Translocation of IgM-Antigen Receptor Associated Signaling Molecules into the Membrane Skeleton During Early B Cell Activation

By

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A thesis submitted in conformity with the requirements for the degree Master of Science
Graduate Department of Immunology
University of Toronto

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Translocation of IgM-Antigen Receptor Associated Signaling Molecules into the Membrane Skeleton During Early B Cell Activation

Lisa Sava Jugloff

Master of Science (1997)

Department of Immunology, University of Toronto

Supervisor: Dr. Jenny Jongstra-Bilen

Committee: Dr. Jan Jongstra and Dr. Stuart Berger

Membrane Immunoglobulin (mIgM) the component of the B cell receptor which provides specificity for antigen has been shown to associate with the cytoskeleton upon receptor cross-linking. In an attempt to understand the role of this interaction during signaling, I have asked the question whether the signaling components of the B cell receptor accumulate in the membrane skeleton along with mIgM. Interestingly, I have shown that tyrosine phosphorylated Igα translocates to the membrane skeleton as part of the B cell receptor complex after mIgM cross-linking. The maximum levels of tyrosine phosphorylated Igα and other substrates correlated with the maximum levels of mIgM in the membrane skeleton fraction. Furthermore, I have demonstrated that the Src family protein tyrosine kinase Lyn and the cytoplasmic tyrosine kinase Syk are found in the membrane skeleton following cross-linking of the B cell receptor. These data suggest that mIgM translocating to the membrane skeleton provides the vehicle for active signaling molecules to be recruited to this subcellular location. This may promote B cell activation events by providing optimal interactions between signaling molecules supported by the cytoskeletal matrix.
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I dedicate this thesis to my brother Denis who has encouraged me to learn and has helped me to achieve my goals.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>vii</td>
</tr>
</tbody>
</table>

## I. Introduction

1. B cell Antigen Receptor - Structure                                    1
2. B cell Antigen Receptor - Signaling                                    4
3. Protein Tyrosine Kinases                                               8
4. B cell Receptor and the Cytoskeleton                                   11
5. Other Cellular Systems and the Cytoskeleton                            13
6. Hypothesis                                                            15

## II. Materials and Methods

1. Cells                                                                 17
2. Antibodies                                                            17
3. Biotinylation of Bal 17.7.1 Cells                                      18
4. IgM Immunoprecipitations from Bal 17.7.1 Lysates                      18
5. Immunofluorescence Microscopy                                         19
6. Subcellular Fractionation of Bal 17.7.1 Cells                         20
7. Western Blotting                                                      22
8. Stock Solutions                                                       24

## III. Results

1. Igα co-caps with mIgM                                                 25
2. Isolation of the membrane skeleton bound mIgM by subcellular fractionations of Bal 17.7.1 Cells 28
3. Maltoside preserves, NP-40 disrupts mIgM/Igα interactions            31
4. Igα translocates to the membrane skeleton as part of the BCR         33
5. Gelsolin releases mIgM and Igα from the membrane skeleton            35
6. The tyrosine phosphorylated form of Igα accumulates in the membrane skeleton with a different kinetics than the bulk of Igα or mIgM 39
7. Rapid accumulation of Lyn and Syk in the membrane skeleton of stimulated cells 45
IV. Discussion

V. Future Directions

1. Activities of Protein Tyrosine Kinases in the Membrane Skeleton

2. Characterization of Other Signaling Molecules in the Membrane Skeleton

3. Subcellular Fractionations on B cells Lacking Igα

4. Disruption of mIgM/cytoskeleton interactions

VI. List of References
List of Figures

Figure 1  Structure of the B Cell Antigen Receptor
Figure 2  Signal Transduction Events Initiated by the B Cell Antigen Receptor
Figure 3  Structure Characteristics of Src-Family and Syk Protein Tyrosine Kinases
Figure 4  Subcellular Fractionation
Figure 5  Igα co-caps with mIgM
Figure 6  Subcellular fractionations isolate membrane skeleton bound mIgM
Figure 7  Maltoside preserves, NP-40 disrupts mIgM/Igα interactions
Figure 8  Igα translocates to the membrane skeleton as part of the B cell receptor complex
Figure 9  mIgM and Igα distribution in the membrane skeleton and membrane soluble fractions following detergent solubilization of the membrane pellet
Figure 10 Gelsolin releases mIgM and Igα from the membrane skeleton
Figure 11 Gelsolin releases actin and Lyn from the membrane skeleton
Figure 12 Kinetics of mIgM binding to the membrane skeleton
Figure 13 Kinetics of tyrosine phosphorylated substrates and Igα accumulating in the membrane skeleton
Figure 14 Kinetics of Lyn and Syk accumulating in the membrane skeleton
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>Abs</td>
<td>antibodies</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAPs</td>
<td>B cell associated proteins</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CDRs</td>
<td>complementarity-determining regions</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence reaction</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>FceRI</td>
<td>high-affinity receptor for immunoglobulin E</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GNEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HF</td>
<td>Hank's balanced salt solution + 2% fetal calf serum</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor family tyrosine-based activation motif</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>lysis buffer</td>
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<tr>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mIg</td>
<td>membrane immunoglobulin</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>m.w.</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonidet P-40</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
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</tr>
<tr>
<td>PI3K</td>
<td>PI-3 Kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC₇</td>
<td>phospholipase C gamma</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
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</table>
Q Glutamine
rpm revolutions per minute
RPMI Roswell Park Memorial Institute culture medium
s second
S Serine
SD standard deviation
SDS-PAGE sodium dodecylsulfate polyacrylamide gel electrophoresis
SH2 src-homology 2 domain (phosphotyrosine binding domain)
SH3 src-homology 3 domain (proline-rich sequence binding domain)
SH4 src-homology 4 domain (membrane association signal)
T Threonine
TBS Tris-buffered saline
TBS-T Tris-buffered saline + 0.1% Tween-20 detergent
TCR T cell receptor
V Valine
Y Tyrosine
Chapter I: Introduction

B cells recognize and respond to foreign antigen through their surface-expressed, clonotypic B cell receptor (BCR). Receptor stimulation can elicit a variety of biological responses including cell activation, proliferation, differentiation, anergy and cell death. These divergent responses are initiated by the immediate BCR recruitment and activation of protein tyrosine kinases. Kinases stimulate at least three pathways of signal transduction including the phosphoinositol, ras and phosphatidylinositol 3-kinase pathways. In addition to signaling events, structural changes involving the cytoskeleton occur. Until recently, the traditional view of the cytoskeleton in B cells is that it solely serves as structural support for the cell, it is involved in cell motility, receptor patching, capping and internalization. However, the BCR association with the cytoskeleton following stimulation adds a further dimension to the role of the cytoskeleton in B cell signaling. The work on other cellular systems including platelets and T cells where the integrin and T cell receptor, respectively, have also been demonstrated to associate with the cytoskeletal matrix, supports the involvement of the cytoskeleton in receptor mediated signal transduction events. I will discuss what is known about the cytoskeleton in B cell activation and further explore the potential role of BCR associations with the cytoskeleton in B cell signaling.

1. B cell Antigen Receptor - Structure

Immature and mature B cells are characterized by their cell-surface bound BCR composed of an antigen binding membrane immunoglobulin (mIg) noncovalently associated with a disulphide-linked heterodimer composed of Igα and Igβ (Figure 1, (1)). The membrane immunoglobulin is a complex of two Ig heavy and two Ig light chains (reviewed in 2,3). Variable
Figure 1 Structure of the B Cell Antigen Receptor

- Antigen binding
- membrane Ig
- Ig-β:Ig-α
- Ig-α:Ig-β
- Signal transduction

= denotes disulfide bonds
regions present in these chains called complementarity-determining regions (CDRs) form pockets to bind antigen. Membrane Ig includes an extracellular domain, a single-pass transmembrane domain and a short cytoplasmic tail. Immature B cells express only mIgM on their surface whereas mature B cells express both mIgM and mIgD. The dual expressed mIgM and mIgD immunoglobulins have the same specificity for antigen. This is a result of the genomic organization of the $\mu$ and $\delta$ constant regions. Constant regions determine the isotype of the immunoglobulin expressed on the B cell surface. In the case of mIgM and mIgD, their constant regions are closely spaced and can be joined to the same upstream variable regions by alternative splicing. Once a B cell is stimulated by antigen, mature mIgM and mIgD expressing cells undergo heavy chain isotype switching. Now B cells can express mIgG, mIgA or mIgE as a result of DNA deletion of intermediary DNA.

Both Ig$\alpha$ and Ig$\beta$ are comprised of an extracellular domain, a single-pass transmembrane domain and a cytoplasmic domain (61 and 48 amino acids, respectively) (4). Ig$\alpha$ is a product of the Mb-1 gene and Ig$\beta$ is the product of the B29 gene (5,6,7). In addition to coupling the BCR into downstream signaling (see below, section 2.), Ig$\alpha$ and Ig$\beta$ are involved in receptor assembly and cell surface expression (8). Receptor expression studies were conducted by separately transfecting the five different heavy chain genes with or without the mb-1 encoding plasmid into the plasmacytoma cell line J558L. J558L transcribes both a $\lambda$ light chain gene and B29 but expresses neither mb-1 or an Ig heavy chain gene. From these studies it was established that cell surface expression of mIgM, mIgA and mIgE all require Ig$\alpha$ and Ig$\beta$. Although both mIgD and mIgG do associate with Ig$\alpha$ and Ig$\beta$, they can be expressed on the cell surface in absence of the heterodimer. Interestingly, it has been demonstrated that anti-Ig$\alpha$ immunoprecipitations from
surface-iodinated splenic B cells lysed in 1% digitonin lysis buffer which preserves mIg/Igαβ interactions removes all the surface mIgM and mIgD (9). Although the exact stoichiometry is not known, these results suggest that all of mIgM and mIgD expressed on the cell surface are in a complex with the Igαβ heterodimer.

Other molecules that interact with mIg were found under conditions that disrupt mIg/Igαβ associations. These ubiquitously expressed intracellular proteins are called BAP's (B cell associated proteins). Among BAPs it was demonstrated that mIgM associates with the known protein prohibitin (p32), which is a regulator of cell growth, a prohibitin-related protein (p37) and an unidentified protein (p41) (10). Other BAP's were described, p29 and p31, that preferentially associate with mIgD (11). The function of the BAP's is still unknown.

2. B cell Antigen Receptor - Signaling

Since the cytoplasmic tails of most membrane immunoglobulin are short (mIgM and mIgD contain only 3 aa), it would seem likely that the long cytoplasmic domains of Igα and Igβ would be involved in signal transduction (reviewed in 2.3). Indeed, Igα and Igβ contain in their cytoplasmic tails the immunoreceptor family tyrosine-based activation motif (ITAM), D/E-X_2-Y-X_2-L/I-X_7-Y-X_2 (12). This motif is present in other signal transducing subunits such as TCR-ζ, TCR-η, CD3γ, CD3δ, CD3ε, FceRIβ, and FcεRIγ (12). Once a receptor is stimulated, the two tyrosines present in the ITAM motif are rapidly phosphorylated (9,13). Indeed, Igα and Igβ were shown to become rapidly phosphorylated following BCR stimulation (9).

