells expressing the remaining Igα and Igβ mutant BCRs was not feasible, a transient expression approach was taken. The cell line J558 C19.5.5 (S.
Figure 3.1. Schematic representation of Igα and Igβ cytoplasmic domain combinations.

(A) A schematic representation of a BCR with the Igα chain highlighted in orange and the Igβ chain highlighted in green. (B) Combinations of mutant Igα and Igβ cytoplasmic tails of interest. Shown are representations of the mutated cytoplasmic domains of each chain, extracellular and transmembrane domains are as shown in (A). Igβ trunc and Igα trunc have cytoplasmic truncations of the indicated chain immediately preceding Y176 and Y195 respectively. Igα QTAT has a 4 amino acid switch within the ITAM from the wild type Igα DCSM to the QTAT normally found in the corresponding region in Igβ. Igβ DCSM is the opposite. atrunc βtrunc refers to cytoplasmic truncations in both chains and Cα and Cβ refers to a cytoplasmic swap of one of the chains.
Figure 3.2. Stable expression of mutant Igα constructs in J558 15-25 cells.
Schematic diagram of mutant BCR with Igα shown in orange and Igβ shown in green (left) and confirmation of expression by western immunoblot using the appropriate J558 cell lines (right). μm3 cells express all BCR components, Igμ, Igλ, Igα, Igβ; C19.5.5 cells express only Igλ and are deficient in Igμ, Igα, and Igβ; L cells express Igλ and Igβ and are deficient in Igμ and Igα; 15-25 cells express Igμ, Igλ, and Igβ and are deficient in Igα. (A) αtrunc cells express a truncated form of Igα and all other WT BCR components. (B) Cβ cells express an Igα with a swap for the cytoplasmic domain of Igβ and all other WT BCR components.
Machtaler and C. Jang, unpublished results), which expresses only the Igλ light chain, was transfected by nucleofection (Chapter 2.3.2) with the Igμ heavy chain gene and cells stably expressing this construct were selected through culture in media containing G418 and verified by western blot analysis (Figure 3.3a). This new cell line, J558 µ (mIgM positive, intracellular only), was used as a recipient for Igα and Igβ mutant constructs.

In order to create the remaining BCR mutant cell lines (Figure 3.1), the appropriate Igα construct and Igβ construct were transfected into J558 µ. It is known that incomplete mIgM BCRs are retained within the ER (Melnick et al., 1992; Melnick et al., 1994) and that only a full 4 chain BCR, where Igα and Igβ heterodimerize in the extracellular and transmembrane regions, is expressed on the cell surface (Dylke et al., 2007). This allows for the distinction between the successfully double transfected population and the untransfected or single transfected population. Analysis by flow cytometry showed BCR surface expression in one of the double mutant pairings assessed (Figure 3.3b) indicating successful double transfection. This was the same in all double mutant pairings assessed (Figure A1).

3.1.4 The effect of cytoplasmic domain truncation on ligand induced BCR internalization

Both the Igα cytoplasmic domain and the Igβ cytoplasmic domain contain internalization signals and each alone is sufficient for internalization (Cassard et al., 1996). There is a general consensus that the ITAMS, as well as the non-ITAM tyrosine residues in the case of Igα, are functionally important regions of the cytoplasmic tails with respect to signaling (Cassard et al., 1996; Hou et al., 2006; Siemasko et al., 2002). With that in mind, the Igα truncation was made immediately preceding the first non-ITAM tyrosine residue (Y176) and the Igβ truncation was made immediately preceding the first ITAM tyrosine residue (Y195), leaving 16 and 24

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Figure 3.3. Establishment of an expression system for Igα and Igβ mutant constructs.
(A) Creation of a recipient cell line J558 μ for Igα and Igβ mutant constructs. J558 C19.5.5 cells, normally expressing only Igλ, were transfected with an Igμ cDNA-containing vector. Transfectants were selected through growth in G418 prior to immunoblotting with anti-Igμ antibodies and any subsequent transfection. (B) Flow cytometry data showing BCR surface expression in J558 μ recipient cells (grey) and in Igα and Igβ transfected cells (red). Cells were incubated with goat anti-mouse IgM on ice for 30 minutes and rinsed once prior to fixation with 8% paraformaldehyde. Cells were then incubated with Alexa Fluor 488-conjugated rabbit anti-goat IgG for 30 minutes then resuspended in FACS buffer. Data is expressed as a histogram with the x-axis reflecting the fluorescent intensity of the cells stained for BCR.
cytoplasmic amino acids respectively of the 61 aa Igα cytoplasmic domain (Sakaguchi et al., 1988; Kashiwamura et al., 1990; Flaswinkel and Reth, 1992) and the 48 aa Igβ cytoplasmic domain (Hermanson et al., 1988).

Clustering BCRs with WT Igα and Igβ chains resulted in the rapid internalization of the surface receptors with the amount slowly decreasing over the course of 60 minutes (Figure 3.4, WT shown in black). Internalization of receptors with truncations in both chains (αtrunc βtrunc) was essentially abrogated (Figure 3.4, αtrunc βtrunc shown in grey) confirming that the sequences in the truncated regions are indeed necessary for receptor internalization. To determine the contribution made by each individual cytoplasmic domain to the internalization of a full 4 chain BCR, constructs with cytoplasmic truncations were used in tandem with the complementary WT chain. Truncation of the Igβ cytoplasmic domain (βtrunc) showed that the Igα cytoplasmic domain alone is sufficient to allow BCR internalization to occur at approximately 72% of the level seen with the WT receptor when the fraction of surface BCR remaining at the cell surface after 60 min was compared (Figure 3.4 and Figure 3.7, βtrunc shown in orange). Conversely, truncation of the Igα cytoplasmic domain (αtrunc) resulted in a severe defect in BCR internalization, showing that the Igβ domain alone is sufficient to allow BCR internalization to occur at only 20% of the level seen with WT receptors (Figure 3.4 and Figure 3.7, αtrunc shown in green). This shows that the Igα cytoplasmic domain makes a larger contribution to receptor internalization than the Igβ cytoplasmic domain.
Figure 3.4. The effect of cytoplasmic truncation on BCR internalization.
(A) Schematic representation of the BCR mutants assessed with Igα shown in orange and Igβ shown in green. (B) Cells were stimulated at 37°C for the indicated time with goat anti-mouse IgM, followed by fixation, and incubation with a fluorescently conjugated rabbit anti-goat secondary Ab. For details see Chapter 2.3.3 and Chapter 2.5.1. Data was collected by flow cytometry. Graph represents data from n≥3 independent experiments.
3.1.5 The effect of cytoplasmic domain duplication on ligand induced BCR internalization

In order to further investigate the roles of the Iga and Igβ cytoplasmic domains, constructs with cytoplasmic swaps were used. The BCRs were still assembled and trafficked normally because of their WT heterodimerization in the extracellular and transmembrane domains, but the cytoplasmic pairing was a homodimer: 2 Iga cytoplasmic domains (Cα) or 2 Igβ cytoplasmic domains (Cβ) (Figure 3.5).

