ABSTRACT

Title of Document: THE ROLE OF MAMMALIAN ACTIN BINDING PROTEIN 1 IN COUPLING BCR SIGNALING AND ANTIGEN TRANSPORT FUNCTIONS

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The B cell receptor (BCR) serves as both signal-transducer and antigen-transporter. Binding of antigens to the BCR induces signaling cascades and antigen-processing and presentation, two essential cellular events for B cell activation. BCR-initiated signaling increases BCR-mediated antigen-processing efficiency by increasing the rate and specificity of antigen transport. Previous studies showed a critical role for the actin cytoskeleton in these two processes. Here I found that actin-binding protein 1 (Abp1/HIP-55/SH3P7) functioned as an actin-binding adaptor protein, coupling BCR signaling and antigen-processing pathways with the actin cytoskeleton. Gene knockout of Abp1 and over-expression of the SH3 domain of Abp1 inhibited BCR-mediated antigen internalization, consequently reducing the rate of antigen transport to processing compartments and the efficiency of BCR-mediated antigen-processing and presentation. BCR activation induced tyrosine phosphorylation of Abp1 and translocation of both

Abp1 and dynamin 2 from the cytoplasm to the plasma membrane, where they colocalized with the BCR and cortical F-actin. The inhibitory effect of a dynamin PRD deletion mutant on the recruitment of Abp1 to the plasma membrane and the internalization of the BCR, co-immunoprecipitation of dynamin with Abp1, and co-precipitation of Abp1 with GST fusion of the dynamin PRD, demonstrate the interaction of Abp1 with dynamin 2. In addition to its role in antigen transport and processing, Abp1 is also important for BCR signal transduction. Splenic B cells from Abp1 knockout mice and A20 B cell line with Abp1 knockdown displayed higher levels of protein tyrosine phosphorylation after BCR crosslinking when compared with wild type mice. BCR-triggered ERK phosphorylation in Abp1-deficient splenic B cells occurred sooner and for a much shorter duration than the wild type B cells, while both Abp1 knockout and knockdown significantly reduced BCR-induced phosphorylation of JNK. These results demonstrate that the BCR regulates the function of Abp1 by inducing Abp1 phosphorylation and actin cytoskeleton rearrangement, and that Abp1 facilitates BCR-mediated antigen-processing by simultaneously interacting with dynamin and the actin cytoskeleton. My results further suggest a negative regulatory role for Abp1 in BCR signal transduction.

THE ROLE OF MAMMALIAN ACTIN BINDING PROTEIN 1 IN COUPLING
BCR SIGNALING AND ANTIGEN TRANSPORT FUNCTIONS

By

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Dedication

This dissertation is dedicated in memory of my late father Opeoluwa Onabajo, who died on April 24 2005, a day after his 62nd birthday. Dad, I wanted to follow in your footsteps when I pursued this career and you told me to "go get them boy" just before boarding the plane, and I haven't done too badly. I miss you and I am sorry I couldn't come for the funeral. I know you are up in heaven smiling down at me today.

Acknowledgements

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List of abbreviations

Abp1	Actin binding protein 1
AP-2	Adaptor protein 2
APC	Antigen presenting cells
Bam32	B cell adaptor molecule of 32 KDa
BCR	B cell antigen receptor
BLNK	B cell linker protein
Btk	Burtons tyrosine kinase
CCP	Clathrin-coated pits
CCV	Clathrin-coated vesicle
CD	Cytochalasin D
CME	Clathrin mediated endocytosis
DAG	Diacylglycerol
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
GED	GTPase effector domain
GST	Glutathione-S-Transferase
HIP-55	HPK1 interacting protein of 55KDa
HPK1	Hematopoetic progenitor kinase
IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine based activation motif

JNK	c-Jun NH2-terminal kinase
LAMP-1	Lysosomal associated membrane protein 1
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
mIg	Membrane immmunoglobulin
MIIC	MHC class II containing compartment
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κ B
N-WASP	Neural Wiskott-Aldrich syndrome protein
PH	Pleckstrin homology
PI	Phophotidylinositol
PI-3-K	Phosphatidylinositol-3-kinase
PIP	Phosphatidylinositol phosphate
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PLCγ2	Phospholipase C-γ
PRD	Proline rich domain
PTK	Protein tyrosine kinase
SH2	Src homology 2
SH3	Src homology 3
shRNA	Short hairpin RNA
siRNA	short interfering RNA

TK   Tyrosine kinase

WT   wild type

XL   Crosslinked

Chapter 1: Introduction

1.1 Humoral immunity

Our body defends us against microorganisms using two major types of immunity, an early, innate immunity that recognizes a limited set of molecular patterns on the surfaces of microbes and a later, more specific adaptive immunity (1). The adaptive immunity can be further subdivided into two main types, humoral immunity and cell-mediated immunity. Cell-mediated or cellular immunity is mediated by T cells, while humoral immunity is mediated by B cells. The B cell produces a protein complex called antibodies (immunoglobulin) in secreted and membrane-bound forms. Antibodies are the primary effectors of humoral immunity. An antibody is a complex made up of two identical light chains and two identical heavy chains. At the N-terminal region of both the heavy and light chain of antibodies the amino acid sequences of antibodies have a high degree of variation. This region, termed the variable region, is responsible for conferring on the antibody its ability to bind to billions of molecules that the body might encounter during the course of its lifetime (2-5). Secreted antibodies reside in most of the body fluids and they can bind to microorganisms and foreign antigens, neutralizing their toxicity, targeting them for destruction by other arms of the immune system. Antibodies also exist in a membrane-bound form on the surface of the B cell. Here, they are termed B cell antigen receptors (BCR). The BCR also has the capability to recognize and bind to foreign antigens. Binding of antigens to the BCR leads to a series of signaling cascades that activate the B cells. These signals also facilitate the rapid internalization of the antigen to compartments within the B cell for processing into peptides and later presented

to T cells on structures called major histocompatibility complex class II (MIIC)

Presentation to T cells enables the B cell to receive additional signals that allow the B cell

to participate more effectively in adapting the immune response to the offending antigen.

1.2 BCR Signaling

The BCR is a multi-subunit protein complex composed of a membrane immunoglobulin

(mIg), which associates non-covalently with the Igα/Igβ heterodimer (Fig. 1.1). The

membrane-bound Ig on the cell surface is responsible for antigen binding, while the

Igα/Igβ propagates intracellular signals (6). The mIg portion of the BCR is made up of

two light chains and two heavy chains, thus creating two antigen binding sites. The Igα

and Igβ each contain one immunoreceptor tyrosine-based activation motif (ITAM) in

their cytoplasmic tails, providing the BCR with signal transduction capabilities (7). Each

ITAM has two copies of the amino acid sequence YXXϕ (D/E XX-YXXϕ-X_{6-8}-YXXϕ),

where Y is a tyrosine, X is any amino acid, and ϕ is a bulky hydrophobic amino acid (8).

The recognition of antigens with repeated epitopes by multiple, individual molecules of

BCR, causes these receptors to aggregate, leading to the activation of signaling cascades

that are mediated by the Igα/Igβ complex. BCR crosslinking by antigen leads to a rapid

translocation of the BCR to cholesterol-rich lipid rafts where it interacts with signaling

proteins (9). In lipid rafts, the ITAMs of the BCR are phosphorylated by the Src-family

kinases, Lyn, Fyn or Blk, which are either constitutively associated or become associated

with lipid rafts upon stimulation (9). The phosphorylated ITAMs serve as docking sites

for the tyrosine kinase (TK) Syk and adaptor proteins, such as B-cell linker protein

(BLNK) (10), Shc (11), Grb2 (12), and Gab (13). The binding of Syk and adaptor

proteins to the phosphorylated ITAMs of the BCR recruits them to the BCR surface

signaling microdomain in the lipid rafts and activates Syk kinase activity (Syk belongs to

a group of TKs which have Src homology 2 (SH2) domains). BCR–induced activation of

Lyn and Syk is required for the activation of Burtons tyrosine kinase (Btk), a Tec family

protein tyrosine kinase (14). Btk and Syk work in concert for optimal phospholipase C

gamma (PLC γ) activation (14, 15). Btk activation is also dependent on the

phosphoinositide 3 kinase (PI-3K)-induced production of phosphatidylinositol 3,4,5-

trisphosphate (PIP$_3$) from phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (16). PI-3K is

itself activated by recruitment to the CD19 co-receptor during BCR signaling (17). The

binding of PH (Pleckstrin Homology) domain of Btk to PIP$_3$ enables Btk recruitment to

the BCR surface signaling microdomain where it is phosphorylated by Lyn. Failure in

binding PIP$_3$ due to mutations in the PH domain of Btk leads to defects in Btk activation

and abnormalities in BCR signaling, consequently resulting in humoral immune

deficiency (18).   Adaptor protein BLNK (B cell linker protein) is phosphorylated by Syk,

and This phosphorylation generates docking motifs for SH2-containing proteins like Vav,

Grb2 and PLC γ2 (10). BLNK, in concert with Btk, activates PLC γ2. PLC γ cleaves

phosphatidylinositol bisphosphate (PIP$_2$) to diacylglycerol (DAG) and inositol

trisphosphate (IP3). IP3 triggers the release of calcium from the endoplasmic reticulum,

and increases calcium influx. DAG in concert with calcium activates protein kinase C

which, in turn, activates nuclear factor kappa B (NF-κB) by phosphorylating the Inhibitor

of κB Kinase (IKK) (19) . Calcium also activates calcineurin which, in turn, activates

nuclear factor of activation of T cells (NFAT). NF-κB and NFAT are transcription factors

that play major roles in B-cell activation (20). Upon activation through BLNK, Vav and

Grb2/SOS (guanine exchange factors) activate Rho and Ras family GTPases, respectively. Activated Ras initiates the Raf/MEKK pathway which results in the eventual activation of MAP kinase ERK (21) while activation of Rho-family GTPase Rac leads to activation of MAP kinases JNK and P38 (22-25). The eventual goal of these kinases is to phosphorylate a number of transcription factors that regulate expression of genes involved in cell survival and proliferation. ERK is responsible for phosphorylating Elk-1 and c-myc (26), JNK phosphorylates c-Jun and activating transcription factor 2 (ATF-2) (24), while P38 phosphorylates ATF-2 (27).