A number of subsequent studies implicated Igα and Igβ as the signaling units of the BCR. A strict correlation was found between the ability of mIgM mutants to associate with Igα and Igβ.
with their ability to transmit anti-IgM stimulated signals (14). Here, wild-type constructs were compared to the mutant constructs including Y^{587}S^{588}:VV, where the two conserved polar residues (YS) in the transmembrane region of human mIgM were mutated to two non-polar valines(VV). Y^{587}:F, a single residue mutation, S^{575}T^{576}:VV, a mutation in another polar region of mIgM, and cyto:Δ a mutation where the cytoplasmic tail of mIgM is deleted and the mIgM receptor becomes PI-linked. In calcium flux assays, cyto:Δ and YS:VV were the only two mutants that failed to transduce signals and failed to associate with Igαβ. However, the other constructs were capable of associating with Igαβ and inducing calcium fluxes characteristic of B cell signaling.

Finally, experiments have demonstrated that chimeric proteins containing the cytoplasmic domains of Igα or Igβ fused with truncated transmembrane CD8 receptors or mutated non-signaling mIgM (YS:VV) which is not capable of associating with the Igαβ heterodimer. mediate signal transduction (15-18). Namely, stimulation of these chimeric receptors triggers BCR signaling events such as tyrosine phosphorylation and calcium elevation.

Signaling through the BCR immediately triggers the phosphorylation of proteins through the activation of a number of protein tyrosine kinases. Targets of BCR induced phosphorylation besides Igα and Igβ include proteins from the three distinct signaling pathways including the phophoinositols, ras and phosphatidylinositol 3-kinase pathways (Figure 2. (reviewed in 2.3)). For example, phospholipase Cγ1 and γ2 (19), SHC, (20), rasGAP (GTPase activating protein) (21,22), p95^{vav} (23), and mitogen-activated protein (MAP) kinase (24) are all phosphorylated. When phospholipase Cγ1 and γ2 are phosphorylated, they are able to catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂). This results in the increase of two breakdown products diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). As a consequence, the serine/threonine kinase protein kinase C (PKC) is activated and intracellular calcium levels are
increased (25).

p21\textsuperscript{ras} is a regulator of cell growth and differentiation. BCR signaling increases the proportion of active or GTP-bound p21\textsuperscript{ras} in B cells (21). p21\textsuperscript{ras}'s activation is dependent on its regulators: rasGAP potentiates an increase in p21\textsuperscript{ras} GTPase activity causing it to hydrolyze its associated GTP to GDP (21), whereas the guanine nucleotide exchange factors (GNEFs) SOS (26) and p95\textsuperscript{av} (23) stimulate the release of GDP from p21\textsuperscript{ras}, allowing GTP to bind. Cross-linking of IgM induces inhibition of rasGAP activity. However, there is no evidence that the induced tyrosine phosphorylation of rasGAP alters its ability to stimulate p21\textsuperscript{ras} GTPase activity (22). In contrast, tyrosine phosphorylation of p95\textsuperscript{av} increases its \textit{in vitro} GNEF activity (23). Phosphorylation of the adaptor protein SHC has been suggested to be involved in recruiting SOS to the membrane associated p21\textsuperscript{ras} through interactions with another adaptor protein GRB-2 which is constitutively associated with SOS (20,26,27,28). Another p21\textsuperscript{ras} regulator includes the phosphatase CD45 (29). The activation of the serine/threonine kinase MAP kinase is important in B cell signaling. By phosphorylating the transcription factors c-myc, c-fos and c-jun, MAP kinase activation ultimately leads to gene expression (30).

In addition, activation of PI3-K occurs upon binding of SH3 domains of the protein tyrosine kinases Lyn and Fyn to a proline-rich region in the noncatalytic 85kDa subunit of PI-3K (31). Interactions with phosphorylated CD19, a B cell costimulatory molecule, also provide a means for activating PI3-K (32). Lipid products of activated PI3-K, specifically PI3,4,5-P\textsubscript{3} have been demonstrated to activate the \(\zeta\) form of PKC (33). Recently, the addition of PI3,4,5-P\textsubscript{3} micelles to platelets has been shown to uncap filamentous (F)-actin (34). The process of uncapping promotes actin assembly.
Figure 2 Signal Transduction Events Initiated by the B Cell Antigen Receptor
3. **Protein Tyrosine Kinases**

As discussed above, signaling through the B cell receptor immediately triggers the phosphorylation of a variety of proteins. However, the various components of the B cell receptor do not possess any intrinsic kinase activity. Therefore, the ability of the B cell receptor to associate with intracellular kinases is crucial in B cell activation events.

The BCR associates with two classes of tyrosine kinases which include the Src-protein-tyrosine kinases (PTKs) Lyn, Fyn, Lck and Blk (35), and the non Src-PTK Syk kinase (36). Src-PTKs possess a highly conserved primary structure (Figure 3, reviewed in 37)). Beginning at the amino-terminus, features include a Src homology 4 (SH4) domain, which allows association with the plasma membrane through sites of myristolation and palmitylation, a 50-80 aa stretch labelled as the unique domain since it is the most divergent sequence among Src members, a Src homology 3 (SH3) domain which binds to proline-rich sequences, a Src homology 2 (SH2) domain which interacts with tyrosine phosphorylated proteins. ATP binding and autophosphorylation sites comprise the catalytic region and finally a negative regulatory domain which includes a site for tyrosine phosphorylation. Unlike Src-PTKs, Syk kinase contains two SH2 domains, lacks the amino-terminus myristolation site and the negative regulatory tyrosine (Figure 3).

It has been demonstrated in *in vitro* binding studies that Src-PTKs interact with the resting BCR mainly through the first ten N-terminal amino acids of the kinase with the four amino acids DCSM present in the Igα ITAM. The equivalent position in Igβ is QTAT which does not bind SRC-PTKs as efficiently (38). Syk has been shown to interact with the resting receptor through the transmembrane/cytoplasmic domains of membrane immunoglobulin (36). As discussed above, tyrosine residues localized in the ITAMs of Igα and Igβ become phosphorylated following BCR
Figure 3 Structure Characteristics of Src-Family and Syk Protein Tyrosine Kinases

Src-Family Protein Tyrosine Kinases

Syk Protein Tyrosine Kinase
ligand-binding. Although the mechanism of ITAM phosphorylation is unclear, the prevailing belief is that the associated Src-PTKs may be the regulatory kinases responsible for phosphorylating these ITAMs. This idea is supported by the observation that cross-linking the transfected BCR on the nonlymphoid cell line AtT20 results in phosphorylation of Igα and Igβ (39). These cells express Src-family PTKs but do not express Syk (40). Whatever is the mechanism of Igαβ phosphorylation, it leads to an increased binding of both Src-PTKs and Syk kinase to the BCR through their SH2 domains (17,38,41). With the increased kinase binding to the cross-linked BCR there is also an enhancement in kinase activation (38,42,43).

In addition to BCR associations, kinases have been demonstrated to interact with other signaling molecules. Lyn, Fyn, and Blk were shown to interact with phospholipase γ2, rasGAP, PI3-K, and MAP kinase in vitro (44). In anti-IgM treated B cells, Lyn was shown to interact with phospholipase γ2, rasGAP (44), and PI3-K (45). Syk has been shown to interact directly with Lyn since Syk and Lyn coimmunoprecipitated from mature and activated B cell lines and gel-purified Syk and Lyn were shown to reassociate in vitro (46). Interestingly, it has been demonstrated that Syk activation is dependent on Src PTKs. Here, wild-type and Lyn-negative DT40 chicken B cell lines were transfected with Syk cDNA. BCR mediated tyrosine phosphorylation of Syk and its in vitro kinase activity were greatly reduced in the Lyn-negative cell lines (47). Cotransfections of Syk and Src-PTK cDNAs into COS cells also demonstrate the requirement of Src-PTKs to induce tyrosine phosphorylation and activation of Syk (48). Another study with COS cells demonstrates that cotransfections of Syk with Blk are required for Syk to associate with the transfected Igα chimera molecule (49). It is also believed that Syk may be the kinase responsible for phosphorylating the downstream signaling targets mentioned above. For
example, comparisons were made between Lyn-negative and Syk-negative B cell lines. It was shown that following anti-IgM stimulation, PLCγ-2 phosphorylation, IP₃ generation and calcium mobilization are dependent on Syk and not on Lyn, whereas Lyn appears to be involved in calcium mobilization independent of IP₃ (48). Furthermore, clustering chimeric receptors composed of a CD16 extracellular domain and a Src family kinase intracellular domain does not initiate a calcium response, whereas clustering of similar chimeras bearing Syk sequences as their cytoplasmic domains does trigger a calcium response in Jurkat cells (50).

4. B cell Receptor and the Cytoskeleton

In addition to signaling events discussed above, signaling through the BCR causes dynamic structural changes within the B cell cytoskeleton. The cytoskeleton is composed of three classes of fibers- the microtubules (20 nm in diameter), the intermediate filaments (10 nm in diameter), and the microfilaments (7nm in diameter). These fibrous proteins form an array throughout the B cell's cytoplasm. Studies have shown that microfilaments are composed primarily of actin and form a complex network called the membrane skeleton which is linked to the inner surface of the plasma membrane (51,52). The cytoskeleton has many functions. For example, it serves as structural support for the cell, is involved in cell motility and is thought to regulate the transduction of signals from the plasma membrane to the nucleus. One of the earliest changes to the cytoskeleton following receptor cross-linking is actin polymerization, as shown in human B cells (53). Within 1 minute, monomeric globular (G)-actin converts to filamentous (F)-actin and reaches a maximum 2-3 minutes after stimulation with anti-IgM antibodies.

Changes to the cytoskeleton are accompanied by alterations in mIgM localization following receptor cross-linking. Prior to cross-linking, mIgM is distributed randomly throughout the
plasma membrane (54). Immunofluorescence studies demonstrate formation of mIgM patches soon after mIgM is incubated with anti-IgM Abs (54). Patching is an energy-independent process which can occur at 4°C and in the presence of the metabolic inhibitor sodium azide. This event is followed by an energy-dependent movement of patches to one pole of the cell termed capping (54, 55). A number of components of the cytoskeleton have been found to co-cap with mIgM, including actin, tubulin (56), myosin (57), α-actinin (58), fodrin (59), and LSP1 (60).

Also shown to occur after receptor cross-linking is the cytoskeletal association of mIg (61-64). Early studies by Braun et al. describe the conversion of mIg from a nonionic detergent-soluble state to a detergent-insoluble state as a consequence of ligand binding. Woda and Woodin provided further evidence for an interaction between mIg and the actin-based microfilaments. Here, cytoskeleton associations could be disrupted by the treatment of insoluble fractions with DNase I which destabilizes F-actin filaments. More recently, we have confirmed the anti-IgM induced actin-based microfilament association of mIgM by electron microscopy, FACS analysis and Western blotting of the detergent-insoluble cytoskeleton (65). Here, electron microscopy was utilized on B cells with mIgM labelled by immunogold. Following mechanical unroofing of the cells, many of the gold particles were found in linear rows juxtaposed to actin filaments lying along the inner surface of the plasma membrane. We found that the mIgM/cytoskeleton interaction which requires cross-linking of mIgM occurs within the first minute of induction and reaches a plateau by 5-10 min. Furthermore, we demonstrated that the cytoskeletal association of mIgM does not require the BCR associated Igαβ heterodimer. Experiments performed by Mr. Jun Young Park with HeLa cells transfected with the mIgM mutant MutA which allows mIgM expression with the absence of Igα/β, indicate that no other B cell specific components are
required for mIgM/cytoskeleton interactions.

Some correlations have been demonstrated between capping, which is a cytoskeleton driven event and the cytoskeletal association of mIg. Only antibodies capable of inducing capping could also stimulate an interaction between mIg and the cytoskeleton (64). Therefore, capped mIg most likely represents cytoskeleton associated mIg. Further studies found a correlation between capping and mitogenicity (66). Although limited, these results suggest a link between mIg association and B cell activation.