Cells with BCRs with a single Igβ cytoplasmic domain (atrunc) were severely defective in their ability to internalize BCRs following receptor stimulation (Figure 3.5, atrunc shown in green). The addition of a second Igβ cytoplasmic domain to BCRs was sufficient to rescue the internalization defect, with the receptors being internalized at 89% of the levels seen in WT BCRs (Figure 3.5 and Figure 3.7, Cβ shown in yellow). Conversely, the addition of a second Iga cytoplasmic domain to BCRs (Cα) caused the receptor internalization to occur at a greater rate and magnitude compared to WT receptors. Internalization occurred at 113% of the level seen with WT BCRs (Figure 3.5 and Figure 3.7, Cα shown in light blue), with the single Iga domain already sufficient to allow for 72% of normal internalization levels. These data further strengthen the argument that the Iga cytoplasmic domain makes a larger relative contribution to BCR internalization than does the Igβ cytoplasmic domain.

3.1.6 The effect of cytoplasmic domain mutation on ligand induced BCR internalization

To determine whether the 4 amino acid motifs within the ITAMs of each Iga and Igβ cytoplasmic domain (Cassard et al., 1996) contribute to the ability of the chain to cause internalization, they were each mutated to the 4 amino acids found in the opposite chain. That is,
Figure 3.5. The effect of cytoplasmic duplication on BCR internalization.
(A) Schematic representation of the cytoplasmic mutants assessed with Igα shown in orange and Igβ shown in green. (B) Cells were stimulated at 37°C for the indicated time with goat anti-mouse IgM, followed by fixation, and incubation with a fluorescently conjugated rabbit anti-goat secondary Ab. For details see Chapter 2.3.3 and Chapter 2.5.1. Data was collected by flow cytometry. Graph represents data from n≥3 independent experiments.
the DCSM normally found on Igα was changed to the QTAT normally found on Igβ (αQTAT),
and vice versa (βDCSM), and internalization of these BCRs was compared to the appropriate
cytoplasmic truncated BCRs, βtrunc and αtrunc respectively.

Mutation of the DCSM residues within the Igα ITAM to QTAT (αQTAT) did not affect the rate
or magnitude of BCR internalization compared to the internalization of BCRs with the single Igα
chain (βtrunc) (Figure 3.6, αQTAT shown in blue, βtrunc shown in orange). The degree of
internalization of βtrunc and αQTAT was essentially identical. However the single Igβ
cytoplasmic domain (αtrunc), which has a severely aberrant phenotype with only 20% of WT
internalization levels, was rescued by mutation of the QTAT residues to DCSM residues
normally found on Igα (βDCSM) (Figure 3.6, βDCSM shown in purple; αtrunc shown in green).
Internalization occurred at 88% compared to levels seen with WT BCRs (Figure 3.6 and Figure
3.7). This shows that the DCSM residues within the Igα ITAM are not necessary, but are
sufficient to promote internalization upon BCR stimulation.

3.2 The effect of Igα and Igβ cytoplasmic domain mutation on BCR trafficking

3.2.1 Rationale

Following internalization, the trafficking of the BCRs and Ag to endocytic processing
compartments is a necessary precursor to peptide loading and presentation on MHC II. Since the
Igα and Igβ cytoplasmic domains have been shown to make differing contributions to BCR
internalization (Chapter 3.1), the logical next question to ask is whether the cytoplasmic domains
also make differing contributions to BCR trafficking to intracellular compartments.
Figure 3.6. The effect of cytoplasmic mutation on BCR internalization.
(A) Schematic representation of the cytoplasmic mutants assessed with Igα shown in orange and Igβ shown in green. (B) Cells were stimulated at 37°C for the indicated time with goat anti-mouse IgM, followed by fixation, and incubation with a fluorescently conjugated rabbit or donkey anti-goat secondary Ab. For details see Chapter 2.3.3 and Chapter 2.5.1. Data was collected by flow cytometry. Graph represents data from n≥3 independent experiments.
Figure 3.7. Comparison between WT and mutant BCR internalization after 60 minutes.
(A) Schematic representations of the cytoplasmic domains of the BCRs assessed with Igα shown in orange and Igβ shown in green. (B) Level of internalization for each mutant after 60 min. Values were normalized to the level of internalization seen in cells with WT BCRs and expressed as a percentage.
Through the use of chimeric receptors, it is known that both the Igα and the Igβ cytoplasmic domains are sufficient for antigen presentation (Lankar et al., 1998; Siemasko et al., 2002) but that the Igα cytoplasmic domain is required for efficient antigen presentation (Lankar et al., 1998). Past investigations of the relationship between BCR structure and intracellular trafficking have focused on the location of the BCR with respect to MHC II (Bonnerot et al., 1995) as opposed to the location of the BCR relative to intracellular compartments.

Canonical endocytosis pathways such as that of the low density lipoprotein (LDL) receptor and the transferrin receptor (TfnR) tell us that following internalization, proteins proceeding through the canonical endocytic pathway move from the plasma membrane, and appear sequentially in early and late endosomes, then in lysosomes. The first intracellular compartments in which the internalized receptors accumulate are early endosomes. These compartments can be identified through the characteristic presence of molecular markers such as the GTPase Rab5 (Zahraoui et al., 1989; Chavrier et al., 1990), early endosome antigen-1 (EEA1) (Mu et al., 1995), and transferrin receptors (TfnR) used in constitutive iron uptake (Harding et al., 1983). Lysosomal compartments are commonly identified by the presence of LAMP-1 (Chen et al., 1988). This is the pathway followed by the BCR upon internalization prior to peptide loading onto MHC II.

3.2.2 The effect of cytoplasmic domain mutations on BCR trafficking to early endosomes

The trafficking of internalized BCR to early endosomes was assessed through the co-immunostaining of BCR and TfnR, and the co-localization of the two was measured using Pearson’s coefficients. The colocalization between BCR and TfnR in WT cells (Figure 3.8) was then compared to Igα and Igβ cytoplasmic truncations (Figures 3.9 and 3.10), Igα and Igβ cytoplasmic duplications (Figures 3.11 and 3.12), and Igα and Igβ cytoplasmic mutations.
Figure 3.8. WT BCR trafficking to intracellular compartments.

(A) Schematic representation of a WT BCR with Igα shown in orange and Igβ shown in green.