There is a group of molecules that exerts regulatory roles on BCR signaling. Notable among these are CD45 and C-terminal Src tyrosine kinase (Csk). CD45 is a phosphatase that prevents hyperphosphorylation of Lyn at its inhibitory tyrosine residue (Y508), which represses the kinase (28). Csk on the other hand exerts a seemingly opposite effect. It is a kinase that phosphorylates this same tyrosine, inhibiting Lyn activation (29). CD45 is partitioned away from the lipid raft signaling microdomains formed after BCR crosslinking, thus effectively shielding it from acting on Lyn (30), which is located within the lipid rafts. In addition, Lyn plays both a positive and negative role in BCR signaling. The resting B cells from Lyn-deficient mice have a hyperphosphorylated status when compared to B cells from normal mice, and Lyn-deficient mice have increased levels of autoantibodies. Lyn generates its inhibitory effect on B-cell signaling by phosphorylating Fc gamma receptor IIB (FcγRIIB) and CD22 on their ITIMs (Immunoreceptor tyrosine based inhibitory motifs). When FcγRIIB and BCR are co-ligated by antibody-antigen complexes, the ITIMs of FcγRIIB are phosphorylated by

Lyn, which enables them to recruit a phosphatase called SHIP (SH2-containing inositol 5` phosphatase (31). SHIP is able to block PIP3 production by hydrolyzing it to PIP2 subsequently inhibiting BCR signaling and B cell activation (32).

1.3 BCR-mediated antigen processing and presentation

When B cells encounter antigens in the body, the antigens are likely to be present in low concentrations, and the number of B cells specific for each antigen is limited. Fortunately, the BCR has adapted to overcome this problem by increasing its rate of internalization once it encounters an antigen that is able to crosslink to multiple BCRs. BCR endocytosis occurs primarily by clathrin-mediated endocytosis (CME) (33), although it may be mediated by other forms of endocytosis such as macropinocytosis. In the absence of antigens, the BCR is constitutively internalized into early endosomes and a large fraction recycles back to the cell surface (8). Following the crosslinking of the BCR with its cognate antigen, signaling cascades lead to an increase in the internalization rate of the BCR. BCR crosslinking also efficiently targets the BCR to the late endosomes (34). Additionally, crosslinking induces a reorganization of the actin cytoskeleton, which is required for the internalization and movement of the BCR into specialized late endosomal compartments (35). In the late endosome, the internalized antigens are fragmented into peptides by proteases that are activated by the acidic environment of the endosomes. The resulting peptides are loaded on to major histocompatibility complex (MHC) class II molecules for presentation of the complex at the surface of B cells (35). The recognition by T cells of MHC class II-presented antigen on the B-cell surface

FIGURE 1.1. Structure of the BCR complex

BCR complex is composed of a membrane-bound immunoglobulin that is responsible for

antigen binding and an Igα/Igβ heterodimer that is responsible for signal propagation.

Membrane immunoglobulin is non-covalently associated with the Igα/Igβ heterodimer.

The Igα/Igβ heterodimer contains ITAMs whose tyrosine is phosphorylated following

BCR crosslinking.

Antigen binding site

Light chain

Igα/Igβ heterodimer

Heavy chain

ITAM
Immunoreceptor
Tyrosine-based
Activation
Motif
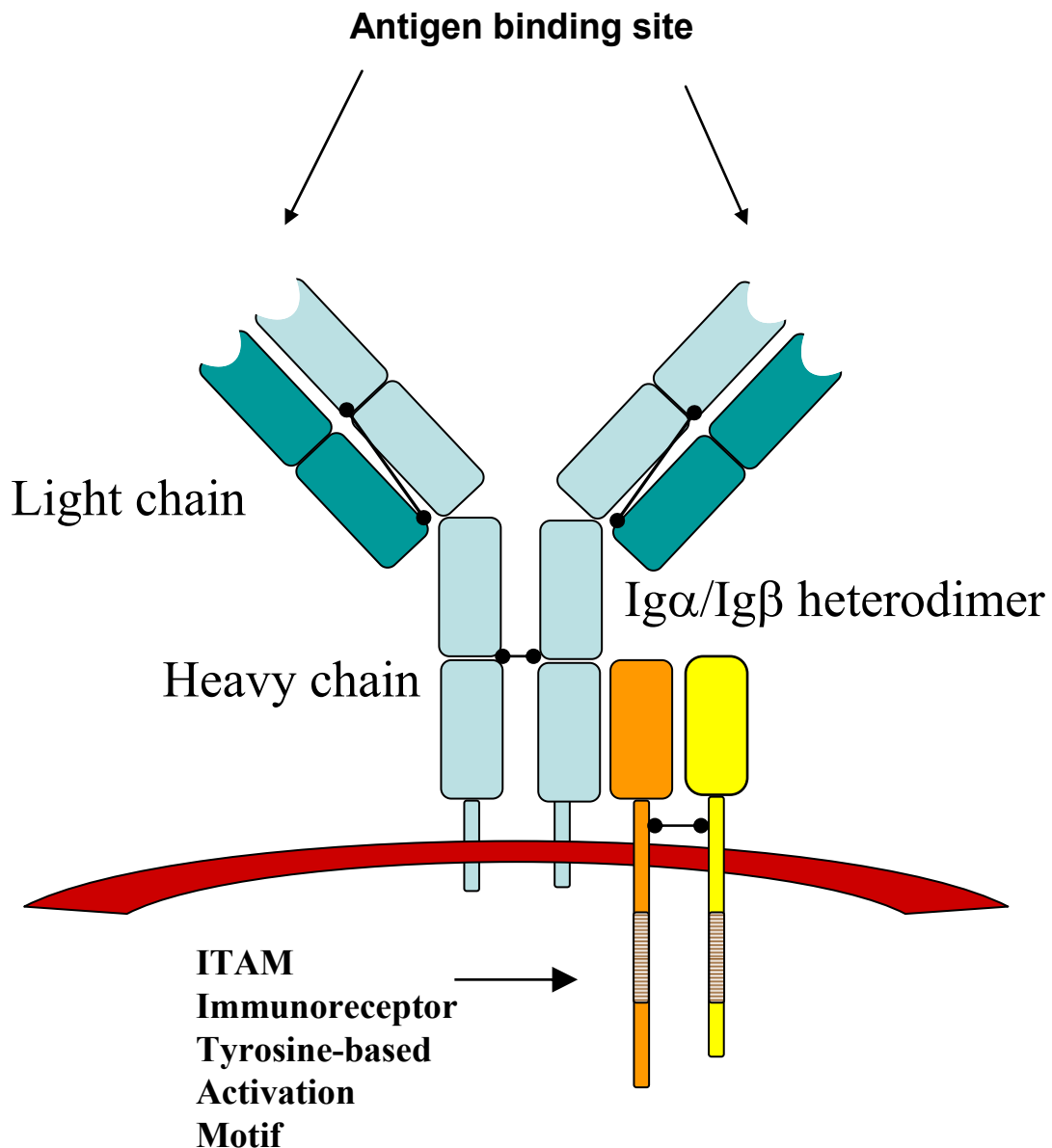
initiates a T cell-dependent antibody response.

1.4 The role of signaling in the intracellular trafficking of the BCR

Recent evidence has shown that the signaling and antigen processing functions of the BCR may be linked. While the BCR internalizes constitutively at a low rate, BCR crosslinking by multivalent antigens leads to a rapid internalization of the BCR and accelerates the transport of the BCR to the MIIC (36). This antigen-induced BCR internalization has been shown to be dependent on BCR-triggered signaling cascades. Tyrosine kinase inhibitors that block BCR-induced signaling inhibit antigen-induced BCR internalization (37). In addition, the Src kinase Lyn, which is activated following BCR crosslinking, was also found to be required for BCR internalization (38). A study from our lab, in collaboration with Dr. Francis Brodsky, showed that crosslinking the BCR induces the recruitment of clathrin to the plasma membrane and BCR-containing vesicles, and the Src-kinase inhibitor PP2 blocks this recruitment (33). The phosphorylation of clathrin in lipid rafts by Src-kinases following BCR crosslinking is required for BCR internalization, which was demonstrated by inhibition of clathrin phosphorylation and BCR internalization by the disruption of lipid rafts. Our lab previously showed that low valency antigens induce a rapid internalization of the BCR and a transient protein tyrosine phosphorylation. However, high valency antigens reduce BCR internalization, extend the residency of the BCR on the cell surface, and increase the level of BCR signaling (34). These results show that BCR signals and BCR endocytosis regulate each other.

The intracellular trafficking of the BCR seems to also be regulated by BCR signaling. Prior to BCR crosslinking, the BCR constitutively traffics to the early endosomes from where it recycles to the plasma membrane. After crosslinking by an antigen, the BCR is targeted from the early endosomes to the late endosomes (39). The targeting of the BCR to late endosomes is disrupted by Src kinase inhibitors that disrupt BCR crosslinking induced signaling (40), emphasizing the need for BCR signaling to target antigen-BCR complexes to the late endosomes. Using chimeric receptors our lab and a number of others have also shown the importance of Igα/Igβ complex in BCR trafficking to the late endosomes following BCR crosslinking (41-43). Specifically, mutating the tyrosines in the ITAMs of Igα/Igβ led to defects in BCR trafficking to late endosomes (43), further indicating that BCR signaling is important for BCR trafficking to the late endosomes.