It is also conceivable that mIgM interactions with the cytoskeleton are involved in receptor internalization. Internalization occurs after the cytoskeletal associated mIgM receptor has capped (55). Similar to mIgM cytoskeletal association, capping (67) and internalization do not require Igαβ associations and can occur in the absence of the heterodimer (68-70). Furthermore, both capping (71) and internalization (71,72) depend upon the activity of protein tyrosine kinases, suggesting cooperations between cytoskeleton and signaling events.

Since our understanding of the functional role of mIg associations with the cytoskeleton is limited, we can turn to other cellular systems that investigate interactions between receptors and the cytoskeleton for significance. Evidence from other cellular systems supports the involvement of the cytoskeleton in receptor mediated signal transduction (discussed below).

5. Other Cellular Systems and the Cytoskeleton

One of the most extensively studied cellular systems for receptor/cytoskeleton interactions are platelets. Once the integrin receptor GPIIb-IIIa present on platelets is stimulated with thrombin, it accumulates in the detergent-insoluble cytoskeletal fraction (73). Signaling molecules such as phospholipase C (74), rasGAP, pp60c-src, pp62c-yes (73), PI3-K (75). Syk (76),
and PTP-1C (77) are also relocalized to the detergent-insoluble cytoskeleton fraction following thrombin stimulation. Activities of phospholipase C (74) and PI3-K (75) were shown to be enriched in the cytoskeleton-rich fractions.

Another receptor that associates with the cytoskeleton is the Epidermal Growth Factor Receptor (EGF) receptor. Both the kinase activity and ligand affinity of the detergent-insoluble cytoskeleton associated EGF receptor is higher than the EGF receptor localized in the detergent-soluble fraction (78, 79). It thus appears the EGF receptor's association with the cytoskeleton plays a role in mediating signal transduction. The specific activities of cytoskeleton bound PI3-K, phosphatidylinositol phosphate kinase and diacylglycerol kinase increased significantly with time of stimulation in human epidermal carcinoma cells expressing EGF receptors (80). It has been suggested that cytoskeleton relocalization of signaling molecules could serve to enhance their activities by providing optimal spacing for interactions and promote downstream signaling events.

Other receptors of the immune system such as the T cell receptor (TCR) (81) and the high-affinity receptor for immunoglobulin E (FceRI) (82) form associations with the cytoskeletal matrix following stimulation. Specifically, the ζ chain of the TCR has been demonstrated to associate with the cytoskeleton through the third ITAM if the distal tyrosine is phosphorylated (83). This cytoskeletal association is correlated with IL-2 production. Also following TCR stimulation both spectrin and PKC translocate from the cytoplasm to the detergent-insoluble cytoskeleton fraction (84). Other examples include molecules that interact with the TCR such as CD45 (85) and CD2 (86). The phosphatase activity of CD45 was regulated by its interactions with the cytoskeleton proteins fodrin and spectrin (85). CD2 was observed to coimmunoprecipitate
with tubulin (86). All of these examples point to cytoskeleton involvement in receptor signaling events.

6. Hypothesis

Based on the evidence from other cellular systems demonstrating that active signaling molecules accumulate in the cytoskeleton fraction following stimulation through various receptors, I propose that this phenomenon is likely to occur in activated B cells. One can envisage the BCR acting as a vehicle to translocate the associated kinases and their downstream targets to the cytoskeleton during mIgM associations with the cytoskeletal matrix. The formation of large aggregates of signal transducing molecules on the cytoskeletal framework of B cells may provide localized concentrations of enzymes, substrates and structural molecules which facilitate the activation of signaling pathways and the reorganization of the cytoskeleton required for B cell activation.

As discussed above, we have demonstrated that mIgM is the only known component of the BCR which associates with the cytoskeleton following stimulation. I propose that in B cells the Igαβ heterodimer will accumulate in the cytoskeleton fraction of stimulated B cells along with cross-linked mIgM. If the accumulation of Igαβ in the cytoskeleton promotes signaling in this fraction I predict that Igα found in the cytoskeleton fraction will be tyrosine phosphorylated. During active signaling processes, the BCR associated tyrosine kinases such as Lyn and Syk should be recruited to the membrane skeleton through interactions between tyrosine phosphorylated Igα and their SH2 domains. In this study I am investigating the presence of tyrosine phosphorylated Igα, Lyn, Syk and of other substrates in the membrane proximal fraction of the cytoskeleton at early time points of anti-IgM stimulation in a transformed B cell line Bal
17.7.1. This work will form the basis for future studies to establish whether the concentration of signaling molecules immobilized on the cytoskeletal matrix enhances B cell activation.
Chapter II: Materials and Methods

1. Cells

The mouse lymphoma line BAL 17.7.1 (mIgM\(^+\), mIgD\(^-\), a gift from Dr. R. Asofsky, Bethesda, MD) and the plasmacytoma line J46 (mIgM\(^-\), Igα\(^-\)) were maintained in culture in RPMI with 10% heat inactivated FCS (Sigma Chemical Co., St. Louis, MO), 2mM glutamine, 100 U/ml penicillin/streptomycin, 0.1 mM nonessential amino acids (Life Technologies Inc., Grand Island, NY) and 4.8 \(\times\) \(10^{-5}\) M β-mercaptoethanol (ICN Biomedicals Inc., Aurora, OH). J46 cells derive from J558L (mIg\(^-\), a gift from R. Tepper, Charlestown, MA) which our laboratory transfected with the mutated μ-heavy chain plasmid mIgM-MutA (87), (a gift from Dr. M.S. Neuberger, Medical Research Council, Cambridge, UK). Cells were tested negative for mycoplasma using a PCR detection assay (developed by Dr. J. Cambier's laboratory, Denver, CO).

2. Antibodies

Several abs were used for Western blot analysis. Purified goat anti-mouse μ Abs were purchased from the Jackson ImmunoResearch Laboratories, West Grove, PA. Rabbit anti-mouse Igα serum (a gift from Dr. J. Jongstra, Toronto, Canada) was affinity-purified in our laboratory by passage over CNBr activated Sephadex columns to which recombinant Mb-1 protein was bound. Mouse anti-rabbit actin mAb 14.3 was a gift from D. Aunis, Strasbourg, France. Rabbit anti-Syk antiserum was generated against a trp-E fusion protein containing the Syk hinge region (a gift from Dr. A. Veillette, Montreal, Canada). Rabbit anti-Lyn antiserum was generated against a fusion protein containing the divergent amino terminal region of murine Lyn (a gift from Dr. J. Cambier, Denver, CO). 4G10 a mouse monoclonal Ab to phosphotyrosine was purchased from Upstate Biotechnology Incorporated Lake Placid, NY. Secondary abs used for Western blotting
included donkey anti-rabbit Ig peroxidase-conjugated Abs (Amersham Corp., Arlington Heights, IL), rabbit anti-goat Ig peroxidase-conjugated Abs (Calbiochem, La Jolla, CA), and goat anti-mouse Ig peroxidase-conjugated (Jackson ImmunoResearch Laboratories) Abs. Rabbit anti-mouse μ sera (a gift from Dr. C. Paige, Toronto, Canada) was used for immunoprecipitations. In the co-capping experiments mouse IgG blocking Abs (Sigma Immuno Chemicals, St. Louis, MO), FITC-conjugated affinity-purified F(ab’)2 fragments of goat anti-mouse μ Abs (Jackson ImmunoResearch Laboratories), affinity-purified rabbit anti-mouse Igα Abs (mentioned above) and Lissamine Rhodamine-conjugated goat anti-rabbit Abs (Jackson ImmunoResearch Laboratories) were used. Cross-linking of the mouse mIgM receptors was performed using affinity-purified F(ab’)2 fragments of goat anti-mouse μ Abs (Jackson ImmunoResearch Laboratories).

3. Biotinylation of Bal 17.7.1 Cells

Biotinylation was performed with NHS-LS-biotin (Pierce, Rockford IL). Cells were washed three times with ice-cold PBS and resuspended (1 x 10^7 cells/ml) in PBS and freshly prepared biotin (final concentration, 100μg/ml in PBS). After end-over-end mixing for 30 min at 4°C, cells were washed three times with PBS and lysed for immunoprecipitations (65).

4. IgM Immunoprecipitations from BAL 17.7.1 Lysates

Biotinylated cells were lysed (10^7 cells/ml) with 0.25% Maltoside (Anatrace, Maumee, OH) or 2% Nonidet P-40 (BDH Inc., Toronto, Canada) containing lysis buffer (LB) with protease inhibitors (14μg/ml aprotinin, 1μg/ml pepstatin, 2μg/ml leupeptin, 1mM PMSF, and 1mM sodium orthovanadate all from Sigma Chemical Co.) for 30 min on ice. After centrifugation at 14,000 x g for 10 min, immunoprecipitations were performed by incubating the detergent-soluble lysates
(3 x 10⁶ cell equivalents) with rabbit anti-mouse μ serum (1:200) or non-immune rabbit serum (1:200) for 2 h at 4°C, followed by incubation with protein A-Sepharose beads (1:20, Sigma Chemical Co.) for 30 min at 4°C. The beads were collected by centrifugation at 14,000 x g for 4 min. Immune precipitates were washed three times with LB containing protease inhibitors and then subjected to a final wash in Tris-buffered saline (20mM Tris-HCl pH 7.2 and 150 mM NaCl) (88). Protein was recovered by addition of Laemmli sample buffer (89) to the washed pellet, followed by immersion in boiling water for 5 min. The sample was centrifuged for 5 min and the supernatant was collected for analysis by SDS-PAGE.

5. Immunofluorescence Microscopy

This analysis was based on methods described by Klein et al. (90, 60). Bal 17.7.1 or J46 cells (1 x 10⁶) were incubated with mouse IgG blocking Abs (10μg/ml) for 10 min at 4°C followed by incubation with FITC-conjugated affinity-purified F(ab')₂ fragments of goat anti-mouse μ antibodies (30μg/ml) for 30 min at 4°C. The cells were washed 2x with HBSS + 2% FCS (HF) and subjected to either capping (20 min, 37°C) or noncapping (20 min, 4°C, in the presence of 0.1% sodium azide) conditions. Immunofluorescence on permeabilized cells was performed on cytospin preparations. Glass slides used in the cytospin preparations were pre-coated with Poly-L-Lysine (Sigma Chemical Co.). Cells (2.5 x 10⁵) were loaded in 1 ml of PBS in each of three slots in an IEC Cytobucket and spun at 4°C for 10 min at 500 rpm. The cells were then permeabilized and fixed with ice-cold methanol for 10 min, air dried for 2 hr, and stained for 30 min with or without affinity-purified rabbit anti-mouse Igα antibodies diluted to 1μg/ml in HBSS. After the cells were washed in PBS, Lissamine Rhodamine-conjugated goat anti-rabbit Abs were used at a dilution of 1:200 to stain for 30 min, followed by a wash in PBS. Photographs were taken by the
Leica DMRB immunofluorescence microscope with photoautomat (model WILD MPS 48) using a 100x PL Fluotar oil immersion objective (Leica, Willowdale, Canada).