(B) Timecourse of WT BCR trafficking to early endosomal (TfnR) and lysosomal (LAMP-1) compartments. Cells were stimulated with Alexa Fluor 633-conjugated anti-mouse IgM for the time indicated prior to fixation with paraformaldehyde. Cells were then permeabilized and incubated with Abs against TfnR and LAMP-1. Scale bar: 10 μm. (C) Measurement of BCR co-localization with TfnR using Pearson’s coefficients. (D) Measurement of BCR co-localization with LAMP-1 using Pearson’s coefficients, for details see Chapter 2.6.2.
Figure 3.9. The effect of an Igα cytoplasmic truncation on BCR trafficking to intracellular compartments. (A) Schematic representation of a BCR with a cytoplasmic Igα truncation (atrunc) with Igα shown in orange and Igβ shown in green. (B) Timecourse of WT BCR trafficking to early endosomal (TfnR) and lysosomal (LAMP-1) compartments. Cells were stimulated with Alexa Fluor 633-conjugated anti-mouse IgM for the time indicated prior to fixation with paraformaldehyde. Cells were then permeabilized and incubated with Abs against TfnR and LAMP-1. Scale bar: 10 μm. (C) Measurement of BCR co-localization with TfnR using Pearson’s coefficients. (D) Measurement of BCR co-localization with LAMP-1 using Pearson’s coefficients for details see Chapter 2.6.2.
Figure 3.10. The effect of an Igβ cytoplasmic truncation on BCR trafficking to intracellular compartments. (A) Schematic representation of a BCR with a cytoplasmic Igβ truncation (βtrunc) with Igα shown in orange and Igβ shown in green. (B) Timecourse of WT BCR trafficking to early endosomal (TfnR) and lysosomal (LAMP-1) compartments. Cells were stimulated with Alexa Fluor 633-conjugated anti-mouse IgM for the time indicated prior to fixation with paraformaldehyde. Cells were then permeabilized and incubated with Abs against TfnR and LAMP-1. Scale bar: 10 μm. (C) Measurement of BCR co-localization with TfnR using Pearson’s coefficients. (D) Measurement of BCR co-localization with LAMP-1 using Pearson’s coefficients for details see Chapter 2.6.2.
Figure 3.11. The effect of 2 Igα cytoplasmic domains on BCR trafficking to intracellular compartments. (A) Schematic representation of a BCR with two Igα cytoplasmic domains (Cα) with Igα shown in orange and Igβ shown in green. (B) Timecourse of WT BCR trafficking to early endosomal (TfnR) and lysosomal (LAMP-1) compartments. Cells were stimulated with Alexa Fluor 633-conjugated anti-mouse IgM for the time indicated prior to fixation with paraformaldehyde. Cells were then permeabilized and incubated with Abs against TfnR and LAMP-1. Scale bar: 10 μm. (C) Measurement of BCR co-localization with TfnR using Pearson’s coefficients. (D) Measurement of BCR co-localization with LAMP-1 using Pearson’s coefficients for details see Chapter 2.6.2.
Figure 3.12. The effect of two Igβ cytoplasmic domains on BCR trafficking to intracellular compartments. (A) Schematic representation of a BCR with two Igβ cytoplasmic domains (Cβ) with Igα shown in orange and Igβ shown in green. (B) Timecourse of WT BCR trafficking to early endosomal (TfnR) and lysosomal (LAMP-1) compartments. Cells were stimulated with Alexa Fluor 633-conjugated anti-mouse IgM for the time indicated prior to fixation with paraformaldehyde. Cells were then permeabilized and incubated with Abs against TfnR and LAMP-1. Scale bar: 10 μm. (C) Measurement of BCR co-localization with TfnR using Pearson’s coefficients. (D) Measurement of BCR co-localization with LAMP-1 using Pearson’s coefficients for details see Chapter 2.6.2.
(Figures 3.13 and 3.14) to determine whether any differences exist in trafficking of BCRs to early endosomes.

Stimulation of WT BCRs with anti-IgM (Chapter 2.3.3) results in clustering of the receptor at the surface and some co-localization with TfnR-containing compartments. Analysis using Pearson’s coefficients shows little change in the co-localization of BCR and TfnR (Figure 3.8c), though the trafficking to this compartment may be masked by the fact that BCR and TfnR co-localize greatly at the cell surface in unstimulated cells.

Trafficking of BCRs with only a single Igα cytoplasmic domain (βtrunc) shows the same minimal changes in BCR and TfnR co-localization with a slight downward trend over time (Figure 3.10c). This was not significantly changed by mutation of the 4 amino acids DCSM to QTAT (Figure 3.13c). Similarly, a truncation of the Igα cytoplasmic domain resulting in a single Igβ cytoplasmic domain (αtrunc) show little change in BCR and TfnR co-localization over time (Figure 3.9c) with the reciprocal QTAT to DCSM amino acid mutation having no significant effect (Figure 3.14c). The mutants with either two Igα or two Igβ cytoplasmic domains show the same minimal changes with respect to BCR co-localization with TfnR over a 60 minute period and at earlier timepoints (Figure 3.11c and Figure 3.12c). These data indicate that none of the BCR mutations tested here resulted in defective trafficking to early endosomes in J558 cells. This also shows that a single cytoplasmic domain is sufficient for normal trafficking to early endosomal compartments and also that unlike with the internalization, the 4 amino acid motif DCSM, does not confer any extra abilities to the Igβ cytoplasmic domain.
Figure 3.13. The effect of mutation of the Igα cytoplasmic domain on BCR trafficking to intracellular compartments. (A) Schematic representation of a BCR with a mutated Igα cytoplasmic domain (αQTAT) with Igα shown in orange and Igβ shown in green. (B) Timecourse of WT BCR trafficking to early endosomal (TfnR) and lysosomal (LAMP-1) compartments. Cells were stimulated with Alexa Fluor 633-conjugated anti-mouse IgM for the time indicated prior to fixation with paraformaldehyde. Cells were then permeabilized and incubated with Abs against TfnR and LAMP-1. Scale bar: 10 μm. (C) Measurement of BCR co-localization with TfnR using Pearson’s coefficients. (D) Measurement of BCR co-localization with LAMP-1 using Pearson’s coefficients for details see Chapter 2.6.2.
Figure 3.14. The effect of mutation of the Igβ cytoplasmic domain on BCR trafficking to intracellular compartments. (A) Schematic representation of a BCR with a mutated Igβ cytoplasmic domain (βDCSM) with Igα shown in orange and Igβ shown in green. (B) Timecourse of WT BCR trafficking to early endosomal (TfnR) and (C) lysosomal (LAMP-1) compartments. Cells were stimulated with Alexa Fluor 633-conjugated anti-mouse IgM for the time indicated prior to fixation with paraformaldehyde. Cells were then permeabilized and incubated with Abs against TfnR and LAMP-1. Scale bar: 10 μm. (D) Measurement of BCR co-localization with TfnR using Pearson’s coefficients. (E) Measurement of BCR co-localization with LAMP-1 using Pearson’s coefficients for details see Chapter 2.6.2.
3.2.3 The effect of cytoplasmic domain mutations on BCR trafficking to lysosomes

Though trafficking to early endosomes appears unaffected, it is possible that mutations in Igα or Igβ chains may result in aberrant trafficking to later endocytic compartments such as lysosomes. BCR trafficking to lysosomal compartments was assessed by co-immunostaining for BCR and the lysosomal membrane protein, LAMP-1, and measured using Pearson’s coefficients.

Stimulation through WT BCRs resulted in clustering at the cell surface followed by receptor internalization. There was a sharp increase in BCR and LAMP-1-labelled compartments, as measured by Pearson’s coefficients (Figure 3.8d) between the 0 min and the 15 min timepoint, with the increase in co-localization continuing over the 60 min timecourse.

Using the Pearson’s coefficients obtained from cells with WT BCRs as a reference, the effects of single cytoplasmic truncations were assayed. A single Igα cytoplasmic domain (βtrunc) was sufficient for trafficking to LAMP-1 positive compartments although the co-localization does not occur to the same magnitude as cells with WT BCRs (Figure 3.10d). The same is true of BCRs with a single Igβ cytoplasmic domain (αtrunc) with the trafficking to lysosomal compartments being even more muted (Figure 3.9d). Cytoplasmic duplications, resulting in either 2 Igα cytoplasmic domains (Figure 3.11d) or 2 Igβ cytoplasmic domains (Figure 3.12d), amplified the response seen with either single domain BCR mutant, but not to the levels seen with WT BCRs.