1.5 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is initiated when adaptor proteins recognize and bind to internalization motifs on the cytoplasmic tails of receptors for various cargos. There are two main classes of adaptor molecules that bind to internalization motifs; tetrameric AP-2 adaptors, and monomeric adaptors such as Dab2 and Epsin (44). The μ2 subunit of AP-2 interacts with YXXϕ internalization motifs on the cytoplasmic tail of receptors (45). Dab2 recognizes FXNPXY internalization signals (46) while Epsin recognizes polyubiquitination signals via ubiquitin-interacting motifs (47). Recruited adaptor proteins bind and sequester cargo receptors while simultaneously binding PIP2 on the plasma membrane (48). After binding of AP-2 to the appropriate cargo receptor, clathrin is recruited to the region. Once the clathrin coat has assembled on the

cytoplasmic side of the plasma membrane, the membrane invaginates to form a coated-pit. The invagination of the coated pit is driven by changes in lipid composition at the membrane, the intrinsic curvature of the clathrin triskelia, as well as forces exerted by the cytoskeleton (49). Notable among proteins involved at this stage are the Bar domain-containing proteins endophilin and amphiphysin. Endophilin can alter lipid compositions at the cell membrane via acyl transferase activity and bind dynamin during endocytic processes (50, 51). Amphiphysin also binds dynamin and, in addition, binds to AP-2 and clathrin (44, 51). It plays a major role in recruiting dynamin to endocytic vesicles during CME (52-54). Dynamin provides the signal and force that pinches off the coated-pit from the cell membrane. It is recruited to the nascent clathrin-coated vesicles (CCVs) just before the pinch off stage (55). Other proteins that are recruited at this dynamin driven-scission stage of CME include N-WASP, Arp2/3 (56), actin (55) and synaptojanin (57). Once the CCVs have pinched off, they shed their clathrin coats due to the activity of Hsc70, an ATPase recruited to the CCVs (58). Following the uncoating stage, the vesicles fuse with other vesicles to form early endosomes (55).

1.6 A role for the actin cytoskeleton during clathrin-mediated endocytosis

Initial evidence for the role of actin in endocytosis came from genetic studies of yeast by a number of groups (59). Mutations in ACT1, the gene that encodes yeast actin, were among the first to be identified as directly impacting the endocytosis of α-factor (a small peptide secreted by yeast during mating) (60). Subsequently, researchers have identified a number of actin-related, actin-binding and actin-regulating proteins that are also involved in yeast endocytosis, and many of these proteins have homologues in mammalian cells

(59), further indicating an essential role for the actin cytoskeleton in regulating endocytosis.

Additional evidence for the role of actin in endocytosis came from the studies of actin cytoskeleton-disrupting agents. Apodaca *et al*. reported that cytochalasin-D (CD), which binds to the barbed end of F-actin and disrupts actin filaments, inhibits apical endocytosis in polarized epithelial cells but has no detectable effect on basolateral endocytosis (61). Fujimoto *et al*. studied the effect of different actin-perturbing drugs on the endocytosis of transferrin and showed that the involvement of the actin cytoskeleton in CME is dependent on cell type and growth conditions (62). There are a number of endocytic pathways in mammalian cells, including the clathrin-mediated endocytosis, the caveolar pathway, a clathrin and caveolae-independent pathway, macropinocytois and phagocytosis (59). A role for the actin cytoskeleton has been determined in most of these pathways (63, 64), and localized polymerization has been shown at sites of caveolae-mediated endocytosis (65, 66), clathrin mediated endocytosis (CME) (55), macropinocytosis (67) and phagocytosis (68) .

The interaction of actin-binding proteins with proteins involved in CME, as well as the effect of actin-regulating proteins on CME has also been used to implicate the actin cytoskeleton in CME. A common structural property of proteins that link the actin cytoskeleton to endocytosis is their multiple protein-protein interaction domains, which provides them with the potential to bind both F-actin and proteins from the endocytic machinery. Cortactin (69) and mammalian actin binding protein 1 (Abp1) (70) bind both

F-actin and dynamin during endocytosis. Huntington-interacting protein 1 (Hip1R) binds to both clathrin and F-actin (71, 72), while syndapins can bind to both N-WASP (an actin nucleation-promoting factor that stimulates Arp2/3 activity (73)) and dynamin (74-76) (Fig. 1.2). Tsujita *et al* describe a group of proteins called pombe cdc 15 homology family (PCH), such as formin-binding protein 17 (FBP17). These proteins were shown to bind to phosphatidylserine and PIP$_2$ in membranes, where they help to deform the membrane during endocytosis. They were also shown to bind to dynamin and recruit and activate N-WASP at the plasma membrane, serving as a link between the endocytosis machinery and the actin cytoskeleton (52). It has recently been shown that another protein, sorting nexin 9 (SNX9), simultaneously binds to dynamin and N-WASP and stimulates Arp2/3-mediated actin assembly, providing another link between the actin cytoskeleton and the endocytosis machinery (77, 78). Buss *et al*. showed that Myosin IV colocalized with clathrin-coated vesicles and binds AP-2 *in-vitro*. Overexpressing the tail region of myosin IV blocked transferrin endocytosis in fibroblasts (79). Myosin IV belongs to a group of proteins that move along actin filaments and help in movement of membrane vesicles within the cytoplasm (80); this could help link the actin cytoskeleton to the inward movement of CCV. It is likely that more protein linkers between the actin cytoskeleton and the endocytosis machinery will be discovered as research progresses in this field.

FIGURE 1.2. Steps during CME and possible links to actin

Clathrin-mediated endocytosis is initiated when adaptor molecules bind to internalization motifs in the cytoplasmic tail of receptors. This is followed by the recruitment of the clathrin and the formation of clathrin-coated pits. Hip1R binds to clathrin and F-actin and may help coordinate the interaction of actin with clathrin at this stage. Dynamin is recruited to the vesicles and is important for the scission of the nascent vesicle from the plasma membrane. Numerous actin-binding proteins that bind the PRD of dynamin may be responsible for linking the actin cytoskeleton to dynamin at this stage. Vesicle uncoating is stimulated by the ATPase activity of Hsc70 which is recruited to the vesicle by auxilin (which binds membrane phospholipids). Uncoated vesicles are able to fuse with other vesicles and be transported to various destinations by interactions with the microtubule network. Interactions between the actin cytoskeleton and microtubule motor proteins (myosins) also facilitate this process.
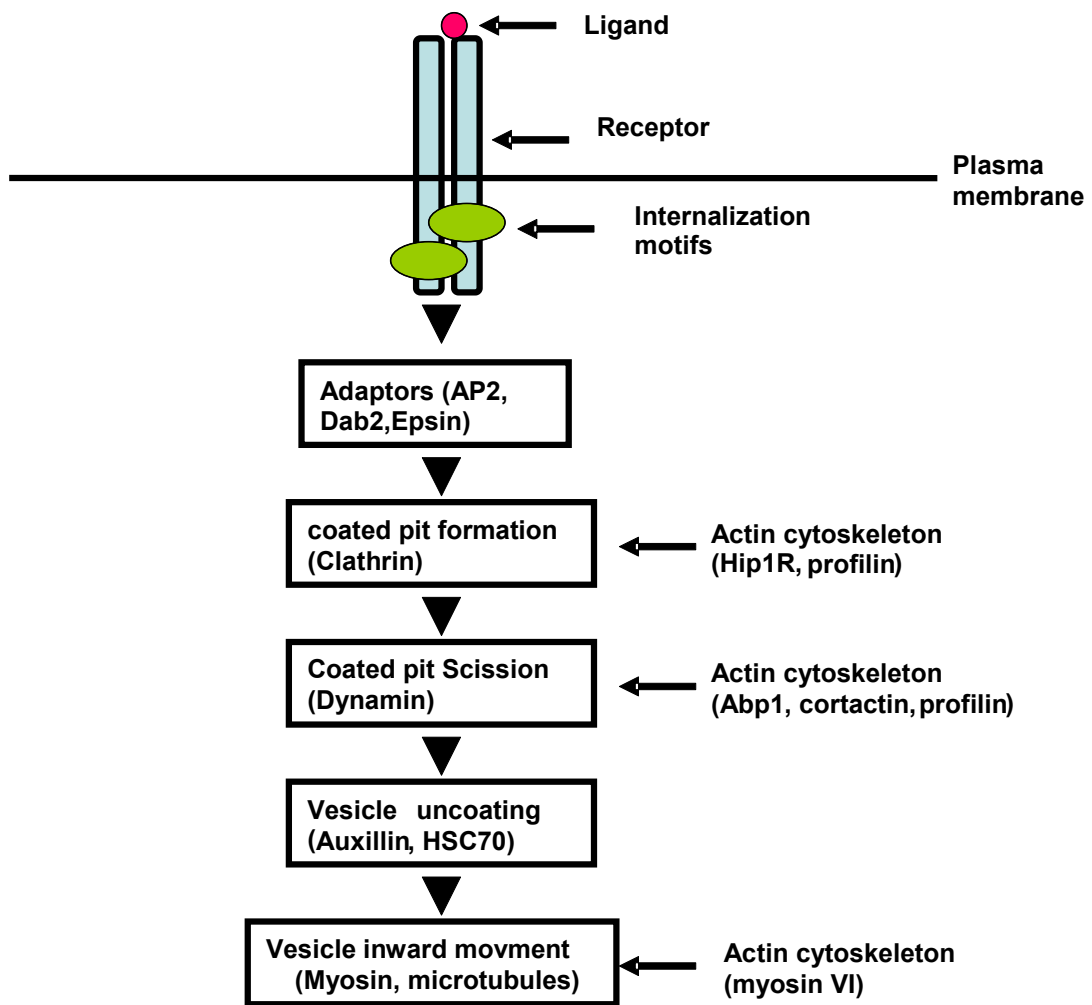
1.7 Regulation of the actin cytoskeleton

The regulation of dynamic actin networks by extracellular signals has been extensively studied during the last decade and has been shown to be largely mediated through the Rho family of GTPases. Rho GTPases (specifically Cdc42, Rac1 and RhoA) are activated in response to a variety of extracellular signals (73). The activation of these GTPases in many cases leads to the activation of the Arp2/3 complex, which in turn regulates actin polymerization. The Arp2/3 complex is required to drive *de novo* actin nucleation, since forming new actin filaments from actin monomers is energetically unfavorable (81). However, the activity of the Arp2/3 complex itself is low without the help of nucleation-promoting factors (NPFs). Examples of NPFs include WASP, N-WASP and WAVE. Rho GTPases are linked to the Arp2/3 complex via these NPFs (81).