6. Subcellular Fractionation of Bal 17.7.1 Cells

The analysis was based on methods described by Klein et al. (90, 60) and Saxton et al. (20). A flow chart of the protocol is depicted in Figure 4. Cells were resuspended to a concentration of 50 x 10⁶/ml in OPTI-MEM (Life Technologies Inc.). Approximately 15 x 10⁶ cells were required for each time point tested. After 10 min at 37°C, cells were stimulated for indicated times by adding 30μg/ml affinity-purified F(ab')₂ fragments of goat anti-mouse μ Abs. Control samples were incubated in the absence of Abs. Reactions were stopped by adding the cells to ice-cold PBS containing phosphatase inhibitors (1mM sodium orthovanadate, 50mM NaF (BDH Inc.) and 10nM sodium molybdate (Sigma Chemical Co.)). The cells were pelleted by centrifuging at 8000 x g for 15 s. Cell pellets were washed once with the same buffer and then resuspended in homogenization buffer (137 mM NaCl, 20mM Tris-HCl, 1mM MgCl₂, 10% glycerol) containing protease and phosphatase inhibitors (14 μg/ml aprotinin, 1μg/ml pepstatin, 2μg/ml leupeptin, 1mM PMSF, 1mM sodium orthovanadate, 50mM NaF and 10nM sodium molybdate) to a concentration of 10 x 10⁶ cells/ml. Cells were homogenized on ice with a hand held Wheaton homogenizer (VWR Scientific, London, Canada) and then viewed under a microscope to assure that more than 95% of the cells were disrupted. Nuclei were removed by centrifuging at 14,000 x g for 15 s at 4°C. Nuclei were washed with LB containing protease and phosphatase inhibitors (see above), solubilized in Laemmli sample buffer and immersed in boiling water for 5 min. The postnuclear supernatant was centrifuged at 100,000 x g (31,500 rpm, SW 60 rotor) for 60 min at 4°C to isolate the membrane fractions. Following the ultracentrifugation, the soluble cytosolic
Figure 4 Subcellular Fractionation

Bal 17.7.1 cells

- Stimulate, homogenize, spin

- SNA
  - Ultraspinn
    - C (cytosolic)
      - (detergent soluble)
    - PM (membrane pellet)
      - Solubilize, spin
        - S1
          - (detergent soluble)
        - P1
          - (detergent insoluble membrane skeleton)
supernatant was removed. The plasma membrane containing pellet was solubilized with either 0.25% Maltoside or 2% Nonidet P-40-containing LB (10 x 10⁶ cell equivalents/ml) with protease and phosphatase inhibitors (see above) for 30 min and centrifuged for 3 min at 14,000 x g. The resulting membrane soluble supernatant fraction was added to Laemmli sample buffer and immersed in boiling water for 5 min. The detergent-insoluble membrane skeleton fraction was washed once with LB containing all inhibitors (see above), resuspended in Laemmli sample buffer (10 x 10⁶ cell equivalents/100μL) and immersed in boiling water for 5 min. Aliquots of total, postnuclear supernatant, cytosolic and the solubilized membrane pellet fractions were withdrawn and prepared for SDS-PAGE analysis by adding to Laemmli sample buffer and immersing in boiling water for 5 min.

To confirm that proteins found in the membrane skeleton are results of interactions with actin based microfilaments, the calcium-dependent, actin severing protein gelsolin was utilized. In these experiments, the plasma membrane containing pellet was solubilized with 0.25% Maltoside containing LB with all inhibitors (see above). 5mM ATP, 0.5mM CaCl₂, and 1μM purified human recombinant gelsolin (a gift from Dr. P. Janmey, Boston, MA).

7. Western Blotting

Samples were separated by SDS-12.5% PAGE and transferred to Immobilon P membranes (Millipore Corp., Bedford MA) as described by Towbin et al. (91). Membranes were soaked in methanol and then blocked in 3% gelatin blocking solution (see stock solutions for composition) for 1 hour. Membranes were washed 1 x 15 min and 2 x 5 min in Tris-buffered saline + 0.1% Tween-20 detergent (TBS-T, see stock solutions for composition). The appropriate primary Ab was diluted in 1% gelatin Ab buffer (see stock solutions for composition) and membranes were
incubated for 1 hour at room temperature. Membranes were washed 1 x 15 min and 2 x 5 min in TBS-T. The corresponding HRP-conjugated secondary Ab was diluted in 1% gelatin Ab buffer and the membranes were incubated for 1 hour at room temperature. Immunopure streptavidin-horseradish peroxidase-conjugated probes were used to detect biotinylated proteins (Pierce). After the incubation, membranes were washed 1 x 15 min and 6 x 5 min followed by 1 min incubation with ECL. Dupont Reflection™ films (Dupont, Boston, MA) were used for autoradiography. To quantitate the amount of membrane IgM associated with the membrane skeleton, a fixed amount of membrane skeleton sample was analyzed with a range of the total or membrane pellet samples representing known cell equivalents as standards. Autoradiograms were scanned using the Bio-Rad Imaging Densitometer (model GS-670, BioRad Laboratories, Richmond, CA). Results are expressed as the mean ± SD, n=3.
8. Stock Solutions

TBS 10x pH 7.6, 1L
Trizma base 24.2g
NaCl 292.4g
adjust pH with concentrated HCl
adjust volume with distilled H₂O

TBS-T, 1L
TBS 10x 100ml
Tween-20 1ml
adjust volume with distilled H₂O

3% Gelatin - Blocking Solution, 500ml
TBS 10x 50ml
Tween-20 500µl
gelatin 15g
10% sodium azide 1ml
adjust volume with distilled H₂O

1% Gelatin - Antibody Buffer, 100ml
TBS 10x 10ml
Tween-20 100µl
gelatin 1g
adjust volume with distilled H₂O

Electrode Running Buffer 10x, 1L
Trizma base 30.3g
Glycine 144.0g
adjust volume with distilled H₂O

Laemmli Sample Buffer 5x, 100ml
glycerol 16.5ml
β-mercaptoethanol 8.5ml
10% SDS 50.0ml
Tris-Cl (pH 6.8) 21.0ml
adjust volume with distilled H₂O, add Bromophenol blue until colour is dark blue

Lysis Buffer
50mM Tris-HCl pH 7.2
150mM NaCl
1mM MgCl₂
2% Nonidet-40 or 0.25% Maltoside
Chapter III: Results

1. Igα co-caps with mIgM

As mentioned in the introduction, although Igα does not have an intrinsic affinity for the cytoskeleton (65) I propose that it will accumulate in this fraction following anti-IgM stimulation when the interactions between mIgM and Igα are maintained. Therefore, it was important to examine if Igα exhibited the same cellular distribution as cytoskeleton associated mIgM. Previously, interactions between mIgM and Igα have only been described by co-immunoprecipitation assays using the detergent-soluble fraction of B cells. The disulfide-linked Igα/β heterodimer co-precipitates with mIgM when B cells are lysed with mild detergents such as CHAPS (2), digitonin and Maltoside (15) which preserves these noncovalent associations. However, these results do not indicate whether Igα interacts with cytoskeleton associated mIgM. By utilizing immunofluorescence microscopy on capped Bal 17.7.1 cells, I could address this issue at the cellular level.

Since mIgM association with the cytoskeleton is suggested to be essential to capping (64), capped mIgM most likely represents cytoskeletal associated mIgM. The time frame of these two events are similar. Capping of mIgM occurs within 15 to 20 minutes after incubation of Bal 17.7.1 cells with anti-IgM Abs at 37°C (54, see Figure 1) a known time point of mIgM/cytoskeleton associations (65).

Bal 17.7.1 cells (IgM-, IgD-) treated with FITC-conjugated anti-IgM Abs illustrate the patched distribution of mIgM on non-capped cells (5a) compared to the polarized localization of mIgM on capped cells (5c). In both non-capping and capping conditions, Lissamine rhodamine-conjugated anti-Igα Abs overlap the staining of mIgM tightly (5b,d). These results demonstrate that Igα co-caps with mIgM. Since the majority of Igα is shown to co-localize with the cytoskeletal...
Figure 5. Igα co-caps with mIgM
Bal17.7.1 or J46 cells were stained with 30μg/ml FITC-conjugated F(ab')₂ fragments of goat anti-mouse μ Abs under noncapping conditions (a) or capping conditions (c,f). Cytospin preparations were then prepared and stained with 1μg/ml rabbit anti-mouse Igα Abs and Lissamine Rhodamine-conjugated goat anti-rabbit Abs at a dilution of 1:200 as described in Materials and Methods (b,d,g) or Lissamine Rhodamine-conjugated goat anti-rabbit Abs at a dilution of 1:200 alone (e,h).
bound mIgM. these results suggest that Igα translocates to the cytoskeleton as part of the B cell receptor complex.

A number of control experiments were conducted to ensure the specificity of Abs. Bal 17.7.1 cells were treated with FITC-conjugated anti-IgM Abs. capped and stained with Lissamine Rhodamine-conjugated Abs without prior incubation with anti-Igα Abs. The secondary stain was not detected (5e). Membrane IgM and Igα stainings were conducted on J46 cells (mIgM+Igα-) as an anti-Igα antibody specificity control. J46 cells derive from the plasmacytoma line J558L (mIgM+Igα-). These cells were transfected in our laboratory with mutated mIgM (MutA) which allows mIgM surface expression in the absence of Igα due to the mutations of the polar amino acids in the IgM-transmembrane domain (65.87). J46 cells were stained with FITC-conjugated anti-IgM Abs under capping conditions (5f) and treated with (5g) or without (5h) anti-Igα Abs followed by staining with Lissamine rhodamine-conjugated Abs. As expected, J46 cells stained for mIgM (5f) but did not stain for Igα (5g,h) indicating that Igα staining overlapping mIgM caps in Bal 17.7.1 cells is specific.

Although Bal 17.7.1 cells express both mIgM and mIgD, the vast majority of Igα was found co-capped with mIgM. This can be explained by the fact that in the subclone 7.1 of Bal 17 cells the expression of mlgD is low as determined by FACS analysis in our laboratory. Using the same anti-IgM and anti-IgD Abs on resting murine B cells, we have found that similar populations of cells stained equally high for both mIgM and mIgD eliminating the possibility that differences observed in Bal 17.7.1 cells were due to inefficient Ab staining for IgD.
2. Isolation of the membrane skeleton bound mIgM by subcellular fractionations of Bal 17.7.1 cells