In order to assess the effect of mutation of the 4 amino acid residues, DCSM and QTAT from Igα and Igβ respectively, to the 4 amino acid residues from the opposite cytoplasmic chain (Figure 3.1), the αQTAT mutant was compared to the βtrunc mutant and the βDCSM mutant was compared to the αtrunc mutant with each pairing having identical BCRs aside from the 4 amino
acid mutation. Neither the mutation of Igα to αQTAT (Figure 3.10d and Figure 3.13d) nor mutation of Igβ to βDCSM (Figure 3.9d and Figure 3.14d) affected the ability of the BCR to traffic to lysosomal compartments compared to the appropriate single chain BCR.

Taken together, these data indicate that both the Igα and the Igβ cytoplasmic domains alone are sufficient for trafficking to lysosomal compartments, and are unaffected by mutation of 4 amino acid residues within their respective ITAMs. The degree of co-localization seen with the duplication mutants compared to the WT hints that although either single cytoplasmic domain may be sufficient for trafficking to lysosomal compartments, the normal rate of trafficking of BCRs to lysosomes may require the additive effects of both the Igα and the Igβ cytoplasmic domains in tandem.

3.3 The effects of Igα and Igβ cytoplasmic domain mutation on BCR signaling

3.3.1 Rationale

The activation of signaling pathways following clustering of BCRs by Ag is a well documented event. The existing mutant BCR internalization studies have been performed using a chimeric receptor system with PDGF receptors or FcγRII receptors in the extracellular and transmembrane domains and either Igα or Igβ but not both in the cytoplasmic domains (Bonnerot et al., 1995; Cassard et al., 1996; Lankar et al., 1998; Siemasko et al., 2002; Granboulan et al., 2003).

Because of this, it has not been possible to examine the signaling capabilities of these mutant Igα and Igβ constructs. In order to assess whether there were differences in signaling capabilities between mutant and WT BCR-expressing cells, both intracellular staining for phosphorylated signaling proteins and flow cytometry (Chapter 2.5.1) were employed. However due to the
number of cells required as well as the time span required to complete all experiments, only stable BCR mutants were assessed.

3.3.2 The effect of Iga and Igβ cytoplasmic domain mutation on BCR-mediated tyrosine phosphorylation

The induction of tyrosine phosphorylation upon stimulation through WT BCRs has been well documented (Gold et al., 1990; Gold et al., 1991). Since there were clear differences in both the rate and magnitude of internalization between WT and mutant cells, it was important to determine whether there was either a correlative decrease in BCR-mediated signaling, or whether the aberrant internalization occurred independent of the signaling. In B lymphocytes, tyrosine phosphorylation is generally used as a broad signaling indicator. BCRs have tyrosines on both the Iga and Igβ chains and there are tyrosine phosphorylation sites on many of the downstream signaling proteins activated immediately downstream from the receptor.

There was a swift induction of total tyrosine phosphorylation upon WT BCR stimulation with a peak at 5 min post stimulation (Figure 3.15). A cytoplasmic truncation of Iga (αtrunc) results in a severe deficiency in tyrosine phosphorylation following the same stimulation (Figure 3.15). Replacement of the Iga cytoplasmic domain with the Igβ cytoplasmic domain (Cβ) was sufficient to restore some tyrosine phosphorylation but not to the levels seen with WT BCRs (Figure 3.15). This was also assessed by western immunoblot (Figure A2). These data indicate that the Igβ cytoplasmic domain is sufficient to allow for a low level of BCR-mediated signaling, but the Iga cytoplasmic domain is necessary for efficient signal transduction.
Figure 3.15. Tyrosine phosphorylation, upon stimulation through the BCR, is affected by mutations in the Igα cytoplasmic domain. (A) Schematic representation of the cytoplasmic mutants used with Igα shown in orange and Igβ shown in green. (B) Cells were stimulated for the times indicated below bars at 37°C with anti-IgM followed by fixation and intracellular staining for phosphotyrosine. Data was collected by flow cytometry. Graph represents data from n≥3 independent experiments.
3.3.3 The effect of Iga and Igβ cytoplasmic mutation on BCR-mediated Akt phosphorylation

Activation of the PI3K pathway is required for activation of mature B cells and also provides pro-survival signals. Additionally, BCR-mediated PI3K signaling has been shown to trigger the formation of a de novo Ag processing compartment (Granboulan et al., 2003). The phosphorylation of Akt is a hallmark of PI3K pathway activation and is therefore used as an indicator. To examine whether there was normal PI3K pathway activation in the BCR mutants, intracellular staining for phosphorylated Akt was performed at specific timepoints. A similar trend was seen with BCR-mediated Akt phosphorylation as with the tyrosine phosphorylation. There was a deficiency in the cells lacking the cytoplasmic domain of Iga (αtrunc) which was somewhat rescued by the replacement of the missing Iga domain with a second Igβ cytoplasmic domain (Cβ) (Figure 3.16). This demonstrates that the Iga cytoplasmic domain is necessary for normal BCR-mediated Akt pathway activation. While this decrease in PI3K signaling correlates with the decrease seen in BCR internalization, it has been previously shown that the internalization of BCR and Ag is not dependent on signaling through the PI3K pathway (Granboulan et al., 2003).

3.3.4 The effect of Iga and Igβ cytoplasmic mutation on BCR-mediated ERK1/2 phosphorylation

Signaling through the Ras/MAPK pathway is induced upon clustering of WT BCRs following antigen binding. Activation of this pathway can be detected by assaying for phosphorylated ERK1/2. Additionally, ERK can be phosphorylated through the action of PKC activated by the calcium flux resulting from PLCγ pathway activation. To examine whether there was normal
Figure 3.16. Akt phosphorylation, upon stimulation through the BCR, is affected by mutations in the Igα cytoplasmic domain. (A) Schematic representation of the cytoplasmic mutants used with Igα shown in orange and Igβ shown in green. (B) Cells were stimulated for the times indicated below the bars at 37°C with anti-IgM followed by fixation and intracellular staining for phospho-Akt (Ser473). Data was collected by flow cytometry. Graph represents data from n≥3 independent experiments.
Ras/MAPK and PLCγ pathway activation in the BCR mutants, intracellular staining for phosphorylated ERK was performed at specific timepoints.