How receptor activation leads to varied responses by the actin cytoskeleton is not yet fully understood, but there is evidence that the activation of different Rho GTPases help to generate these varied responses. RhoA helps regulate the formation of stress fibers, Rac1 regulates lamellipodia formation, and Cdc42 regulates filopodia formation (69, 73). The small GTPases that are involved in directly regulating the actin cytoskeleton during endocytosis are not yet known, although a role had been described for Rac and Rho whose mutants were shown to block endocytosis of transferrin (82) and low density lipoproteins (83).

A number of groups, including our own, have shown that BCR crosslinking can create dynamic changes in the actin cytoskeleton (22, 35, 84-87). Hao *et al*, showed that the

actin cytoskeleton undergoes an initial decrease in F-actin levels followed by an increase

in F-actin levels at localized sites within the cell after BCR crosslinking of DT40 cell

lines as well as primary splenocytes (87), indicating that BCR crosslinking induces

dynamic changes in the actin cytoskeleton. Our lab also has shown a similar

accumulation of F-actin with the BCR after BCR crosslinking (35), however the link

between the two is yet to be fully elucidated. Among the potential candidates that could

link BCR signaling to actin cytoskeletal rearrangements is Bam32 (B-cell adaptor

molecule of 32 kDa). It contains an N-terminal SH2 domain, C-terminal PH domain, and

a number of tyrosine residues, one of which has the consensus tyrosine-phosphorylation

motif (YXXP) (88). It is regulated by BCR signaling in a PI-3K-dependent manner and

by NFAT activity (88). Following BCR crosslinking, Bam32 is recruited to the plasma

membrane via the interaction of its PH domain with phosphatidylinositides on the plasma

membrane (88). Bam32 also regulates the reorganization of the actin cytoskeleton by

activating Rac1 in response to the BCR crosslinking (85), indicating that Bam32 might

serve to link signals generated during BCR signaling to the reorganization of the actin

cytoskeleton following BCR crosslinking. Not surprisingly, Bam32 knockout mice had a

marked decrease in the rate of BCR internalization, probably due to defects in the BCR

induced reorganization of the actin cytoskeleton (86). In support of this, preliminary data

from our lab shows BCR signaling-dependent activation of Vav, the GEF of Rho-family

GTPases and WASP, an actin nucleation promoting factor (S. Sharma, unpublished

observations). The activation of Bam32, Vav and WASP by BCR crosslinking provides

further evidence for a connection between BCR signaling and the actin cytoskeleton.

Vascotto *et al*. have recently provided further evidence for how BCR signaling might

regulate the actin dependent trafficking of the BCR. In one study, they observed that

myosin II, an actin motor protein, is regulated by BCR signaling and is required for the

interaction of BCR internalized antigens and MHC class II molecules in late endosomes.

Pharmacologic inhibition or siRNA depletion of Myosin II in splenic B cells led to

defects in antigen presentation and activation of T cells (89). The same group showed

that BCR crosslinking-induced actin reorganization was drastically altered, and the

trafficking of the BCR into late endosomes is inhibited in a Syk-deficient B cells line

(84). These further indicated a three way connection between the actin cytoskeleton, BCR

signaling, and antigen transport pathways.

1.8 The actin cytoskeleton and the intracellular trafficking of the BCR

BCR internalization is dependent on the actin cytoskeleton (35). Work in our lab showed

that Cytochalasin D (CD), an actin disrupter, Latrunculin, an actin depolymerizer, and

Jasplakinolide, an F-actin stabilizer, inhibited the antigen-enhanced internalization of the

BCR. In contrast, constitutive BCR internalization was not affected by CD treatment,

implying that actin involvement in BCR internalization is regulated by signals generated

by BCR crosslinking (35). In CD-treated cells, cortical actin was visualized as reduced

patches close to the plasma membrane that colocalized with BCR, implying that the BCR

was trapped in vesicles that were associated with the actin cytoskeleton. Electron

microscopy studies showed that the BCR accumulated in elongated clathrin-coated pits in

CD-treated cells, indicating that BCR-containing clathrin-coated pits failed to pinch off.

An interesting finding was the fact that, unlike previous reports that showed that CD had

an inhibitory effect on BCR signaling measured by observing of proliferation (actin is

required for cytokinesis during proliferation and this is not a BCR signaling mediated

defect) (90), we observed an elevation of phosphorylation after BCR stimulation in CD-

treated cells compared to untreated cells. Taken together, these results show that the actin

cytoskeleton is important for the rapid internalization of the BCR following antigen

crosslinking, however, the mechanism remains to be elucidated.

1.9 Dynamin and clathrin-mediated endocytosis

Dynamin is a 100 kDa GTPase that is required for pinching off of coated pits formed at

the surface of the plasma membrane. It consists of four main domains, an N-terminal

GTPase domain, a PH domain, a GTPase effector domain (GED,) and a C-terminal PRD

(91). The GTPase of dynamin has a higher GTP hydrolysis activity than small GTPases

such as Ras(91). Its GTPase activity is greatly enhanced (up to 15 fold) by self

oligomerization, as in the case of dynamin being recruited to nascent clathrin-coated pits,

where it oligomerizes to form tight spirals that constrict to pinch off of these vesicles (91,

92). The GED domain regulates dynamin GTPase activity by binding directly to the

GTPase domain of dynamin. This was demonstrated by the fact that addition of the

GED domain alone induced the assembly of dynamin and increased its GTPase activity

by 50 fold (93). The PH domain of dynamin is required for binding to $PI(4,5)P_2$. This

binding has been shown to be important for dynamin recruitment to the plasma

membrane, and mutations in the PH domain disrupt receptor-mediated endocytosis (94,

95). The PRD region contains SH3 domain-binding sites that are defined by the PXXP

motif (where X stands for any amino acid) and interacts with numerous proteins

necessary for a variety of cellular processes including clathrin-mediated endocytosis (96).

These proteins include amphiphysin (53), endophilin (96), PLCγ (97), Grb (98), Cortactin (99), Vav (100), and Abp1(70). The PRD of dynamin is required for the recruitment of dynamin to clathrin-coated pits via interactions with the SH3 domain of amphiphysin (101). Amphiphysin may direct dynamin to coated pits by binding to both dynamin and the alpha adaptin of AP-2 (102). Dynamin also binds to endophilin, a protein recruited to the plasma membrane during receptor-mediated endocytosis (RME) (96). Endophilin converts lysophosphatidic acid (LPA), an inverted cone-shaped lipid, to phosphatidic acid (PA), a cone-shaped lipid, inducing plasma membrane curvature during RME (50). In addition to interactions between the PH domain of dynamin and the plasma membrane, the PRD of dynamin, which interacts with the SH3 domains of amphiphysin and endophilin, helps direct the recruitment of dynamin to the plasma membrane during RME.

Dynamin is known to be regulated by signaling during RME. The recruitment of dynamin1 to the plasma membrane in the neurological synapse upon depolarization of the synapse has been shown to be dependent on dynamin dephosphorylation (103). The dephosphorylation of dynamin during depolarization of the synapse is mediated by the calcium-dependent phosphatase, calcineurin (104). Upon repolarization of the membrane, dynamin is phosphorylated by protein kinase C (PKC) and relocates to the cytosol (105). Ahn *et al*. showed that dynamin 2 can also be tyrosine phosphorylated at positions 231 and 597 by Src kinases in response to β adrenergic stimulation, and this phosphorylation was shown to be important for the internalization of the β_2 adrenergic receptor (106). During internalization of the FcγRI in mast cells, dynamin 2 is dephosphorylated in

response to the aggregation of FcγRI, and this aggregation enhanced the rapid internalization of the FcγRI via CME (107). It is unclear whether dynamin is phosphorylated during BCR crosslinking induced signaling events. Previous studies carried out in our lab have, however, shown that dynamin 2 is recruited to the cell surface upon BCR crosslinking and that this recruitment is inhibited by Src kinase inhibitors (B. Brown, unpublished observations), implying that the function of dynamin 2 is regulated by Src kinases in B cells. In contrast, a recent study reported that dynamin was not phosphorylated in T cells following TCR activation (100), implying that the function of dynamin is regulated by different mechanisms in different types of cells.