The membrane skeleton is defined as the portion of the cytoskeleton proximal to the plasma membrane which requires high g forces to be sedimented (92). It is here that interactions between transmembrane cell surface proteins such as the B cell receptor and components of the cytoskeleton occur. In order to study the accumulation of BCR components and associated proteins to the cytoskeleton, I isolated the membrane skeleton of Bal 17.7.1 cells. To confirm the subcellular fractionation protocol was indeed purifying distinct portions of the B cells and to quantitate the yields of each fraction, I compared all of the fractions for the presence of membrane and intracellular IgM. I used the membrane form of IgM as a membrane marker while the intracellular form of IgM was used as a cytoplasmic marker. The anti-IgM Abs I utilize for Western blotting are able to detect these two forms of IgM. By analyzing Bal 17.7.1 lysates on a 10% gel the higher band (membrane form of IgM) was determined as 87 kDa and the lower band (intracellular form of IgM) was determined as 82 kDa. The identification of these two bands was previously established in the laboratory by immunoprecipitation experiments where B cells were either metabolically or surface labelled (65). As shown in Figure 6.1 I analyzed all of the fractions obtained throughout the procedure together with total, unfractionated cells with anti-IgM Abs by Western blotting. Percentages given are based on three independent experiments from Bal 17.7.1 cells stimulated for 10 min at 37°C. After homogenization, 16% ± 5.3 of IgM (membrane and intracellular forms) is recovered in the nuclear pellet (lane N) presumably representing protein from remaining intact cells. As follows, the postnuclear supernatant (lane SNA) recovered 78% ± 4.4 of IgM (membrane and intracellular forms). After the ultracentrifugation of SNA, a distinct separation of the cytoplasmic and membrane...
Figure 6. Subcellular fractionations isolate membrane skeleton bound mlgM
Unstimulated (A) or stimulated (B, 10 min with 30 μg/ml F(ab')₂ fragments of goat anti-mouse μ Abs at 37°C) Bal 17.7.1 cells were homogenized and subcellular fractions obtained as described in Materials and Methods. Total, nuclear (N), postnuclear supernatant (SNA), cytosolic (C), membrane pellet (PM), 2% NP-40 insoluble membrane skeleton (P1) and 2% NP-40 soluble membrane fractions (S1) were analyzed (2 x 10⁵ cell equivalents) by Western blotting with goat anti-mouse IgM Abs at a dilution of 1:2000 followed by rabbit anti-goat Ig HRP-conjugated Abs at a dilution of 1:25000. Numbers on the right of each panel correspond to the positions of the molecular mass markers in kDa (Bio-Rad low m.w. prestained SDS-PAGE standards).
fractions of the material occurs. 76% ± 3.5 of the cytoplasmic form of IgM was recovered in the cytosolic supernatant fraction (lane C) and no membrane form was detected, whereas 75% ± 4.4 of the membrane form of IgM was recovered in the membrane pellet fraction (lane PM). Whether the fractions were isolated from stimulated (A) or unstimulated (B) cells, the distribution of IgM was similar in these fractions. The membrane pellet fraction (lanes PM) from unstimulated or stimulated cells was solubilized with 2% NP-40 containing LB. In unstimulated cells mIgM is not expected to associate with the membrane skeleton. From three independent experiments from unstimulated cells, 72% ± 1.8 of total mIgM is found in the membrane soluble fraction (S1) and less than 5% of mIgM is detected in the membrane skeleton fraction (P1). In contrast, in stimulated cells 54% ± 3.5 of total mIgM is found in the membrane skeleton detergent insoluble fraction (P1) and 21% ± 1.3 of total mIgM is found in the membrane soluble fraction (S1). However, if we take into account the 75% total yield of the membrane pellet fraction and the 25% loss, the amount of total mIgM found in S1 fractions from unstimulated cells is 96% and the actual binding of mIgM in the membrane skeleton fraction is 72% of total mIgM. The mIgM found in the membrane skeleton fraction of stimulated cells should represent mIgM associating with actin containing membrane skeleton. Further experiments were conducted confirming cytoskeletal associations. These results will be discussed in further detail (see Figures 10,11).

Our previous studies yielded average values ranging from 40 to 80% of total mIgM being recovered in detergent-insoluble cytoskeleton-rich pellets following cross-linking of mIgM (65). In these experiments I lysed stimulated cells in 1% NP-40 containing lysis buffer and the detergent-insoluble bulk cytoskeleton fraction was analyzed by Western blotting. Although the methods of analysis are different, the values of cytoskeletal bound mIgM I obtained through subcellular
fractionations are comparable to my previous data (65). In the subcellular fractionations, 2% NP-40 was required to solubilize the membrane pellet fractions in order to optimally disrupt mIgM/Igα interactions (see Results Section 4, Figures 8 and 9).

3. Maltoside preserves, NP-40 disrupts mIgM/Igα interactions

Based on our hypothesis and supported by the co-capping experiments (see Figure 5), we predicted that Igα will be found in the membrane skeleton only under conditions in which interactions between IgM and Igα are preserved. It was therefore important to find the conditions under which IgM/Igα interactions remain intact.

Immunoprecipitations were conducted to compare the properties of the two detergents, NP-40 and Maltoside. NP-40 is a non-ionic detergent known to disrupt mIgM/Igα interactions (1). Maltoside, a milder detergent, preserves these noncovalent associations (15). I tested a range of Maltoside concentrations (0.125% to 1%) to establish the minimum detergent conditions that would lyse Bal 17.7.1 cells as observed by light microscopy in order to preserve maximum interactions between mIgM and Igα. I found that 0.25% Maltoside is the minimum Maltoside concentration which totally lyses Bal 17.7.1 cells, such that only nuclei remain after 30 min at 4°C. Next, I verified mIgM/Igα interactions under these lysis conditions as compared to NP-40 lysis by immunoprecipitations. I biotinylated Bal17.7.1 cells prior to immunoprecipitations in order to detect interactions between the membrane forms of IgM and Igα. The results indicate that anti-IgM Abs immunoprecipitate the membrane form of IgM from cells lysed in either 2% NP-40 or 0.25% Maltoside to the same extent (Figure 7a, lanes 1-3). However, a biotinylated protein with molecular weight of 35kDa, as revealed by streptavidin-HRP, was found to co-immunoprecipitate with mIgM only in Maltoside lysates
Figure 7. Maltoside preserves, NP-40 disrupts mIgM/Igα interactions
Bal17.7.1 cells were biotinylated, lysed with either 2% NP-40 (lanes 1, 2) or 0.25% Maltoside (lanes 3, 4) and immunoprecipitated with rabbit anti-mouse μ sera (lanes 1, 3) or non-immune serum (lanes 2, 4) both at dilutions of 1:200. Immunoprecipitates were analyzed by Western blotting with a) HRP-conjugated streptavidin at a dilution of 1:2500 or b) rabbit anti-mouse Igα Abs at a dilution of 1:2000 followed by donkey anti-rabbit Ig HRP-conjugated Abs at a dilution of 1:10000. Position of the m.w. markers in kDa are shown between the figures. Arrow head (7a) indicates Igβ band.
(Figure 7a, lane 3) and not in NP-40 lysates (Figure 7a, lane 1). The same samples were analyzed with anti-Igα Abs by Western blotting which identified the 35kDa protein in anti-IgM immunoprecipitates from Maltoside lysates as Igα (Figure 7b). As controls, the NP-40 and Maltoside lysates were incubated with non-immune rabbit serum which did not immunoprecipitate IgM or Igα (Figure 7a, lanes 2, 4). The results confirm that NP-40 disrupts whereas Maltoside maintains interactions between mIgM and Igα. These two different lysis buffers were then utilized for membrane pellet solubilizations. A comparison of their effects on mIgM and Igα localization within the soluble and insoluble (membrane skeleton) was studied.

4. Igα translocates to the membrane skeleton as part of the BCR complex

The results of Figure 8 compare mIgM and Igα distributions in the membrane skeleton (P1) and membrane soluble fractions (S1) following either 2% NP-40 or 0.25% Maltoside solubilization of the membrane pellet (PM). In stimulated cells (Figure 8b), the localization of mIgM to the membrane skeleton fraction is independent of the detergent used to solubilize the membrane pellet (lanes P1). However, the localization of Igα following membrane pellet solubilization is dependent on the detergent utilized at this step. Maltoside solubilization, which preserves mIgM/Igα interactions, results in both mIgM and Igα being recovered in the membrane skeleton fraction (lanes P1) of stimulated cells. In contrast, NP-40 solubilization disrupts mIgM/Igα interactions. Although cytoskeleton associated mIgM translocates to the membrane skeleton fraction (lane P1), most of Igα is localized in the membrane soluble fraction (lane S1) of stimulated cells. It should be noted that the S1 lanes of anti-IgM panel in Figure 8b are anomalous results. Subsequent experiments indicate that approximately one third of the amount of mIgM found in the P1 fraction is present in the S1 fractions of stimulated cells (see Figure 9).
Figure 8. Igα translocates to the membrane skeleton as part of the BCR complex

The membrane pellet (PM) from unstimulated (A) or stimulated (B. 10 min with 30μg/ml F(ab')2 fragments of goat anti-mouse μ Abs at 37°C) Bal 17.7.1 cells were solubilized with LB containing 2% NP-40 or 0.25% Maltoside yielding membrane skeleton (P1) and membrane soluble (S1) fractions. The above and cytosolic (C) fractions were analyzed (2 x 10⁶ cell equivalents) by Western blotting with anti-IgM and anti-Igα Abs as described in Figures 6 and 7. Position of the m.w. markers in kDa are shown on the right.
I also analyzed the distribution of mIgM and Igα in the fractions from unstimulated cells (Figure 8a). I found that both mIgM and Igα were found in the membrane soluble fractions (lanes S1) following either NP-40 or Maltoside detergent solubilization of the membrane pellet.

Figure 9 summarizes the recoveries of mIgM and Igα found in the membrane skeleton and membrane soluble fractions following detergent solubilization of the membrane pellet. A fixed amount of membrane skeleton (P1) and membrane soluble (S1) samples (2 x 10⁶ cell equivalents) were analyzed together with membrane pellet (PM) samples representing a range of known cell equivalents as standards. The fractions were analyzed by Western blotting with anti-IgM and anti-Igα Abs followed by densitometric analysis. Percentages generated are mean values from three independent experiments conducted on Bal 17.7.1 cells stimulated for 10 min at 37°C. Membrane pellet fractions from stimulated cells solubilized with Maltoside or NP-40 resulted in 76% ± 5.3 and 72% ± 2.2 respectively, of membrane pellet mIgM accumulating in the membrane skeleton fraction.

71% ± 1.8 of membrane pellet Igα was found in the membrane skeleton after Maltoside solubilization compared to 27% ± 3.1 found in the membrane skeleton following NP-40 solubilization. These results indicate that Igα localization to the membrane skeleton fraction correlates with its association with mIgM. Therefore, in the cell Igα most likely translocates to the membrane skeleton as part of the B cell receptor complex. Co-capping experiments support this conclusion (see Results Section 1. Figure 5).

5. Gelsolin releases mIgM and Igα from the membrane skeleton

The calcium-dependent actin severing protein gelsolin was utilized in the subcellular fractionation experiments to confirm mIgM/cytoskeletal interactions in the membrane skeleton. Gelsolin is a 90 kDa protein which shortens actin filaments only in the presence of micromolar
Figure 9. Distribution of mIgM and Igα in the membrane skeleton and membrane soluble fractions following detergent solubilization of the membrane pellet
Bal 17.7.1 cell were stimulated (10 min with 30μg/ml F(ab')2 fragments of goat anti-mouse μ Abs at 37°C), homogenized and subcellular fractions obtained as described in Materials and Methods. Membrane pellets were either solubilized with 2% NP-40 or 0.25% Maltoside. The percent of total membrane pellet mIgM (white bars) and Igα (black bars) found in the membrane skeleton (P1) and membrane soluble (S1) fractions were determined. Values are expressed as the mean ± SD, n = 3.
calcium ion concentrations (93). Its ability to sever actin was utilized at the stage of membrane pellet solubilization in the subcellular fractionation protocol. The rationale for including gelsolin in the Maltoside solubilization buffer was based on the assumption that mIgM found in the membrane skeleton fraction of stimulated cells was due to indirect or direct interactions with actin. If actin is severed, the filamentous network responsible for mIgM/cytoskeleton interactions will be destroyed. As a result, mIgM will be released from the membrane skeleton and localized to the membrane soluble fraction.

The results generated for Figure 10 are based on averaged values from two independent experiments conducted on Bal 17.7.1 cells stimulated for 10 min at 37°C. A fixed amount of membrane skeleton (P1) and membrane soluble (S1) samples (2 x 10^5 cell equivalents) generated in the presence or absence of gelsolin were analyzed together with membrane pellet (PM) samples representing a range of known cell equivalents as standards. The fractions were analyzed by Western blotting with anti-IgM and anti-Igα Abs followed by densitometric analysis.