Stimulation through WT BCRs resulted in a swift, but small increase in ERK1/2 phosphorylation which was also closely mirrored by the response of cells with BCRs with 2 Igβ cytoplasmic domains (Cβ) (Figure 3.17). However, a truncation of the Igα cytoplasmic domain (αtrunc) resulted in increased and sustained ERK phosphorylation over a 60 min period compared to the WT and Cβ cells (Figure 3.17). This suggests that the Igα cytoplasmic domain may play more of an inhibitory role in ERK phosphorylation.
Figure 3.17. ERK phosphorylation, upon stimulation through the BCR, is affected by mutations in the Igα cytoplasmic domain. (A) Schematic representation of the cytoplasmic mutants used with Igα shown in orange and Igβ shown in green. (B) Cells were stimulated for the times indicated below the bars at 37°C with anti-IgM followed by fixation and intracellular staining for phospho-p44/42 MAPK. Data was collected by flow cytometry. Graph represents data from n≥3 independent experiments.
The process of uptake in general is important for the proper function and survival of cells regardless of the specific cell type. Some specific cells such as macrophages and neutrophils employ phagocytosis while all cells make use of pinocytosis at the very least. This allows cells to take up macromolecules from the extracellular environment to fulfill their iron (transferrin receptor) or cholesterol (LDL receptor) requirements for example, and it is also a process used by pathogens to gain entry into cells. Internalization is often but not always clathrin-dependent. Endocytosis can also occur via caveolae, though BCR internalization is generally thought to be mainly mediated by clathrin since the phosphorylation of the clathrin heavy chain has been shown to be necessary for BCR internalization (Stoddart et al., 2002).

The internalization of BCR-antigen complexes is a necessary precursor to B cell activation, antigen presentation to helper T cells and the resulting immune response. Constitutive internalization of BCRs from the surface occurs, allowing for the internalization of monovalent Ag as well as membrane and receptor turnover and recycling which may also have Igα and Igβ structural requirements. However this discussion is restricted to the effects of Igα and Igβ mutations on the rapid and augmented internalization response induced by BCR cross-linking.

While it has been shown that WT heterodimerization of Igα and Igβ in the extracellular and transmembrane domains is required for surface expression of the BCR, it has been noted that mutations in the cytoplasmic domains of these proteins does not disrupt trafficking of the receptor to the cell surface (Dylke et al., 2007). This has allowed for the creation of Igα and Igβ cytoplasmic domain mutants while maintaining the BCR surface expression that is absolutely
required for BCR-mediated B cell activation. Although several groups have previously undertaken the task of determining the necessity and sufficiency of regions within the cytoplasmic domains, all cytoplasmic mutants have previously been assessed by homotypic interactions using chimeric receptor systems (Bonnerot et al., 1995; Cassard et al., 1996; Granboulan et al., 2003; Lankar et al., 1998). We have undertaken this study using the 4 chain BCR, which is more reflective of the situation occurring upon BCR stimulation of B lymphocytes.

By comparing the panel of 7 BCRs, with varying combinations of Igα and Igβ cytoplasmic mutations (Figure 3.1), to WT BCRs, we were able to show that the Igα cytoplasmic domain makes a larger contribution for ligand-induced internalization than does the Igβ cytoplasmic domain. The results obtained with our single truncation mutants (αtrunc and βtrunc) confirm previous reports that the individual cytoplasmic domains are sufficient for receptor internalization (Bonnerot et al., 1995; Cassard et al., 1996; Granboulan et al., 2003; Patel and Neuberger, 1993). In fact, the data acquired from the cytoplasmic duplication mutants, Cα and Cβ (Figure 3.5), should parallel the results obtained using chimeric receptors as well as further illustrating the role each single domain plays in receptor internalization. It was initially surprising to see that Cα BCRs were internalized at both an increased rate and magnitude compared to cells with WT BCRs, but is consistent with the idea that Igα cytoplasmic domain is the principal mediator of the receptor internalization as shown with the single domain truncation mutants (αtrunc and βtrunc).

The 4 amino acid motif, DCSM, from within the Igα ITAM has been shown to be important in BCR-mediated signaling studies, specifically it is sufficient to switch the signaling phenotypes of Igα and Igβ. Mutant Igβ constructs containing this motif show that DCSM confers the ability
to flux calcium and produce IL-2 in response to BCR stimulation, effects normally attributed to
the Igα chain (Cassard et al., 1996), but which other processes may also be affected by this motif
have not been identified. Here we show that the DCSM motif confers not only signaling abilities
but is also sufficient to increase the rate of internalization of a deficient mutant to WT levels
(Figure 3.6). However it is curious that the removal of this amino acid motif from the Igα
cytoplasmic domain (αQTAT) has no effect on the rate of internalization compared to the single
Igα cytoplasmic domain (βtrunc). It would be interesting to insert this motif onto the cytoplasmic
domain of a protein which is not normally internalized such as a FcγRII to see whether it is then
internalized.

Having established the relative contribution each cytoplasmic domain is making to receptor
internalization, the next step should be to dissect the mechanism behind these results. Are the
defects seen in internalization due to a conformational defect from a missing or mutated
cytoplasmic domain, or are they due to actual signals found within the cytoplasmic domain?
Clearly the Igα cytoplasmic domain is dominant over the Igβ cytoplasmic domain in this process
for which there are several possible explanations. It is possible that the Igα cytoplasmic domain
allows for better translocation of BCRs into regions where clathrin-coated pits will form or even
recruiting clathrin or the appropriate adaptors itself (Figure 4.1a). But perhaps the more
attractive explanation, and one that is known to be true, at least in part, is that the Igα
cytoplasmic domain has the capacity to recruit different signaling proteins than the Igβ
cytoplasmic domain. It is already known from signaling studies that Igα interacts with BLNK on
the non-ITAM tyrosine Y204 (Engels et al., 2001; Kabak et al., 2002) and that the DCSM motif
allows for interactions with the Src-family kinase Fyn (Cassard et al., 1996). Since the process of
BCR internalization has been shown to be both signaling-dependent and actin-dependent, the Igα.
Figure 4.1. Models of Igα control of BCR internalization.

(A) The Igα cytoplasmic domain allows for better translocation of BCRs to regions of clathrin-coated pit formation. The absence or mutation of the Igα cytoplasmic domain prevents this translocation thereby inhibiting receptor uptake. (B) The Igα cytoplasmic domain recruits specific signaling proteins initiating a signaling cascade which activates and recruits cytoskeletal nucleators and other proteins necessary for receptor internalization. (C) The Igα cytoplasmic domain interacts directly with the actin nucleators and other necessary proteins as well as signaling which initiates receptor internalization. Igα is shown in orange, Igβ is shown in green. Yellow circles represent phosphorylated tyrosine residues, black triangles represent clathrin, blue squares and grey shapes represent involved proteins and red lines represent polymerized actin filaments.
Actin Nucleators

Clathrin and Adaptor Proteins

INTERNALIZATION

SIGNAL
cytoplasmic domain may be more efficient at recruiting the signaling proteins required for signal transduction which subsequently activates and recruits cytoskeletal nucleators and other proteins necessary for internalization (Figure 4.1b). Alternately, the Igα cytoplasmic domain, including the DCSM motif, may directly recruit these actin nucleators and other proteins while simultaneously contributing to signaling (Figure 4.1c). Though the DCSM motif contains a serine residue, it has not been reported to be phosphorylated, nor does the motif suggest any binding domain interactions according to various bioinformatics websites including Phosphosite (www.phosphosite.org), SwissProt (www.expasy.ch/sprot/), Pfam (http://pfam.sanger.ac.uk/), SMART (http://smart.embl.de/), and PROSITE (http://www.expasy.org/prosite/). So while our results clearly show that this motif confers internalization abilities, precisely how it affects internalization remains unclear.