1.10 The role of dynamin in linking the actin cytoskeleton to the endocytosis machinery

Previous studies have revealed that dynamin may provide a major link between the endocytosis machinery and the actin cytoskeleton (63) (Fig. 1.3). A number of actin-binding proteins have been shown to have the ability to bind to the PRD of dynamin via their SH3 domains. Examples of such proteins include cortactin that binds and activates the Arp2/3 complex (108), syndapins that interact with N-WASP (74, 76), profilin that binds to actin monomers and promotes ATP exchange on monomeric actin, facilitating filament elongation, and also binds to clathrin (109), and Abp1 that binds F-Actin (70). In addition to serving as a link between the endocytic machinery and the actin cytoskeleton, dynamin is suggested to have a role in linking extracellular signals to the actin cytoskeleton. Dynamin has been shown to regulate the subcellular localization of

Rac to the leading edge of lamellipodia (F actin-based structures), and induces cell spreading in response to PDGF in rat fibroblasts and NIH3T3 cells (110). It was shown in these experiments that dynamin knockdown using siRNA as well as dominant negative transfections of a Dynamin K44A (a mutation that inactivates the GTPase of dynamin) were able to mislocalize Rac and hence interfere with Rac-induced actin reorganization at the leading edge of lamellipodia. Dynamin was also shown to be involved in the reorganization of the actin cytoskeleton at the immunological synapse formed between a T cell and an antigen-presenting cell (APC) (100). In T cells, dynamin was shown to be important for recruiting Vav (100). Vav is a GEF that activates Cdc42, which regulates WASP, a key activator of actin polymerization in the T cell synapse (111). Further evidence for the regulatory role of dynamin in the actin cytoskeleton was provided by studies of actin pedestal formation induced by enteropathogenic *E-coli* invasion of epithelial cells. Interaction of the bacteria with the epithelium generates actin-rich pedestals by bacteria-secreted factors. Bacteria-induced actin pedestal formation was abolished when dynamin expression was knocked down using siRNA (112), implicating dynamin in the regulation of this actin-based structure.

1.11 Abp1 in Endocytosis

Mammalian actin-binding protein 1 (Abp1), which is also known as SH3P7 and HIP-55, was discovered in a screen for novel proteins containing SH3 domains (113). Abp1 belongs to the debrin family of actin-binding proteins (114). It is a 55 kDa phosphoprotein with multiple sequence motifs for protein-protein interactions (114), including two N-terminal actin-binding domains, a PRD, and a C-terminal SH3 domain.

FIGURE 1.3. Dynamin is a major link between CME and actin

Dynamin is recruited to nascent clathrin coated vesicles (CCVs) by interactions between its PH domain and PI(4,5)P at the plasma membrane as well as interactions between its PRD with amphiphysin (binds AP-2) and endophilin (binds lipids at the plasma membrane). The PRD of dynamin mediates the interaction between dynamin and the actin cytoskeleton by binding to a number of SH3 domain-containing actin-binding proteins (Abp1, cortactin, profiling, and syndapins).
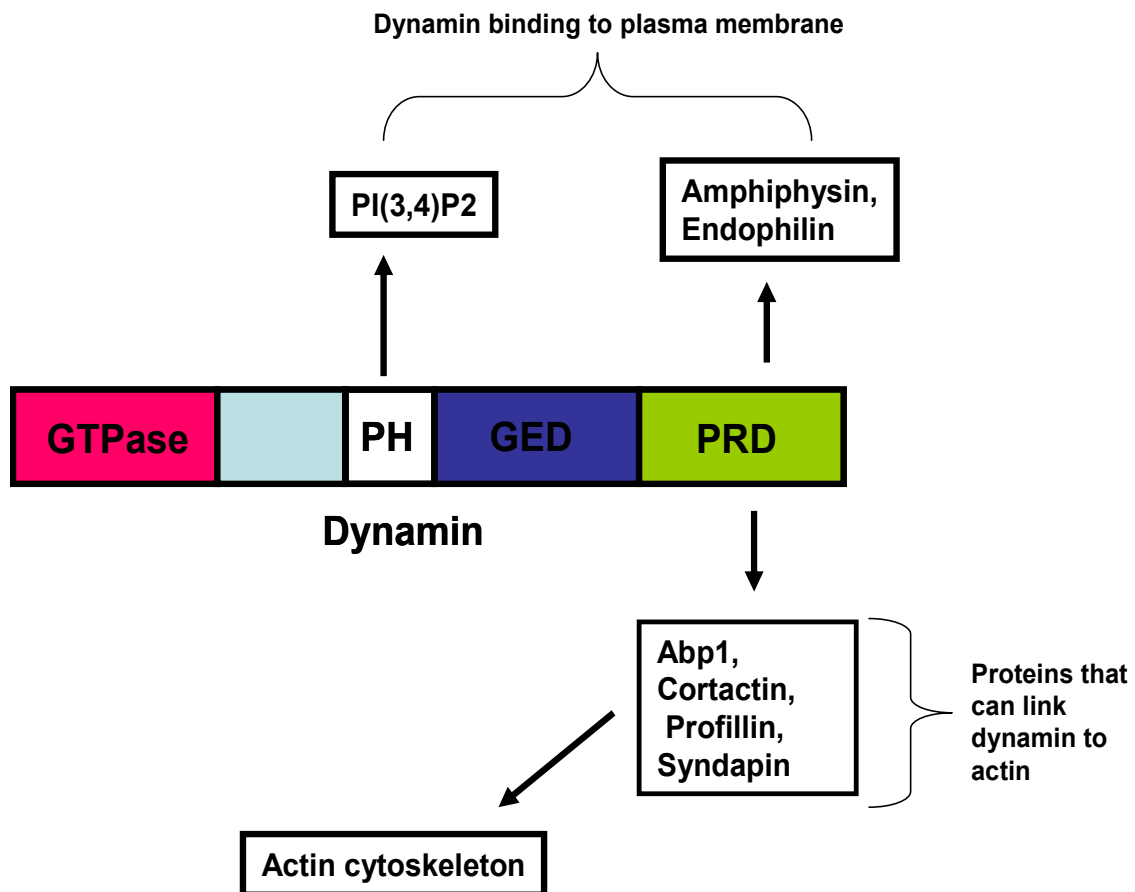
Abp1 was first discovered in yeast and originally characterized as a protein important in actin-dependent endocytosis (59). Sequence analysis revealed that mammalian Abp1 had a significant sequence homology to yeast Abp1 (21% in the actin-binding domain and 40% in the SH3 domain) (115). Yeast Abp1 has the ability to bind to the Arp2/3 complex and increase the affinity of this complex for F-actin. Abp1 binds directly to F-actin in mammalian systems (114). Abp1 is expressed in most tissues of the mouse that have been tested, including testis, spleen, brain, thymus, heart, and lungs, while very little was detected in ovaries and muscle (114). In addition, a number of human cell lines, including Ramos B cells and Jurkat, express the human homolog of Abp1 (114). Mammalian Abp1 lacks the acidic motif required for interacting with the Arp2/3 complex (115, 116), and may, therefore, not be directly involved in regulating the actin cytoskeleton. However, Abp1 was recently shown to interact with and activate N-WASP with the help of Cdc42 in neuronal cells (117), implying that at least in some mammalian systems, Abp1 may be involved in actin regulation.

Abp1 is able to interact with the PRD of dynamin via its SH3 domain, and this interaction has been shown to be important for receptor-mediated endocytosis of transferrin (70). The ability of Abp1 to bind to both F-actin and dynamin implies that it could provide a physical link between the endocytic machinery and the actin cytoskeleton. Abp1 has been shown to be phosphorylated at tyrosines 337 and 347 of the PRD by Syk and Lyn *in vitro* upon BCR crosslinking (114). These findings led us to hypothesize that Abp1 plays a role in linking the BCR signaling and endocytosis pathway to the actin cytoskeleton.

FIGURE 1.4. Actin binding protein 1 (Abp1) and its interactions

Abp1 is a 55 kDa phosphoprotein with multiple sequence motifs for protein-protein interactions, including two N-terminal actin-binding domains, a PRD, and a C-terminal SH3 domain. The PRD of Abp1 contains two consensus tyrosine phosphorylation sites which are substrates for Src kinases in B cells. The SH3 domain of Abp1 has been shown to bind dynamin and facilitate the internalization of transferrin as well as the binding of HPK1 (a MAP4K in the JNK activation pathway) in T cells and hence, serves an adaptor function in T cell signaling.
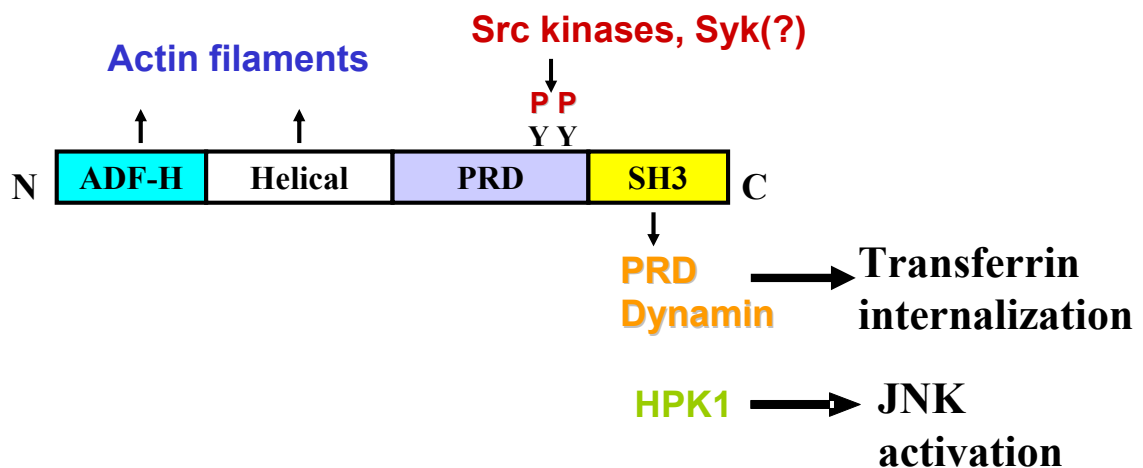
1.12 Role of Abp1 in Lymphocyte activation

Abp1 (HIP55), was identified as a key regulator of hematopoetic progenitor kinase 1 (HPK1) in T cells (118, 119). HPK1 belongs to a group of kinases referred to as germinal center kinases and a subgroup of the STE20-related proteins (120). HPK1 is a MAP4K that is able to activate the c-Jun N-terminal kinase (JNK) pathway by activating MAP3Ks that include MEKK1, MLK3, and TAK1 (121). Activation of JNK leads to the phosphorylation of c-jun, a component of the AP-1 transcription factor that regulates cell proliferation and apoptosis (24). HPK1 was recently shown to be a negative regulator of T-cell receptor signaling by inhibiting TCR-mediated production of IL-2 (121). In 293T cells, Abp1 was shown to associate with HPK1 in immunoprecipitation studies. This interaction was meditated by the binding of the SH3 domain of Abp1 with one of the four PRDs of HPK1 and the interaction was shown to be important for JNK activation (118, 119).