When the membrane pellet of stimulated cells is solubilized with Maltoside containing LB, 78% of membrane pellet mIgM and 71% of membrane pellet Igα were found localized in the membrane skeleton fraction. Significant amounts of mIgM and Igα were released from the membrane skeleton following membrane pellet solubilization with gelsolin-containing Maltoside-LB. Namely, 42% of IgM and 51% of Igα accumulating in the membrane skeleton were released from the membrane fraction following the treatment. The reason why only half of mIgM and Igα were shown to be released from the membrane skeleton following gelsolin treatment of the membrane pellet may be that the complex network of cytoskeletal elements may not allow all parts of actin filaments to be equally accessible to gelsolin severing. Indeed, it appears that based on two
Figure 10. Gelsolin releases mIgM and Igα from the membrane skeleton
Bal 17.7.1 cell were stimulated (10 min with 30μg/ml F(ab')2 fragments of goat anti-mouse μ Abs at 37°C), homogenized and subcellular fractions obtained as described in Materials and Methods. Membrane pellets were either solubilized with 0.25% Maltoside or 0.25% Maltoside + 1 μM gelsolin. The percent of total membrane pellet mIgM (white bars) and Igα (black bars) found in the membrane skeleton (P1) and membrane soluble (S1) fractions were determined. Values are expressed as the mean of two experiments.
independent experiments, an average value of 21% of actin still remains in the membrane skeleton from gelsolin treated membrane pellets as compared to actin present in the membrane skeleton prepared in the absence of gelsolin (Figure 11a). These results indicate that gelsolin does not completely sever all of the actin filaments of the membrane skeleton and it is possible that some mIgM is localized partly in those less accessible areas. Based on these considerations and our previous findings of mIgM in the membrane skeleton by electron microscopy (65), we conclude that the majority of mIgM found in the membrane skeleton is a result of associations with actin based microfilaments and not detergent insolubilities. Similarly, gelsolin was utilized by another group which examined PKCe interactions with actin (94). Here, gelsolin treatment of insoluble cytoskeleton-rich fractions reduced but did not completely abolish PKCe interactions with the cytoskeleton.

6. The tyrosine phosphorylated form of Igα accumulates in the membrane skeleton with a different kinetics than the bulk of Igα or mIgM

A time course study was conducted to examine the kinetics of Igα accumulation in the membrane skeleton. Based on previous data examining the accumulation of mIgM in detergent-insoluble cytoskeleton (65). I chose to stimulate Bal 17.7.1 cells with anti-IgM antibodies for 0.5, 1, 2, 5, and 10 min. The results presented in Figures 12 and 13 represent one typical experiment out of three.

I examined the distribution of mIgM in the membrane skeleton and membrane soluble fractions at the various time points of stimulation (Figure 12). Unstimulated Bal 17.7.1 cells were used as a control. The highest increase in membrane IgM levels in the membrane skeleton as compared to controls is detected by 0.5 min of stimulation. Membrane IgM levels further increase
Figure 11. Gelsolin releases actin and Lyn from the membrane skeleton
Membrane skeleton (P1) fractions from stimulated (10 min with \(30\mu g/ml F(ab')_2\), fragments of goat anti-mouse \(\mu\) Abs at \(37^\circ C\)) Bal 17.7.1 cells following 0.25% Maltoside or 0.25% Maltoside + gelsolin membrane pellet solubilization were analyzed by Western blotting with a) mouse anti-actin Abs at a dilution of 1:200 followed by goat anti-mouse Ig HRP-conjugated Abs at a dilution of 1:5000 or b) rabbit anti-mouse Lyn Abs at a dilution of 1:200 followed by donkey anti-rabbit Ig HRP-conjugated Abs at a dilution of 1:25000. Position of the m.w. markers in kDa are shown on the right.
Figure 12. Kinetics of mIgM binding to the membrane skeleton
Unstimulated (lane 5) or stimulated (lanes 6-10 for indicated time points with 30 μg/ml F(ab')2 fragments of goat anti-mouse μ Abs at 37°C) Bal 17.7.1 cells were homogenized and subcellular fractions obtained as described in Materials and Methods. Total (lanes 1-4), 0.25% Maltoside insoluble membrane skeleton (P1)(a,b lanes 5-10) and 0.25% Maltoside soluble membrane (S1) fractions (c,d lanes 5-10) were analyzed (2 x 10^5 cell equivalents) by Western blotting with a.c) goat anti-mouse IgM Abs as described in Figure 6 or b.d) mouse anti-actin Abs as described in Figure 11. Position of the m.w. markers in kDa are shown on the right.
by 1 min with maximum levels reached at 2-5 min and are maintained for up to 10 min, the longest point tested (Figure 12a, lanes 6-10). The accumulation of mIgM in the membrane skeleton fractions are paralleled by a decrease in the amount of mIgM found in the membrane soluble fractions (Figure 12c lanes 6-10). To ensure the fractions had equal loads, I compared actin levels in the samples (Figure 12b,d). As shown in Figure 12, the membrane skeleton (b, lanes 5-10) and the membrane soluble fractions (d, lanes 5-10) have equal loads.

As a first step to examine the role of Igα in the membrane skeleton, the phosphorylation state of Igα in the membrane skeleton was studied at different times of stimulation. Although it is well established that Igα becomes phosphorylated on tyrosine residues after receptor cross-linking, it has always been an analysis of Igα in the detergent-soluble fraction (9). Here for the first time the phosphorylation state of Igα in the membrane skeleton fraction is explored.

The same samples were analyzed with anti-phosphotyrosine Abs. As shown in Figure 13a, the tyrosine phosphorylated 35 kDa protein is most likely Igα because it overlapped precisely with the 35 kDa protein revealed by specific anti-Igα antibodies (Figure 13b). In unstimulated cells, a basal level of tyrosine phosphorylated Igα is observed in the membrane soluble fraction (Figure 13a, lane 1). Tyrosine phosphorylated Igα is not found in the membrane skeleton of unstimulated cells (Figure 13a, lane 2). However, once Bal 17.7.1 cells are stimulated tyrosine phosphorylated Igα is present in the membrane skeleton fractions (Figure 13a, lanes 4.6.8.10.12). At 2-5 min of anti-IgM stimulation, when overall tyrosine phosphorylation levels reach maximal levels, maximum levels of tyrosine phosphorylated Igα are found in the membrane skeleton fraction (Figure 13a, lanes 8.10). This was not due to an increase in bulk Igα levels, which remained constant between 0.5 and 10 min (Figure 13b, lanes 4.6.8.10.12). The presence of Igα in the membrane soluble fractions decreases
Figure 13. Kinetics of tyrosine phosphorylated substrates and Igα accumulating in the membrane skeleton
0.25% Maltoside soluble membrane (S1) (lanes 1, 3, 5, 7, 9, 11) and 0.25% Maltoside insoluble membrane skeleton (P1) (lanes 2, 4, 6, 8, 10, 12) fractions from unstimulated (lanes 1, 2) or stimulated (0.5, 1, 2, 5, 10 min with 30µg/ml F(ab')₂ fragments of goat anti-mouse µ Abs at 37°C lanes 3-12) Bal 17.7.1 cells (1 x 10⁶ cell equivalents) were analyzed by Western blotting with a) 4G10, a mouse monoclonal Ab to phosphotyrosine at a dilution of 1:1000 followed by goat anti-mouse Ig HRP-conjugated Abs at a dilution of 1:5000 or b) anti-Igα Abs as described in Figure 7. Position of the m.w. markers in kDa are shown on the right.
with increasing time of stimulation (Figure 13b, lanes 3, 5, 7, 9, 11). The tyrosine phosphorylated Igα levels in S1 fractions follow the same pattern as tyrosine phosphorylated P1 samples but with a less dramatic increase at 2-5 min. At 10 min, the tyrosine phosphorylated levels of Igα and of other proteins decline in both P1 and S1 fractions (Figure 13a, lanes 11, 12). The tyrosine phosphorylated Igα found in the membrane soluble fractions of stimulated cells may represent Igα in association with mIgM that did not associate with the membrane skeleton. As shown in Figures 9 and 12 a portion of mIgM remains in the membrane soluble fraction following stimulation. The detergent lysis may disrupt the weakly associated mIgM/cytoskeleton complexes yet not effect the more stably associated complexes. In all of the above experiments equal protein loads in each fraction was assured by Coomassie Blue staining (not shown) and Western blotting for actin levels (Figure 12b,d).

Interestingly, this kinetics study suggests that Igα is maximally tyrosine phosphorylated following translocation to the membrane skeleton. Thus, there may be an active signaling process occurring in the membrane skeleton as mIgM/Igα are localized to this subcellular location. With increasing time of stimulation the accumulation and organization of signaling molecules to the membrane skeleton could be occurring. When one compares the literature of tyrosine phosphorylation events occurring in the detergent-soluble fraction of stimulated B cells with events in the membrane skeleton, similarities can be drawn. As Gold et al. have demonstrated, there is an increase in Igα’s tyrosine phosphorylation state with time of stimulation. The increase occurred at 1 min. and was maximal at 5 min. Here Gold stimulated Bal 17 cells and then lysed them in conditions that disrupted mIgM/Igα interactions (1% Triton X-100) which was followed by anti-Igα immunoprecipitations (9). It is clear that the Igα analyzed in these time course studies was originally
removed from mIgM thus from the membrane skeleton due to lysis conditions.

Phosphorylated proteins with approximate molecular weights of 32-37, 40-45, 50-56, 65, 69-73, 81, 88, 100, 115, and 126 have been detected in the Triton X-100 or NP-40 detergent soluble fractions from stimulated B cells (2). Note that these detergents disrupt mIgM/Igα interactions and may also disrupt other signaling molecules from their associations with the cytoskeleton. Again, what is known of tyrosine phosphorylated proteins in literature may indeed represent phosphorylation events that are originally occurring in the membrane skeleton. In my time course experiments (Figure 13a), there was a significant increase in tyrosine phosphorylated proteins of similar molecular weight in both S1 and P1 fractions. The increased tyrosine phosphorylation was observed within 0.5 min of stimulation which reached its maximum by 5 min (Figure 13a).

The phosphorylated proteins present in the membrane skeleton may either represent phosphorylation in this fraction of existing or translocated proteins or the translocation of already phosphorylated proteins after anti-IgM cross-linking. Although we do not know the identity of all the tyrosine phosphorylated proteins in the membrane skeleton fraction, we did confirm the accumulation of BCR-associated Igα (see above), and Lyn and Syk kinases (see below).

7. Rapid accumulation of Lyn and Syk in the membrane skeleton of stimulated cells

The results of Figure 13 raise the question of whether known B cell signaling events are occurring in the membrane skeleton fraction. Does Igα accumulation in the membrane skeleton also recruit associated protein tyrosine kinases? If so, they could be responsible for the increase in Igα's phosphorylation and/or other signaling events occurring in the membrane skeleton. As mentioned above, phosphorylated Igα that is immunoprecipitated from the soluble fraction of stimulated cells may include a pool of Igα that was originally localized in the membrane skeleton. If this is true, any
protein co-immunoprecipitated with phosphorylated Igα such as PTKs (reviewed in 2) may have also originally resided in the membrane skeleton.

It is widely known that membrane IgM cross-linking leads to enhanced binding of Src and Syk family kinases to the B cell receptor (38,42). Since tyrosine phosphorylated Igα is found in the membrane skeleton after anti-IgM cross-linking, the presence of protein tyrosine kinases known to interact with Igα were assessed in the membrane soluble and membrane skeleton fractions. The Src family protein tyrosine kinase Lyn and the non-Src family protein tyrosine kinase Syk were chosen for analysis.