In terms of trafficking, it is known that prior to peptide-MHC II complex expression on the cell surface, BCR-ligand complexes are found within the MIIC compartment along with MHC II. How the ligand-receptor complexes arrive at their destination is less clear and it is this aspect that we have addressed in this study.

The results show very little change in the co-localization of any of the mutant BCRs with TfnR. This is likely due to the large degree of co-localization between the two receptors at the cell surface which may mask any real trends with respect to BCRs in early endosomes. Without this masking effect, we would likely see an early increase in BCR-TfnR co-localization followed by a decrease over time as the BCR proceeds through the endocytic pathway. But based on our observations it appears that so long as the receptors are internalized in the first place, trafficking to early endosomes appears to proceed unimpeded by any mutations tested herein.
We also show that both the Igα cytoplasmic domain and the Igβ cytoplasmic domain are individually capable of directing internalized BCRs to lysosomal compartments albeit with differing efficiencies (Chapter 3.2). Although the co-localization of BCR and LAMP-1-positive compartments appears to be hindered in certain mutants, the slower increase in co-localization is reflective of the slower rate of internalization in these cells. We would not expect to see the same amount of BCR-LAMP1 co-localization with mutants severely defective in internalization, as we would with WT BCRs simply because of the fact that a substantially smaller amount of BCR has been internalized at any given timepoint. The mutants with the most severe internalization phenotypes are also the mutants with the least amount of BCR-LAMP-1 co-localization after 60 min however it is likely that given enough time these mutants would catch up. Ideal trafficking analysis would involve comparing the co-localization in cells that had internalized identical quantities of their receptors.

Our results are in opposition to previous reports that BCRs with 2 Igα cytoplasmic domains are retained within the early endosomes (Luisiri et al., 1996) or were not able to enter the MIIC compartment (Siemasko et al., 1999) indicating that the Igβ cytoplasmic domain is necessary for normal trafficking. Data obtained using βtrunc and Ca BCRs refute these claims which can be interpreted several ways. The fact that βtrunc BCRs, which are not full cytoplasmic truncations, are still found in lysosomal compartments could mean that the signal which allows trafficking to lysosomes is found N-terminal to Y195. However the fact that Ca BCRs, which do not have any part of the Igβ cytoplasmic domain, are found within lysosomal compartments suggests that using a chimeric receptor system may not be equivalent to using a 4 chain BCR. It has also been reported that the di-ubiquitynation of Igβ is a prerequisite for transport into LAMP-1 positive compartments (Zhang et al., 2007). While the cytoplasmic truncation of the Igβ retains 2 amino acid residues which may be ubiquitinated, the data obtained from our Ca mutant which has 2 Igα
cytoplasmic domains, is inconsistent with this claim since this mutant had the most normal trafficking phenotype. Again, it is possible that the results are an effect of the chimeric system.

One caveat of the system we have used to acquire trafficking data is that we are unable to examine trafficking to the MIIC compartment. The J558 cell lines were derived from a plasma cell myeloma. In a physiological setting, plasma cells no longer respond to activating signals, no longer express MHC II and therefore no longer interact with T cells. In an ideal situation we would examine whether BCR-ligand complexes traffic to MIIC however because J558 cells do not express MHC II they will not contain MIIC compartments. It is still useful to know whether they traffic to LAMP-1 positive compartments since it would reveal any mutations in Igα or Igβ resulting in either exclusion from these compartments or retention in earlier endocytic compartments and in fact, several other groups have even taken the same approach in different cell lines (Brown and Song, 2001). It is possible that transfection of J558 cells with MHC II may result in some co-localization of MHC II with LAMP-1 containing compartments since the other characteristic proteins found in MIICs are generally speaking, ubiquitously expressed and the inability of plasma cells to act as APCs is likely a direct effect of the lack of MHC II expression rather than a defect in the antigen processing pathway itself. If MHC II is indeed the only missing component of the antigen presentation system, we would expect co-localization with LAMP-1 and a normally functioning MIIC. However this would require verification of the presence of all other required components in J558 cells.

The question of how internalized BCRs, found first in early endosomes, arrive in other compartments remains elusive. While these results show that BCRs can be found within lysosomal compartments, it is not clear how they get there. It is known that unligated BCRs taken up through constitutive endocytosis can be found in early/sorting endosomes and are
quickly recycled back to the surface. The signal that allows for the differential subcellular localization of these BCRs is unknown at this time. Because the BCRs that are recycled and the BCRs that are degraded are structurally identical, it is unlikely that a direct sorting signal exists within the cytoplasmic domain. Crosslinking of BCRs is known to not only initiate signaling pathway activation, but also to increase production and trafficking of MHC II to MIICs and enzymes such as cathepsin S to facilitate Ag loading in the MIIC (Forquet et al., 1999; Lankar et al., 2002; Siemasko et al., 1998; Zimmermann et al., 1999). It is not unreasonable to speculate that the signaling initiated by crosslinked BCRs also directs newly internalized receptors to proceed through the endocytic pathway instead of recycling to the cell surface by default (Figure 4.2). Whether this occurs automatically or whether there is a signal within the cytoplasmic domains which the activated B cell can recognize is unclear. It is also possible that the signaling may allow for a recruitment cascade of proteins to the early endosomal membrane allowing for the maturation of the compartments within which the internalized BCRs are found (Figure 4.2).

The activation of signaling pathways initiated upon BCR stimulation is one of the more commonly studied aspects of the BCR. We were curious as to the effects of the Igα and Igβ mutations on signaling pathway activation and so attempted to look at their effects on the broad signaling capacities of these BCRs. Our results showed that the total tyrosine phosphorylation initiated upon BCR stimulation is defective in the mutants assessed (αtrunc and Cβ). This result is not altogether unexpected given that the Igα cytoplasmic domain has 4 tyrosine residues and the Igβ cytoplasmic domain has only 2 so not only the phosphorylation of the Igα and Igβ themselves should be defective, but the signaling initiated from them as well. Our data illustrates the fact that if the membrane proximal events are muted, signaling amplification cannot completely compensate. Immunoprecipitation of WT and Cβ BCRs followed by immunoblotting for phosphorylated tyrosine supports this (S. Machtaler, unpublished data). The results showing
Figure 4.2. Model of BCR trafficking.
The default for internalized BCRs is recycling back to the cell surface unless a signal is generated by BCR cross-linking which causes the recruitment of the appropriate proteins for compartment maturation and progression through the endocytic pathway. Igα is shown in orange, Igβ is shown in green. EE: early endosome; LE: late endosome; L: lysosome. Red arrows indicate maturation over time.
the levels of phosphorylated Akt (Figure 3.16) following stimulation are also consistent with this idea but are however inconsistent with a previous report. This report suggested that PI3K pathway activation was mediated by Igβ not Igα and that the Igβ-mediated PI3K signaling was necessary for entry into LAMP-1 positive intracellular compartments (Granboulan et al., 2003). If this is true we would not expect any change in the levels of Akt phosphorylation in the αtrunc mutant given that it retains a WT Igβ chain, and we would expect an increase in the amount of Akt phosphorylation in the Cβ mutants since it has a second Igβ cytoplasmic domain. Instead we see that both are decreased. Additionally, the Cα BCR mutant which has 2 Igα cytoplasmic domains, shows the Igβ cytoplasmic domain is not in fact required for entry into LAMP-1 positive compartments.