Abp1 is recruited to the T-cell:APC interface in T cells in an antigen-dependent manner where it localizes with lipid rafts (122). Abp1 is phosphorylated on tyrosines 334 and 344 by Zap70 in Jurkat T cells (119). Abp1 is important for TCR-mediated activation of T cells. T cells showed defects in proliferation, cytokine production, and upregulation of activation markers, notably CD69, in Abp1-knockout mice (Abp1$^{-/-}$). Abp1$^{-/-}$ mice displayed a marked reduction in humoral immune response to T-dependent antigens (123). Defective TCR-induced activation of Lat, PLCγ1, JNK and HPK1 were also observed in Abp1$^{-/-}$ mice (123). These point to a role for Abp1 in antigen-induced signaling in T cells.

The role for Abp1 in the signal transduction of B cells has yet to be studied. The fact that it is a substrate for Src kinases, as well as a link to kinases involved in later stages of BCR signaling, raises the possibility that Abp1 serves as an adaptor protein that links upstream to downstream signaling events of the BCR for B cell activation. The ability of Abp1 to interact with the actin cytoskeleton allows it to link the actin cytoskeletal dynamics regulated by BCR activation with BCR-mediated signaling events.

1.13 Significance

In this thesis, I have investigated the interaction between the actin cytoskeleton, BCR signaling and antigen processing pathways. I have identified Abp1 and dynamin 2 as molecular links between the actin cytoskeleton, BCR signaling and antigen processing pathways. I have studied the interrelationship between dynamin and Abp1 and the role of these two proteins in regulating BCR-mediated signal transduction and antigen processing and presentation.

The internalization, processing, and presentation of antigens, by B cells, is a critical step in mounting an effective immune response to pathogens, and this study will provide further insights into how this processes are regulated. When the processes are not properly regulated, B-cell malfunction may lead to serious consequences. This is underscored by a variety of pathological conditions including autoimmune and allergic disorders, some of which can be traced to defects in B-cell regulation. The effector function of the B cell is mediated by the BCR, with which it binds antigens. The BCR has

two functions, first as an antigen-binding receptor and second as a signal transducer that activates the B cell. After binding the antigen the BCR transports it to endosomal compartments that enable the processing and presentation of the antigen. The antigen, when presented to T cells enables additional signals to be supplied to the B cell by T cells. Defects at the stage of antigen processing and presentation or during signal transduction can have significant consequences for the immune system either by attenuating the immune system or overstimulating the immune system. Both scenarios are undesirable events. Understanding both the antigen processing and presentation functions of the BCR and the signaling functions of the BCR as well as the links that may exist between them will enable us appreciate how these events are regulated. This can help us develop effective ways to alter the function of the B cell and help control the immune response.

Chapter 2: Actin binding protein 1 regulates BCR-mediated antigen processing and presentation in response to BCR activation

2.1 Abstract

The B cell receptor (BCR) serves as both signal-transducer and antigen-transporter. Binding of antigens to the BCR induces signaling cascades and antigen-processing and presentation, two essential cellular events for B cell activation. BCR-initiated signaling increases BCR-mediated antigen-processing efficiency by increasing the rate and specificity of antigen transport. Previous studies showed a critical role for the actin cytoskeleton in these two processes. Here we found that actin-binding protein 1 functioned as an actin-binding adaptor protein, coupling BCR signaling and antigen-processing pathways with the actin cytoskeleton. Gene knockout of Abp1 and over-expression of the SH3 domain of Abp1 inhibited BCR-mediated antigen internalization, consequently reducing the rate of antigen transport to processing compartments and the efficiency of BCR-mediated antigen-processing and presentation. BCR activation induced tyrosine phosphorylation of Abp1 and translocation of both Abp1 and dynamin 2 from the cytoplasm to plasma membrane, where they colocalized with the BCR and cortical F-actin. Mutations of the two tyrosine phosphorylation sites of Abp1 and depolymerization of the actin cytoskeleton interfered with BCR-induced Abp1 recruitment to the plasma membrane. The inhibitory effect of a dynamin PRD deletion mutant on the recruitment of Abp1 to the plasma membrane, co-immunoprecipitation of dynamin with Abp1, and co-precipitation of Abp1 with GST fusion of the dynamin PRD

demonstrate the interaction of Abp1 with dynamin 2. These results demonstrate that the BCR regulates the function of Abp1 by inducing Abp1 phosphorylation and actin cytoskeleton rearrangement, and that Abp1 facilitates BCR-mediated antigen-processing by simultaneously interacting with dynamin and the actin cytoskeleton.

2.2 Introduction

B cell-mediated antibody responses constitute one of the major components of the immune system. B cells are activated through two separate stages of signals, and the B cell antigen receptor (BCR) plays an essential role in the generation of both stages of signals. The binding of antigens to the BCR induces signaling cascades that provide the first stage signals for B cell activation (14, 124). Subsequently, the BCR internalizes the antigens to the endosomal system, where the antigens are processed and loaded onto MHC class II molecules. The interaction between B cells and T cells in the context of antigenic peptide-MHC class II complexes triggers the second stage of signals. The induction of affinity maturation and the establishment of B cell memory require both stages of signals (125, 126).

The BCR serves as both signal transducer and antigen transporter. Binding of the BCR to multi-valent antigens not only induces signal transduction, but also triggers rapid internalization of the BCR and accelerates targeting of the BCR to antigen processing compartments (127, 128). The BCR increases the antigen processing and presentation efficiency of B cells by increasing the kinetics and specificity of antigen uptake and transport to antigen processing compartments (127, 128), allowing B cells to present an antigen even when the antigen is sparse. BCR signaling and antigen

processing/presentation functions have been shown to be interrelated. BCR signaling

blockage by tyrosine kinase inhibitors (40, 129), mutations of the tyrosine

phosphorylation sites in the Igα chain of the BCR (43, 130, 131), and loss-function

mutants for Lyn or Syk (38, 84, 132) inhibit accelerated antigen transport and lower the

antigen-presenting efficiency of B cells. Our lab previously showed that crosslinking the

BCR induced the recruitment of clathrin to the cell surface and BCR-containing vesicles

and the tyrosine phosphorylation of clathrin in lipid rafts, both of which were required for

BCR internalization (33). The exact mechanisms underlying the interaction of BCR

signaling and antigen transport pathways have not been well studied.

The involvement of the actin cytoskeleton in BCR-mediated activation of B cells has

long been suggested. Early studies showed that antigen binding induced the translocation

of the BCR and tyrosine kinases Lyn and Syk to the detergent-insoluble cytoskeletal

fractions (133-135), reorganization of the actin cytoskeleton (90, 136-139), and transient

increases in F-actin levels in B cells (35, 87). Hao and August recently showed that

disruption of the actin cytoskeleton altered BCR-induced activation of the MAP kinase

ERK and transcription factors SRF, NFAT, and NF-κB (87). We previously

demonstrated that the dynamic property of the actin cytoskeleton was required for signal-

stimulated BCR internalization. BCR endocytosis is blocked at the pinching-off step

during clathrin-coated vesicle formation in the absence of the functional actin

cytoskeleton (35). Stoddart *et al*. (140) suggested an actin cytoskeleton-dependent and

clathrin-independent BCR internalization pathway in DT40 chicken B cells. It has

recently been reported that myosin II, an actin motor, is activated upon BCR engagement

and facilitates BCR-driven antigen processing and presentation by interacting with MHC class II–invariant chain complexes (141). The findings that the actin cytoskeleton undergoes reorganization in response to BCR signaling and this reorganization is required for signal-induced BCR internalization suggest a role for the actin cytoskeleton in cross talk between BCR signaling and antigen-processing pathways.

Actin-binding protein 1 (Abp1, SH3P7 or HIP-55) is a multi-domain protein that contains two independent F-actin-binding domains (ABDs), a proline-rich domain (PRD), and a Src homology 3 (SH3) domain (114, 115, 118). The SH3 domain of Abp1 is closely related to the SH3 domain of murine cortactin, an F-actin binding protein and a substrate of Src kinase. Thus, Abp1 is able to simultaneously interact with the actin cytoskeleton and molecules of other pathways (70, 118, 142, 143). Abp1 was first cloned from yeast (144) and named Abp1p. Its mammalian homologue was cloned later by several different research groups (114, 115, 118). Abp1 was found to bind F-actin and be capable of directly (in yeast) (116) or indirectly (in mammal) (115, 145) regulating the ability of the Arp2/3 complex to assemble branched actin filament networks. In yeast, Abp1p directly interacts with RVS167/amphiphysin, an endocytosis machinery protein, and was recruited to cortical actin patches partially coinciding with sites of endocytosis (146). In mammalian cells, Abp1 accumulates in lamellipodia in response to growth factors or the expression of dominant-active Rac1 (115) and is involved in transferrin receptor (TfR) endocytosis by a direct interaction with dynamin (70), a GTPase that drives the release of the nascent clathrin-coated vesicles (91). The role of Abp1 in TfR internalization was further confirmed in Abp1-deficient embryonic fibroblasts (147) and in cells where Abp1

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was knocked down by siRNA (148). Recently established Abp1-knockout mice exhibited a moderate reduction in synaptic endocytosis and a dramatic defect in the reformation of fusion-competent vesicles in synapses of hippocampus neurons (147). Abp1-deficiency also caused abnormal structure and function of multiple organs, including the spleen, heart and lung in both heterozygous and homozygous mice (147).