The Src family protein tyrosine kinase Lyn contains a Src Homology 4 (SH4) domain which signifies a membrane association signal (37). Therefore, Lyn is expected to be present in the membrane pellet fraction of Bal 17.7.1 cells. Studies in the literature also indicate that the percentages of total cellular Src family protein tyrosine kinases which associate with the B cell receptor are extremely low (1-3% (35)). Therefore, if Lyn does accumulate in the membrane skeleton fractions through associations with Igα, the levels of Lyn in this fraction are expected to be low. The results presented in Figure 14a(i,ii) are two typical experiments out of three. The results show that after solubilization of the membrane pellet, the majority of Lyn is found in the detergent-soluble membrane fractions in both unstimulated and stimulated cells (Figure 14a, lanes 1,3,5,7,9,11) as compared to the corresponding membrane skeleton fraction (Figure 14a, lanes 2,4,6,8,10,12). A basal level of Lyn is detected in the detergent-insoluble membrane skeleton fraction in unstimulated cells (Figure 14a, lane 2). However, in stimulated cells there is an increase in the presence of Lyn in the membrane skeleton fractions (Figure 14a, lanes 4,6,8,10,12), particularly the p53 form. The two forms of Lyn detected (53kDa and 56kDa) represent alternatively spliced mRNAs. The proteins
Figure 14. Kinetics of Lyn and Syk accumulating in the membrane skeleton
0.25% Maltoside soluble membrane (S1) (lanes 1, 3, 5, 7, 9, 11) and 0.25% Maltoside insoluble
membrane skeleton (P1) fractions (lanes 2, 4, 6, 8, 10, 12) from unstimulated (lanes 1, 2) or
stimulated (0.5, 1, 2, 5, 10 min with 30μg/ml F(ab')2 fragments of goat anti-mouse μ Abs at 37°C
lanes 3-12) Bal 17.7.1 cells (1 x 10⁶ cell equivalents) were analyzed by Western blotting with a)
rabbit anti-mouse Lyn Abs as described in Figure 11, i and ii correspond to two independent
experiments. b) rabbit anti-mouse Syk Abs at a dilution of 1:200 followed by donkey anti-rabbit Ig
HRP-conjugated Abs at a dilution of 1:25000. Position of the m.w. markers in kDa are shown on the
right. Arrow head indicates the faster migrating Syk band.
differ in the presence or absence of a 21 amino acid sequence located in the N-terminus of the Lyn PTK (96,97). It is still unknown as to whether or not the two forms of Lyn possess distinct functions in B cell signaling events. According to the literature, Lyn is known to associate with the resting BCR mainly through its first ten N-terminal amino acids and then associates with the stimulated, phosphorylated BCR through its SH2 domain (17.38). The Lyn that accumulates in the membrane skeleton fraction following stimulation may represent Lyn in association with phosphorylated Igα. The increase is most predominant at the earlier time points, 0.5 to 2 min of stimulation (Figure 14a, lanes 4.6.8) and decreases at later time points (Figure 14a, lanes 10.12). The gradual decline in the amount of Lyn found in the membrane skeleton fraction may reflect a repositioning of Lyn away from the BCR allowing other molecules to associate with the BCR. In a study by Saouaf et al., the abundance of immunoprecipitated Lyn from the detergent-soluble fraction was also found to decrease with time (95). Although the results are similar, one must realize the extreme differences in what is actually being analyzed. In the Saouaf et al. study, the stimulated B cells were lysed with NP-40 prior to anti-Lyn immunoprecipitations. These conditions disrupt associations between mIgM/Igα and presumably kinase associations with the cytoskeleton via Igα. The pool of Lyn that was immunoprecipitated in these studies included membrane soluble and membrane skeleton Lyn. The membrane skeleton pool of Lyn is significantly masked by the membrane soluble pool.

As discussed in section 5, a significant portion of mIgM and Igα were released from the membrane skeleton following membrane pellet treatment with the actin severing protein gelsolin. Thus their localization in the membrane skeleton fraction was due to cytoskeletal interactions and not detergent insolubilities. Similarly, as shown in Figure 11b, gelsolin treatment of the membrane pellet releases approximately 50% of Lyn from the membrane skeleton fraction as compared to the
Lyn present in this fraction in the absence of gelsolin. This is similar to the amount of mIgM and Igα released by gelsolin (see Figure 10).

It has also been observed that binding of Src family kinases to the B cell receptor increases their activities (38, 42, 43). Studies done on protein tyrosine kinases demonstrate a parallel increase in their activities with levels of tyrosine phosphorylation (95). Although activity measurements were not conducted, the same anti-Lyn Western blot was stripped and reprobed with anti-phosphotyrosine Abs while anti-phosphotyrosine blots (Figure 13a) were reprobed for Lyn to assure the position of Lyn among tyrosine phosphorylated bands in P1 and S1 fractions (not shown). It appears that Lyn is already tyrosine phosphorylated in the S1 fraction of unstimulated cells (Figure 13a, lane 1). In the P1 fraction a minor increase in a tyrosine phosphorylated band co-localizing with p53Rhn was observed particularly at 2-10 min of stimulation (Figure 13a, lanes 8,10,12) after maximum levels of the protein were reached (see Figure 14a). The intensity of the tyrosine phosphorylated band co-migrating with p56Rhn remained constant in all S1 fractions and this band was absent in the P1 fractions. These results suggest that the small fraction of tyrosine phosphorylated p53Rhn found in the membrane skeleton becomes further tyrosine phosphorylated and is possibly active.

In addition to Lyn, the non-Src family protein tyrosine kinase Syk was also examined. In contrast to Lyn, Syk does not contain a SH4 domain and is found to be localized in the cytosolic portion of a B cell (37). Indeed, I found most of Syk to be localized in the cytosolic fraction of Bal 17.7.1 cells (not shown). In unstimulated cells, a small fraction of Syk is found in the S1 fraction of the membrane (Figure 14b, lane 1). This may represent Syk interacting with the resting B cell receptor (36). In stimulated cells there is a decrease in Syk levels in S1 fractions (Figure 14b, lanes 3,5,7,9,11) which is followed by a increase in the amount of Syk in the P1 fractions (Figure 14b.
Figure 14b also shows that another faster migrating form of Syk was detected only in the P1 samples of stimulated cells. This may represent a non-phosphorylated form since only the upper Syk band in the membrane skeleton overlapped with a tyrosine phosphorylated protein in the P1 fractions of stimulated cells (Figure 13a, lanes 4.6.8.10.12). Stripping and reprobing of the Syk blots with anti-phosphotyrosine Abs confirmed this result (not shown). The induction of tyrosine phosphorylation of the Syk band increases moderately after 0.5 min of stimulation in S1 fractions (Figure 13a, lanes 1.3.5.7.9.11) while it is more predominantly increased in the P1 fractions especially between 2-10 min (Figure 13a, lanes 8.10.12). These results suggest that following stimulation tyrosine phosphorylated, presumably active Syk accumulates rapidly in the membrane skeleton and this pool becomes increasingly phosphorylated with increasing times of stimulation.

To confirm the identity of the tyrosine phosphorylated proteins found in the membrane skeleton fractions as Lyn and Syk kinases, immunoprecipitations with specific Abs need to be conducted (see Section V. Future Directions).
Chapter IV: Discussion

Evidence shows that mIg accumulates in the cytoskeleton fraction following receptor cross-linking but its role in B cell signalling is not well understood (61-64). Based on previous reports of receptors and their associated active signalling molecules accumulating in the cytoskeleton following stimulation, we hypothesized that mIgM/cytoskeleton interactions are involved in signal transduction. Previously our lab took the approach of identifying what component of the BCR mediates mIgM translocation to the cytoskeletal matrix to further our understanding of this phenomenon. We demonstrated that the accumulation of mIgM in the cytoskeletal fraction of stimulated B cells does not require the signalling transducing unit of the B cell receptor, the Igαβ heterodimer (65). Although Igαβ is not required for mIgM/cytoskeleton interactions to occur, we predicted that in the cell the whole BCR complex including the kinases associated with the Igαβ heterodimer would accumulate in the cytoskeletal fraction.

In this study I have demonstrated that Igα translocates to the membrane skeleton as part of the BCR complex after mIgM cross-linking. The results of the time course study suggest that Igα levels of tyrosine phosphorylation reach a maximum after Igα has translocated to the membrane skeleton. Furthermore, I have shown that the Src family PTK Lyn and the cytoplasmic PTK Syk are found in the membrane skeleton following the cross-linking of the BCR. In combination these results suggest that the membrane skeleton is a region of the B cell where active signaling processes are occurring. Traditionally, analysis of mIgM and its associated signaling molecules have been conducted on detergent soluble fractions of stimulated B cells. Conditions in many of these studies were harsh enough to disrupt signaling molecules' associations with the cytoskeletal matrix (see Results Section 6.7). As a consequence of this approach, these investigations may have focused on
the entire cellular pool of signaling molecules. By doing so, a population of signaling molecules that were originally co-localized with mIgM in the cytoskeletal matrix may have been overlooked. Here, through subcellular fractionations we are presenting novel information to the field of B cell signaling by showing that mIgM translocating to the membrane skeleton provides the vehicle for the associated signaling molecules to be recruited to this subcellular location. It is tempting to speculate that these events represent the beginnings of signaling cascades occurring on the cytoskeletal matrix.

In support of B cell signaling events occurring on the cytoskeletal matrix, the co-capping immunofluorescence studies on anti-IgM stimulated Bal 17.7.1 cells demonstrate that Igα distribution overlaps with capped, cytoskeleton associated mIgM (Figure 5). Interestingly, other signalling components have been found to co-localize with mIg during the capping process, including tyrosine phosphorylated proteins (98), p21<sup>ras</sup> (99), and calmodulin (100). This supports the idea that the cytoskeleton may provide the structural matrix to concentrate signalling molecules. By demonstrating co-capping of IgM and Igα at the cellular level, this led me to believe that Igα would be found in the cytoskeleton compartment as part of the B cell receptor complex following stimulation.

Through subcellular fractionations I was able to demonstrate the phenomenon of mIgM translocation to the membrane skeleton in stimulated Bal 17.7.1 cells (Figure 6). The purity of the fractions was confirmed by the separation of intracellular form of IgM from the membrane form of IgM. Following the ultracentrifugation, the lower 82 kDa band representing the intracellular form of IgM was localized to the cytoplasmic supernatant fraction and the higher 87 kDa band of membrane IgM was localized to the membrane pellet. Also, the distribution of IgM was similar in the unstimulated and stimulated cells up to the membrane pellet fraction indicating that cross-linking
induced aggregation of mIgM did not affect the distribution of the membrane form of IgM. Following membrane pellet solubilization, mIgM accumulated in the soluble fraction (S1) in unstimulated cells and in the membrane skeleton fraction (P1) of stimulated cells. As shown in Figure 11, the gelsolin treatment released 80% of the membrane skeleton localized actin, indicating the presence of F-actin in this fraction. Furthermore, analysis of the membrane skeleton fraction for talin, a known membrane skeleton protein in platelets (73), indicated that approximately 80% of membrane localized talin was found in the membrane skeleton fraction of stimulated Bal 17.7.1 cells (data not shown). This data supports the notion that the P1 fraction contains the membrane proximal portion of the cytoskeleton.