The most surprising result from our signaling studies was the elevated and sustained level of ERK phosphorylation seen in the αtrunc mutant (Figure 3.17). It is possible that the increase in ERK phosphorylation is due to the fact that the Igα cytoplasmic domain normally recruits negative regulators of BCR signaling and the αtrunc mutant is unable to do so. This is a particularly attractive hypothesis since many of the ITIM-containing negative regulators of BCR signaling are phosphorylated by the Src-family kinases. If the additional recruitment of Src-family kinases by their SH2 domains, particularly Lyn, is defective upon signal initiation because of the missing Igα cytoplasmic domain, there may not be an adequate level of phosphorylation of CD22 (Cornall et al., 1998) which recruits the SH2 domain-containing protein tyrosine phosphatase-1 (SHP-1) (Blasioli et al., 1999), or phosphorylation of FcγRIIB which then recruits SH2 domain-containing inositol phosphate 5'-phosphatase (SHIP) and may also recruit SHP-1 and SHP-2 (Famiglietti et al., 1999; Hippen et al., 1997; Nakamura et al., 2000). Alternately the absence of the Igα cytoplasmic domain may indirectly affect the activation of negative regulators of ERK itself which include the MAPK phosphatase (MKP)1, MKP2, and
MKP3 (Brondello et al., 1997; Camps et al., 1998; Slack et al., 2001) or protein phosphatase 2A (PP2A) (Alessi et al., 1995; Sohaskey and Ferrell, 1999).

There are two schools of thought regarding the relationship between BCR signaling and BCR internalization. One says that the internalization and signaling are mutually exclusive events, that any given BCR is involved in one or the other but not both (Hou et al., 2006), the other says that one of the functions of receptor internalization is attenuation of signaling (Gazumyan et al., 2006; Stoddart et al., 2005). The increase in ERK phosphorylation in the αtrunc mutant would seem to support the latter, however a clearer picture will require the assessment of additional BCR mutants, in particular the βtrunc mutant.

There is a growing movement in the BCR field of study attempting to identify links between the BCR-mediated functions and elements that were initially studied as separate entities such as actin cytoskeletal rearrangements and BCR-mediated signaling. Where mutations in the Igα and Igβ cytoplasmic domains have predictable effects on BCR-mediated signaling pathway activation, it is clear that they will also affect function, though the exact mechanisms remain elusive. This is partly due to the fact that signaling through the BCR mediates so many events and the outcome may vary depending on the developmental stage of the cell, the extracellular environment, and the type of antigen bound by the receptor. We are beginning to see that actin binding proteins such as (actin-binding protein 1) Abp1, and ubiquitination by proteins such as Cbl and the E3 ubiquitin ligase Itch may be involved in classical BCR-mediated events (Jacob et al., 2008; Onabajo et al., 2008; Zhang et al., 2007). Though the details are not yet well understood, it will be interesting to see how all of these processes are interconnected and what other players are involved.
The current models of actin-dependent clathrin-mediated endocytosis identifies the yeast equivalents of Huntingtin-interacting protein-1 related (HIP1R) and epidermal-growth-factor-receptor-pathway substrate 15 (EPS15) as likely candidates linking the clathrin coat to actin since they have both been shown to bind clathrin and actin and colocalize with the two at sites of internalization (Engqvist-Goldstein et al., 1999; Engqvist-Goldstein et al., 2001; Henry et al., 2002). The actin-related protein-2/3 (Arp2/3) complex-activators neuronal Wiskcott-Aldrich syndrome protein (N-WASP) and cortactin have been shown to be localized at sites of internalization (Cao et al., 2003; Kaksonen et al., 2005) and it is known that Arp2/3 mutation blocks endocytosis in yeast (Martin et al., 2005). The current model postulates that N-WASP and cortactin activate the Arp2/3 complex at sites of internalization causing Arp2/3-dependent actin branch formation with the force for movement generated by myosin proteins. The polymerizing actin is linked to the forming vesicle by HIP1R and EPS15, and Abp1 may function to initiate a negative feedback loop turning off actin-mediated internalization (Kaksonen et al., 2006). With the exception of the clathrin heavy chain, the effects of BCR crosslinking on any of these proteins has not yet been investigated, nor have the converse effects of mutation or deficiency in any of these proteins on BCR internalization been studied, but it is not unreasonable to believe that BCR internalization occurs by this process as well.

This thesis has taken a systematic approach to looking at the contributions of the Igα and Igβ cytoplasmic domains in the processes of receptor internalization, intracellular trafficking, and signaling pathway activation. In it we show that the Igα cytoplasmic domain makes a larger relative contribution to BCR internalization and that it is necessary for normal levels of BCR-mediated signaling. We also show that the presence of both cytoplasmic domains is necessary for normal trafficking dynamics to LAMP-1 positive compartments. While this study has provided answers to several questions surrounding Igα, Igβ, and the first steps in Ag
presentation, it is important to realize that these experiments employed antibody stimulation to mimic soluble Ag binding. It is entirely possible that responses may differ in the case of BCR binding to small particulate or membrane bound antigen, and further studies are necessary to assess whether this is indeed the case.
References:


Figure A1. Surface expression of BCRs in double transfectants.

(A) J558 μ (B) J558μm3 (C) βtrunc (D) αtrunc βtrunc (E) αQTAT (F) βDCSM. J558 μ cells were transfected with the Igα and Igβ constructs shown in (C) – (F). Cells were incubated with Alexa Fluor 633-conjugated goat anti-mouse IgM for 20 min on ice prior to analysis by flow cytometry. Data shown in dot plot format with both axes measuring fluorescent intensity. Green dots represent unstained cells. Yellow dots represent cells stained with fluorescent Ab.
Figure A2. The effect of Igα cytoplasmic domain mutation on tyrosine phosphorylation induced upon BCR stimulation.

Timecourse of comparing total tyrosine phosphorylation upon BCR stimulation in either (A) μm3, atrunc, and 15-25 cells, or (B) μm3, Cβ, and 15-25 cells. Cells were stimulated with goat anti-mouse IgM for the indicated times at 37°C. Total cell lysate was run on an SDS-PAGE gel then transferred to a nitrocellulose membrane. Membranes were incubated with the appropriate dilutions of 4G10 then goat anti-mouse IgG conjugated HRP prior to incubation with ECL. Results were visualized using autoradiography film. For details see Chapter 2.4.2.
Appendix 2  Connexin 43 (Cx43)

Appendix 2.1 Introduction

Gap junctions (GJ) are intercellular connections which are composed of the apposed hemichannels of 2 adjacent cells. These junctions allow the rapid transfer of small molecules between cells conveying electric, metabolic, and immunologic information. Each hemichannel in turn is composed of 6 subunits called connexins of which there are 21 mammalian isoforms. Connexins make up connexons and are classified by molecular weight and have a well conserved structure with 4 membrane-spanning domains, 2 extracellular loops with conserved cysteine residues and often a large, C-terminal cytoplasmic domain containing multiple regulatory sites. Related families of GJ proteins also exist, called pannexins in mammalian cells and innexins in lower invertebrates.