In lymphocytes, antigen engagement of the BCR or TCR induces tyrosine phosphorylation of Abp1, probably by Lyn, Syk or Zap70 (114, 119). Abp1 was found to be recruited to the immunological synapse of T cells and bind to phosphorylated Zap70 in response to TCR stimulation (122). RNAi knockdown of Abp1 inhibited TCR-induced activation of hematopoetic progenitor kinase 1 (HPK1) and the MAP-kinase JNK (118, 119). T cells from Abp1-knockout mice showed similar TCR signaling defects (123). While T and B cells appeared to develop normally, T cells in Abp1$^{-/-}$ mice exhibited reduced T cell proliferation and IL-2 secretion. These defects were accompanied by reduced T cell-dependent antibody responses (123). While the role of Abp1 in B cells has not yet been examined, the data accumulated to date suggest a potential role for Abp1 in interaction with both antigen receptor signaling and antigen transport pathways.

In this study, we examined the relationship of Abp1 with BCR signaling and antigen processing and presentation pathways. We demonstrate that the BCR regulates the function of Abp1 by inducing Abp1 phosphorylation and actin cytoskeleton rearrangement, and that Abp1 facilitates BCR-mediated antigen processing by interacting with dynamin and the actin cytoskeleton.

2.3 Materials and Methods

2.3.1 Mice, cells and cell culture

B cell lymphoma A20 IIA1.6 cells (H-2^d, IgG$_{2a}^+$, FcγRIIB$^-$) were cultured in DMEM supplemented with 10% FBS. C57BL6 mice which were 6~8 weeks old were purchased from Taconic (Hudson, NY). Abp1 knockout mice (Abp1$^{-/-}$) were generated and crossed into a C57BL/6 background as previously described (123). To isolate splenic B cells, single-cell suspensions of splenocytes were subjected to density-gradient centrifugation (2300 x g) in Ficoll (Sigma-Aldrich, St Louis, MO) to obtain mononuclear cells, treated with anti-Thy1.2 mAb (BD Biosciences, San Jose, CA) and guinea pig complement (Rockland Immunobiochemicals, Gilbertsville, PA) to remove T cells, and panned for 2 h to remove monocytes.

2.3.2 DNA constructs and transfection

The cDNA of myc-tagged full length (myc-Abp1), actin-binding domains (ABDs), PRD-SH3 domains (PRD-SH3), and SH3 domain (SH3) of Abp1 were cloned into a pRK5 plasmid as previously described (115). Mutations of tyrosines 337 and 347 to phenylalanine (myc-Abp1 Y337FY347F) were generated using the Stratagene quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by sequencing. DNA constructs were introduced into A20 B cells by electroporation using a Nucleofection kit (Amaxa, Gaithersburg, MD).

2.3.3 Analysis of the movement of the BCR from the cell surface to late endosomes

B cells were incubated with Alexa Fluor (AF) 488-conjugated F(ab')$_2$-goat anti-mouse

IgG or IgM (Invitrogen, Carlsbad, CA) for 20 min at 4°C to label the surface BCR. Cells

were washed and adhered to poly-lysine-coated slides (Sigma-Aldrich) for 40 min at 4°C

and then chased at 37°C for varying lengths of time to allow for BCR internalization. At

the end of each time point, cells were fixed with 4% paraformaldehyde, permeabilized

with 0.05% saponin, and incubated with a mAb specific for lysosome-associated

membrane protein 1 (LAMP-1) (ID4B, ATCC, Manassas, VA) and an AF633-conjugated

secondary antibody. Myc-Abp1 was detected using Cy3-anti-c-myc mAb (Sigma-

Aldrich). Endogenous Abp1 was detected using rabbit anti-Abp1 antibody (70) and an

AF546-conjugated secondary antibody (Invitrogen). Cells were mounted with gel mount

(Biomeda, Foster City, CA) and analyzed using a laser-scanning confocal fluorescence

microscope (LSM 510; Zeiss, Oberkochen, Germany). For quantitative analysis of

images, the cellular distribution of the BCR was divided into three different categories:

the BCR mainly distributed on the cell surface without colocalization with LAMP-1,

extensively colocalized, and partially colocalized with LAMP-1 at the perinuclear region

of cells. Cells were categorized by visual inspection. Over 100 cells from three

independent experiments were analyzed for each time point, and the data were plotted as

percentages of the total number of cells in the images. To quantify the levels of

colocalization between the BCR and LAMP-1, the correlation coefficients of the staining

for the BCR and LAMP-1 in individual cells were determined using the LSM510

software.

2.3.4 Analysis of BCR internalization

Splenic B cells were incubated with biotinylated $F(ab')_2$ fragments of goat anti-mouse IgM (20 μg/ml; Jackson ImmunoResearch) for 30 min at 4^oC to label the surface BCR. After washing off unbound antibodies, cells were chased at 37^oC for 0, 2, 5, and 20 min. The chase was terminated by adding ice-cold DMEM containing 6 mg/ml BSA. The biotinylated antibodies remaining on the cell surface were stained with PE-streptavidin (5 μg/ml; Qiagen, Valencia, CA) at 4^oC. The cells were then fixed and analyzed using a flow cytometer (FACSCalibur, BD Bioscience). The data was plotted as a percentage of the mean fluorescence intensity of cell-surface PE-streptavidin at time 0. To depolymerize the actin cytoskeleton, cells were treated with 5 μm latrunculin (Calbiochem, San Diego, CA) for 30 min at 37^oC before the internalization assay, and latrunculin was also included in the incubation medium during the internalization assay.

2.3.5 Antigen presentation Assay

Splenic B cells were incubated sequentially with the following antibodies and reagents at 4^oC. Anti-CD32/CD16 mAb (BD Biosciences) was used to block FcγII/IIIRs. A peptide (a.a. 52-68) of MHC class II I-E α chain fused with red fluorescence protein (EαRFP) was used as the antigen (a gift from Dr. Mark Jenkins, University of Minnesota). An equivalent concentration of rabbit anti-RFP (Rockland Immunochemicals) was used to bind to RFP and rabbit anti-mouse IgM (5 μg/ml, Jackson ImmunoResearch) to cross-link the BCR. Goat anti-rabbit IgG (Fc) (5 μg/ml, Jackson ImmunoResearch) was used to target the Eα-RFP-anti-RFP antibody complex to the BCR. B cells were allowed to internalize the antigen-antibody complex for 10 min at 37^oC, washed, and incubated at

34

37°C for 14 h. After washing, cells were incubated with anti-CD32/CD16 mAb and biotin-conjugated mAb Y-Ae (eBioscience, San Diego, CA), followed by PE-streptavidin to label Eα-I-A^{b} complexes (149, 150). Cells were fixed and analyzed using a flow cytometer. The surface expression level of MHC class II was monitored before and after the incubation with the antigen-antibody complex using PE-anti-mouse MHC class II (Miltenyi Biotec, Auburn, CA) by flow cytometry.

2.3.6 Analysis of cellular distributions of Abp1, F-actin, and dynamin 2

A20 B cells and splenic B cells were incubated with Cy5-conjugated Fab fragments of rabbit anti-mouse IgG+M to label the BCR and activated by F(ab')$_2$ donkey anti-mouse IgG+M (20 μg/ml; Jackson ImmunoResearch). Cells were permeabilized and stained with goat-anti-Abp1 antibody for endogenous Abp1, Cy3-anti-myc antibody for transfected Abp1, anti-dynamin 2 antibody (BD biosciences), or AF488-phalloidin (Invitrogen) for F-actin. Goat anti-mouse Abp1 antibody was generated by immunization of a goat with GST-Abp1 fusion proteins by Alpha Diagnostics International (San Antonio, TX) and purified using a protein G-sepharose column. To disrupt the actin cytoskeleton, cells were pretreated with 5 μM latrunculin for 30 min at 37°C. Cells were analyzed using a confocal fluorescence microscope. The recruitment of Abp1 to the cell surface was quantified by visually inspecting five randomly selected fields (~100 cells) from each of three independent experiments. Correlation coefficients between the staining of Abp1 and BCR in individual cells were determined using the LSM510 software to quantify the extent of the colocalization. Over 100 cells from two or three independent experiments were analyzed for each time point.

To further analyze the cellular distribution of Abp1 in relation to dynamin 2, A20 cells were co-transfected with plasmids of myc-Abp1 and either GFP-dynamin 2 (GFP-Dyn) or GFP-dynamin 2 with its PRD deleted (GFP-ΔPRD) (kind gifts from Dr. Mark A. McNiven at Mayo Clinic, Rochester, MN). The BCR was labeled using AF633-Fab-goat anti-mouse IgG for 15 min at room temperature on polylysine coated slides. The cells were then activated with rabbit anti-mouse IgG (20 µg/ml) for 5 and 30 min at 37°C, followed by fixation and permeabilization. Transfected Abp1was stained with Cy3-anti-myc antibody, and cells were analyzed using a confocal fluorescence microscope. Correlation coefficients between the staining of Abp1 and GFP-Dyn at the cell surface area were determined using the LSM510 software. Over 30 cells from three independent experiments were analyzed for each condition.