Taking advantage of the mild detergent Maltoside that preserves mIgM/Igα associations (Figure 7), I was able to show that tyrosine phosphorylated Igα accumulates in the cytoskeleton compartment of the membrane fraction following stimulation as part of the B cell receptor complex (Figures 8.9.13). Interestingly, it appears that maximum tyrosine phosphorylation is reached after Igα has translocated to the membrane skeleton. This finding which is the first documented evidence of a signaling component of the BCR to accumulate in the membrane skeleton following receptor stimulation supports the idea of active signaling processes occurring on the cytoskeletal matrix. These signaling processes are most likely triggered by mIgM translocating to the cytoskeleton. For example, the highest levels of mIgM binding to the cytoskeleton (2 and 5 min) occur when maximum levels of tyrosine phosphorylated Igα and of overall protein tyrosine phosphorylation are also found in the membrane skeleton (Figures 12.13). Subsequently, I found that Lyn and Syk kinases which are known to interact with tyrosine phosphorylated Igα are recruited to the membrane skeleton compartment within 30 sec of anti-IgM stimulation when significant amounts of mIgM and
Igα are already found in this fraction (Figures 12, 13, 14). This is consistent with our hypothesis that cytoskeletal association of mIgM could provide the mechanism to recruit the signalling molecules to the membrane skeleton.

Although it seems that a small fraction of Lyn and Syk kinases are found in the membrane skeleton. this may represent a significant population since only small amounts of kinases appear to associate with tyrosine phosphorylated ITAM's of Igαβ at any time of induction (35). The fact that tyrosine phosphorylated bands overlapping with Lyn and Syk kinases were found following BCR induction suggests that these kinases are in an active form. further supporting the view that membrane skeleton bound kinases are involved in BCR signalling. One can speculate that the active Igα associated kinases in the membrane skeleton allow further phosphorylation of Igα and other substrates localized in the fraction.

The following arguments point out that mIgM, Igα, Lyn, Syk and tyrosine phosphorylated substrates found in the membrane skeleton of stimulated cells are a result of interactions with the actin based microfilaments and not due to unspecific sedimentation of large aggregates of signalling molecules. Using the actin severing protein gelsolin we showed that significant amounts of mIgM, Igα and Lyn were released from the membrane skeleton fraction as shown in Figures 10 and 11b. However, some mIgM, Igα and Lyn still remained in the membrane skeleton fraction probably due to gelsolin's inability to completely seve all of the actin (Figure 11a). If the accumulation of Lyn and Syk kinase in the membrane skeleton was simply due to trapping within the aggregated BCR. one would expect Lyn and Syk to continue to accumulate in this fraction with time of cross-linking. However. this does not occur. Syk localization to the membrane skeleton fraction remained constant throughout all points of stimulation (Figure 14, lanes 4, 6, 8, 10, 12) despite increasing amounts of
mIgM and Igα between 30 sec and 2 min of stimulation (Figures 12, 13). In the case of Lyn, the increase was most predominant at 0.5 and 1 min of stimulation and then decreased (Figure 14, lanes 4, 6, 8, 10, 12). Other tyrosine phosphorylated proteins peaked at 2 to 5 min of stimulation and decreased at 10 min (Figure 13). This evidence supports the notion that mIgM translocation to the membrane skeleton involves time dependent signaling process rather than random accumulations of signaling molecules due to detergent insolubilities or trapping effects by increasing cross-linking of the BCR.

What about the downstream consequences of the translocation of mIgM-associated signaling molecules into the membrane skeleton? One can envisage that the anchored mIgM/Igα complexes provide optimal positioning for the associated kinases to efficiently phosphorylate their substrates and continue the downstream signaling cascade. An example of a downstream signaling cascade is the p21ras pathway. This is based on the fact that p21ras co-caps with surface immunoglobulin in mouse splenic B cells (99). Interestingly, p21ras activation induced by BCR cross-linking was shown to be regulated by protein tyrosine kinases (29). PTKs are known to phosphorylate regulators of p21ras such as rasGAP (21, 22), SOS (20) and p95VAV (23) and adaptor molecules such as SHC (20) (see Introduction, Section 2).

Furthermore, evidence suggests that p21ras regulatory proteins can be recruited to the vicinity of cytoskeleton associated mIgM and the Igα associated PTKs. For example, studies have shown that Lyn kinase interacts with ras.GAP through the amino-terminal 27 residues of its unique domain (44). Ras.GAP may be a molecule that couples Igα associated tyrosine kinases to Ras activation. One can speculate that the unidentified tyrosine phosphorylated proteins found to co-cap with surface immunoglobulin (98) may represent the tyrosine phosphorylated proteins I found in the membrane.
skeleton fractions following BCR stimulation (Figure 13). These tyrosine phosphorylated proteins may be intimately involved in recruiting signaling molecules to the membrane skeleton through interactions with SH2 domains. As shown in the integrin signaling pathway the GRB-2 SH2 domain binds directly to tyrosine phosphorylated Focal Adhesion Kinase (FAK) which is localized in cytoskeleton-rich focal adhesions (101,102). Furthermore, through their SH2 domains SHC or GRB-2 could interact with the capped, tyrosine phosphorylated proteins and direct the GRB-2/SOS complex to the vicinity of the cytoskeleton associated mlgM.

Alternatively, SH3 domains of GRB-2 could aid in the localization of SOS to co-capped p21ras and mlgM. For example, in microinjection studies it was shown that the SH3 domain of GRB-2 was responsible for targeting GRB-2 to membrane ruffles (103). Although the guanine nucleotide exchange factor SOS is translocated from the cytosolic fraction to the membrane following EGF stimulation in fibroblasts (26) a recent study in B cells showed that BCR cross-linking does not increase the amount of membrane-associated SOS (20). However, the fraction examined in the B cell study was the membrane soluble fraction and not the membrane skeleton fraction. One could then predict that SOS is accumulated in the membrane skeleton fraction of stimulated B cells where capped p21ras is likely to accumulate.

Furthermore, studies have shown that activation of Raf-1, a serine-threonine kinase, depends on its recruitment by Ras to the membrane skeleton in COS cells (104). Activation of Raf-1 leads to the phosphorylation and activation of MEK (MAP kinase kinase or MAPKK) which then phosphorylates and activates mitogen activated protein kinase (MAPK). Recently, active MAPK was shown to be associated with the microtubule cytoskeleton suggesting unknown substrates are microtubule-associated (105). Inside the nucleus, MAP kinases regulate gene expression by
phosphorylating a number of transcription factors including c-jun, c-fos, and c-myc (30). Based on these findings it appears that the progression of the p21ras pathway is closely linked with the interactions of several of its constituents with the cytoskeleton. BCR association with the cytoskeleton may provide the first linkage point between the p21ras pathway and the cytoskeleton.

BCR cross-linking also increases the association of ras.GAP with tyrosine phosphorylated proteins with molecular weights of p62 and p190 (106). p190 has been shown to stimulate the GTPase activity for Rac and Rho (107,108). Rac and Rho have been implicated in cytoskeletal rearrangements such as membrane ruffling (109) and actin stress fibre formation (110), respectively, through microinjection assays. Expression of the GAP N-terminal region, which possesses the ability to bind to p190 in fibroblasts correlated with disruption of the actin cytoskeleton and cellular adhesion (104). These molecules may be involved in the cytoskeletal reorganizations inherent in B cell activation events such as receptor capping, internalization, mobility and cell-to-cell contacts. The interaction of rasGAP with Lyn (44) in the membrane skeleton may provide the first signals for these rearrangements.

Our knowledge of the role of the cytoskeleton in B cell signaling is currently limited. Evidence from other cellular systems such as the platelet and T cell supports the involvement of the cytoskeleton in receptor mediated signal transduction. To gain a similar understanding in B cells, it was crucial to first define signaling molecules translocating to the cytoskeleton together with mIgM. My findings of the co-localization of mIgM associated tyrosine phosphorylated Igα with protein tyrosine kinases and other phosphorylated substrates in the membrane skeleton are the first indications of the involvement of mIgM/cytoskeleton interactions in B cell signaling.
Chapter V: Future Directions

1. Activities of Protein Tyrosine Kinases in the Membrane Skeleton

In this study we suggested that Lyn and Syk kinases accumulating in the membrane skeleton are active. This is based on the co-migration of tyrosine phosphorylated bands with the kinases. The activities of protein tyrosine kinases Lyn and Syk found in the membrane skeleton will be compared to their activities in cytosolic or membrane soluble fractions. Kinases will have to be extracted from the membrane skeleton pellets and immunoprecipitated by the appropriate antibody. Extraction conditions will have to be determined which may require harsh detergents such as deoxycholate and/or sonication. Extraction of Syk kinases from platelet membrane skeleton was performed by incubations with 0.5M NaCl (74). In vitro kinase assays and exogenous substrate assays will be performed. The comparison of PTK activities in the various fractions will determine if there is an active pool of kinases accumulated in the membrane skeleton fraction.

2. Characterization of Other Signaling Molecules in the Membrane Skeleton

To further characterize the signaling molecules present in the cytoskeleton following anti-IgM cross-linking, the presence of p21ras, p21ras regulatory molecules ras.GAP and SOS in membrane skeleton and membrane soluble fractions from unstimulated and stimulated cells will be analyzed. Detection of the adaptor proteins SHC and GRB-2 will also be studied.

If p21ras is localized in the membrane skeleton compartment, p21ras activation will be compared from membrane skeleton extracts, membrane soluble and cytosolic fractions by determining GTP/GDP+GTP ratios.

3. Subcellular Fractionations of B cells Lacking Igα

To verify whether protein tyrosine kinases are translocated to the membrane skeleton through
associations with Igα. Subcellular fractionations will be conducted on B cells expressing mIgM ± Igα. A mutated form of mIgM (MutA) has been transfected into J558L cells (mIgM⁺; Igα⁻) in our laboratory which allows the surface expression of mIgM in absence of Igα. Cross-linking of MutA induced its association with the cytoskeleton. These cells will be compared to J558L cells transfected with mIgM (MutA) and Igα. MutA has the ability to associate with Igα despite the mutations of the polar residues in the IgM transmembrane domain. Subcellular fractionations will be conducted on these two cell lines with and without anti-IgM stimulation. Membrane skeleton and membrane soluble fractions will be analyzed by Western blotting for the presence or absence of Lyn and Syk kinases. If Igα is required, Lyn and Syk will only accumulate in the membrane skeleton fractions of the stimulated cell line that expresses Igα.

4. Disruption of mIgM/cytoskeleton interactions

An ongoing project of the lab is to generate a mutated mIgM receptor which will not bind to the cytoskeleton. Anti-IgM induced events in the mutated mIgM transfectant that lacks IgM/cytoskeleton interactions will be compared to those induced in wild type mIgM transfectants. This system will allow us to determine whether mIgM/cytoskeleton interactions are the vehicle for which signaling molecules such as Igα associated PTKs or p21ras and its regulatory molecules are recruited to the membrane skeleton. One can then examine the consequences of mIgM/cytoskeleton disruption on various signaling events such as activation of protein tyrosine kinases and p21ras activation. Cytoskeletal driven events such as capping and internalization will also be compared in the wild type and mutated forms of mIgM. Ultimately these investigations will determine the role of ligand-induced mIgM interactions with the cytoskeleton in B cell signaling and cytoskeletal events.
List of References


70. Patel, K.J. and Neuberger, M.S. (1993). Antigen presentation by the B cell antigen receptor is driven by the α/β sheath and occurs independently of its cytoplasmic tyrosines. Cell 74, 939-946.