The Cx43 isoform (Figure A3) is ubiquitously expressed but is the predominant isoform found in hematopoietic cells. Mice with a Cx43 deficiency show many defects and die postnatally of ventricular arrhythmia (Reaume et al., 1995). In terms of the immune system, a Cx43 deficiency (Cx43-/-) results in compromised B and T cell development, a reduction in the number of CD4 T cells, a reduction in the number of IgM-positive B cells (Cancelas et al., 2000; Montecino-Rodriguez et al., 2000). Heterozygous mice (Cx43+/-) exhibit a similar but less severe initial phenotype, which is gradually normalized over the course of one month suggesting gene dosage effects (Montecino-Rodriguez et al., 2000). Additionally, Cx43+/- mice treated with the thymidylate synthase inhibitor 5-fluorouracil (5-FU) or irradiation show only a 20% recovery in bone marrow cellularity compared to the WT 70% (Montecino-Rodriguez and Dorshkind, 2001). Taken together this suggests that Cx43 plays a critical role in hematopoiesis.
Figure A3. Schematic representation of Cx43.

Cx43 subunits contain 4 membrane-spanning domains with 2 extracellular loops (EL) containing conserved cysteine residues shown in yellow, one cytoplasmic loop (CL) and a large cytoplasmic tail (CT) with multiple phosphorylation sites shown in red and binding domains (not shown). Tyrosine phosphorylation sites found at positions 247 and 313, threonine phosphorylation site at 326 and serine phosphorylation sites at positions 306, 314, 328, 330, 365, 368, 369, and 373. Numbers indicate positions within the amino acid sequence. Figure not drawn to scale.
Generally speaking the role of Cx43, and gap junctions in general, in immune cells is not well understood. Its upregulation in multiple immune cell types upon exposure to inflammatory factors has been reported but not explored (Eugenin et al., 2003). In dendritic cells, gap junction communication has been shown to be sufficient to immunologically couple adjacent cells, allowing for degraded antigen from one cell to be presented on the MHC molecules of the neighbouring cell (Neijssen et al., 2005). In terms of cancer, Cx43 has been associated with tumor suppression, though the mechanism for this is also unclear.

While gap junction communication is generally thought to be important in the cell-cell communication of sessile cells, it has recently been shown that Cx43 plays a role in glioma motility (Bates et al., 2007), and neuronal cell migration during development (Elias et al., 2007). Additionally, the dye transfer that occurs between lymphocytes and endothelial cells during transepithelial migration suggests that Cx43 may play a role in B cell migration though the mechanism of how this communication is used remains unclear and the role of the gap junction proteins is unknown (Oviedo-Orta et al., 2002).

The Matsuuchi Lab is interested in the role of gap junction proteins in processes involved in immune cell interactions, and lymphoid tumor spread and establishment. We (C. Jang, S. Machtaler and M. P. Dang-Lawson) have been working on the collection of preliminary data for a joint project with the Naus lab. In the following section a summary of the work I have done is presented.
Appendix 2.2 Results

The presence or absence, as well as the level of expression of Cx43 in various primary cells and tissue culture cell lines were assessed by western immunoblot. Results show that Cx43 is expressed at varying levels in all immune cells tested, with slight variations in molecular weight, except for the J558μm3 plasma cell line (Figure A4). Because of our research on the BCR-induced changes in signaling pathways and cell morphology, the effect of BCR stimulation on Cx43 was assessed by western immunoblot. The immunoblot of the timecourse showed a shift in molecular weight with a peak in both WEHI-231 and A20 cells at 15 min post-stimulation (Figure A5).

The complete absence of Cx43 in J558 cells means that the effect of Cx43 and any other Cx43 mutants can be assessed in a normally null cell system. To that end, J558μm3 cells were transfected with a Cx43-GFP construct. In addition, B lymphoma WEHI-231 cells which have endogenous expression of Cx43 were also transfected. The intracellular localization of the transfected Cx43-GFP was assessed by confocal microscopy and showed that in both cell lines, the Cx43-GFP was located in a punctuate distribution (Figure A6) with the occasional cell having a large intracellular aggregate. In order to determine the precise intracellular localization of Cx43, immunostaining of intracellular compartments was employed. Cx43-GFP co-localizes with early endosomal markers and with the ER, but does not co-localize with lysosomal markers (Figure A7).

Because it is one of our goals to understand the role of Cx43 in cytoskeletal dynamics, it’s effect on the internalization of the BCR, a cytoskeletal-dependent event, was assessed. J558μm3 cells transfected with Cx43-GFP were compared to WT J558μm3 cells. Cx43 did not have an effect
Figure A4. Cx43 expression in immune cells.
(A) Cx43 expression in lymphoma and myeloma culture cell lines and (B) Cx43 expression in primary cells and immune cell lines. Total cell lysate (60 μg protein) was run on an SDS-PAGE gel then transferred to a nitrocellulose membrane. Membrane was probed with an appropriate dilution of Cx43 antibody followed by an appropriate dilution of HRP-conjugated rabbit antibody prior to incubation with ECL. Results were imaged with autoradiography film. Blot was subsequently reprobed for β-actin. For details see Chapter 2.4.2.
Figure A5. The effect of BCR stimulation on Cx43.

An appropriate number of J558 μm3 cells, WEHI-231 cells, and A20 cells were stimulated for the indicated times as described in Chapter 2.3.4. Astrocyte lysate (5 μg) was added as a control and 30 μg total cell lysate from each sample was run on an SDS-PAGE gel then transferred to a nitrocellulose membrane. Membrane was probed with an appropriate dilution of Cx43 antibody followed by an appropriate dilution of HRP-conjugated rabbit antibody prior to incubation with ECL. Results were imaged with autoradiography film. Blot was subsequently reprobed for β-actin. For details see Chapter 2.4.2.
Figure A6. Expression of Cx43-GFP in transfected WEHI-231 and J558μm3 cells.
Live cells were imaged by confocal microscopy to assess Cx43-GFP expression and localization.
Images are 3D reconstructions.
Figure A7. Intracellular localization of Cx43-GFP in unstimulated J558μm3 cells. (A) and (B) Unstimulated J558μm3 cells expressing Cx43-GFP were fixed with paraformaldehyde, permeabilized, and stained with Abs against early endosomal compartments (TfnR) and lysosomal compartments (LAMP-1), respectively. (C) Live, unstimulated J558μm3 cells expressing Cx43-GFP were incubated with ER-Tracker for 20 min at 37°C prior to imaging. Scale bars: 10 μm. Image shown in (C) acquired by S. Machtaler.
on BCR internalization (Figure A8) but in work not shown in this thesis, has been shown to have an effect on cell spreading and may have an effect on other cytoskeletal-mediated events (S. Machlater and L. Matsuuchi, unpublished data).
The effect of Cx43 on BCR internalization

Figure A8. The effect of Cx43 on BCR internalization.
J558μm3 cells transfected with Cx43-GFP (shown in green) were stimulated in parallel with WT J558μm3 cells (shown in black) at 37°C for the indicated times with goat anti-mouse IgM followed by fixation and incubation with a fluorescently conjugated secondary Ab. For details see Chapter 2.3.4 and Chapter 2.5.1. Data was collected by flow cytometry. Graph represents data from n>3 independent experiments.