2.3.7 Analysis of tyrosine phosphorylation of Abp1

Untransfected A20 cells and A20 cells transfected with plasmids myc-Abp1 were activated by crosslinking the BCR with goat anti-mouse IgG (20 µg/ml) for indicated times and lysed in a lysis buffer containing 0.5% Triton X100, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl$_2$, 1 mM EGTA, 1 mM Na$_3$VO$_4$, and protease inhibitors (Sigma). Lysates were subjected to immunoprecipitation using rabbit-anti-Abp1 antibody for endogenous Abp1 and anti-myc mAb (Bethyl Labs, Montgomery, TX) for transfected Abp1. The immunoprecipitates were analyzed by SDS-PAGE and Western blotting, probing with anti-phospho-tyrosine mAb (4G10, Upstate Biotech, Lake Placid,

NY). The blots were stripped and reblotted with guinea pig anti-Abp1 (115) or anti-myc antibodies.

2.3.8 Co-immunoprecipitation and coprecipitation of Abp1 and dynamin 2

A20 cells were activated with rabbit anti-mouse IgG for indicated times at 37°C and lysed with the 0.5% Triton X100 lysis buffer. The lysates were subjected to immunoprecipitation using goat-anti-Abp1 antibody and protein G sepharose beads, and the immunoprecipitates were analyzed using SDS-PAGE and Western blotting. The presence of dynamin 2 in the anti-Abp1 immunoprecipitates was detected using anti-dynamin 2 antibody (BD Biosciences), and Abp1 was detected using guinea pig anti-Abp1 antibody.

The DNA construct of GST-dynamin PRD fusion protein (GST-dynamin PRD, a gift from Dr. Mark McNiven, Mayo Clinic, Minnesota) was expressed in the *E. coli* BL21 strain. The bacteria were grown at 37°C in LB medium supplemented with 100 µg/ml ampicillin until OD_{600} = 1.0. The synthesis of the fusion protein was induced by 1.0 mM IPTG (US Biological, Swampscott, MA) for 3 h. Cells were harvested, washed and lysed using a lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 1 mg/ml lysozyme, and protease inhibitor cocktail) and three freeze-thaw cycles. The lysate was centrifuged at 70,000 x g for 25 min at 4°C, and supernatant was loaded onto a GSTrap FF column (GE Healthcare, Uppsala, Sweden). The column was washed with PBS and stored in PBS containing 0.05% Sodium Azide. A20 cells transfected with either myc-Abp1 or myc-Abp1-ABDs were activated with goat anti-mouse IgG (20

μg/ml) for indicated times at 37°C and lysed with the Triton X100 lysis buffer. The

lysates were incubated with GST-dynamin PRD beads overnight, and the precipitates

were analyzed using SDS-PAGE and Western blotting. The presence of myc-Abp1 in the

precipitates was detected using anti-myc mAb (BD Biosciences) and a HRP-conjugated

secondary antibody.

2.3.9 Statistics

P values were generally obtained by conducting 2 tailed, paired students t test on data

from at least 3 independent experiments. To determine the significance between data of

correlation coefficients, the mean correlation coefficients of six individual fields

randomly selected from at least two independent experiments were acquired, and p values

were determined using the 2 tailed, paired students t test.

2.4 Results

2.4.1 Abp1 is required for BCR-mediated antigen uptake

To test whether Abp1 plays a role in BCR-mediated antigen transport, the functions of

Abp1 were disrupted by gene knockout and over-expression of dominant negative

mutants. The Abp1 knockout mouse model was previously developed by Han *et al.*

(123), and the deletion of the Abp1 gene (Fig. 2.1A, *top panel*) and the absence of Abp1

protein expression (Fig. 2.1A, *bottom panels*) were confirmed by PCR analyses of

genomic DNA and Western blot analyses of splenic B cell lysates. The movement of the

BCR from the cell surface to the LAMP-1^{+} compartment was followed by

immunofluorescence microscopy (Fig. 2.1B). Based on the cellular distribution pattern of the BCR and LAMP-1, cells were categorized into three groups: (1) BCR colocalizing with LAMP-1 extensively in the perinuclear region, (2) BCR remaining on the cell surface and periphery with no significant colocalization with LAMP-1, and (3) BCR partially colocalizing with LAMP-1. The numbers of cells in these three categories were plotted as percentages of the total number of cells in the field (Fig. 2.1C). After a 30 min chase at 37°C, the surface labeled BCR was extensively colocalized with the LAMP-1 in over 60% of wt splenic B cells, compared to just ~30% of Abp1$^{-/-}$ splenic B cells (Fig. 2.1B-C). In more than 60% of Abp1$^{-/-}$ splenic B cells, the BCR remained at the cell surface and periphery after a 30 min chase (Fig. 2.1B-C), indicating that Abp1-deficiency dramatically slowed BCR-mediated antigen transport. This is further supported by the quantitative analysis of colocalization between the BCR and LAMP-1 staining. While BCR crosslinking increased the correlation coefficients between the BCR and LAMP-1 in both wt and Abp1$^{-/-}$ splenic B cells, the increase in wt B cells was significantly greater than that in Abp1$^{-/-}$ B cells (Fig. 2.1D). To analyze the effect of Abp1-deficiency on the kinetics of BCR internalization, the surface BCR of splenic B cells from both wt and Abp1$^{-/-}$ mice were labeled with biotin-F(ab')$_2$-anti-mouse IgM at 4°C and chased for 0, 2, 5 and 20 min at 37°C. Biotin-anti-mouse IgM remaining at the cell surface after the chase was detected with PE-streptavidin and quantified using flow cytometry. As shown in Fig. 2.1E, Abp1-deficiency significantly decreased the kinetics of BCR internalization. Furthermore, Abp1-deficiency and F-actin depolymerization by latrunculin-treatment inhibited BCR internalization to a similar extent. These data demonstrate that Abp1-

deficiency inhibits BCR-mediated antigen uptake, consequently reducing the rate of antigen transport to the antigen processing compartment.

To determine which domain of Abp1 is important for BCR-mediated antigen transport, we introduced plasmids of myc-tagged full length Abp1 (myc-Abp1), two actin binding domains (ABDs), myc-Abp1 with two tyrosine phosphorylation sites mutated (Y337FY347F), PRD and SH3 domains (PRD-SH3), or SH3 domain (SH3) of Abp1 (70) into A20 B cells by transient transfection (Fig. 2.2A). The movement of the BCR from the cell surface to late endosomes was analyzed using immunofluorescence microscopy. After 30 min chase, the BCR in more than 50% of cells that underwent electroporation but did not express proteins from transfected plasmids colocalized with LAMP-1 extensively in the perinuclear location (Fig. 2.2A-B). Overexpression of full-length myc-Abp1, myc-Abp1 ABDs, or myc-Abp1 Y337FY347F did not alter the extent of the colocalization between the BCR and LAMP-1 (Fig. 2.2Aa-Al and 2B), indicating that they had no significant effect on the movement of the BCR to the LAMP-1$^+$ compartment. In contrast, only 10-20% of cells that expressed myc-Abp1 PRD-SH3 or myc-Abp1 SH3 showed colocalization of the BCR with LAMP-1, and in ~70% of those cells, the BCR remained on the cell surface and periphery, displaying no significant colocalization with LAMP-1 after the 30 min chase (Fig. 2.2Am-At and 2.2B). This indicates that over expression of the SH3 domain of Abp1 inhibits the movement of the BCR from the cell surface into late endosomes and suggests a role for the SH3 domain of Abp1 in BCR-mediated antigen transport.

FIGURE 2.1. Abp1 gene knockout reduces the rates of BCR internalization and movement from the plasma membrane to late endosomes.

(*A*) Abp1 gene knockout in mice was confirmed using PCR (top panel) and Western blot (bottom panel). (*B*) Splenic B cells from wt and Abp1$^{-/-}$ mice were incubated with Alexa Fluor (AF)-488-F(ab')$_2$-goat anti-mouse IgM at 4°C for labeling and crosslinking the surface BCR and then chased at 37°C for 30 min. Cells were fixed, permeabilized, and labeled with anti-Abp1, anti-LAMP-1, and fluorochrome-conjugated secondary antibodies. Cells were analyzed using a confocal fluorescence microscope. Shown are representative images of three independent experiments. Bar, 5 μm. (*C*) Cells were categorized by visual inspection into three different categories: cells showing extensive colocalization, no colocalization, and partial colocalization between the BCR and LAMP-1. Cells from more than ten randomly selected fields containing at least 15 cells per field from three independent experiments were inspected. Shown are the average percentages (±S.D.) of cells in each of the three categories. **, *p<0.01*. (D) Shown are correlation coefficients between the staining of the BCR and LAMP1 in ~100 individual cells of three independent experiments. Black bars represent mean correlation coefficients. *, *p<0.05,* paired 2 tailed student t test of mean correlation coefficient from six fields of view from three independent experiments. (*E*) The surface BCR was labeled with biotin-F(ab')$_2$-goat anti-mouse IgM at 4°C and chased at 37°C for indicated times. Biotin-anti-mouse IgM left on the surface after the chase was detected by PE-streptavidin and quantified using a FACS Calibur. For latrunculin (LAT) treatment, cells were incubated with 5 μM latrunculin before and during the analysis. The data were plotted as the

percentages of the surface-labeled BCR at time 0. Shown are the averages (±S.D.) of three independent experiments. *, *p<0.05*, **, *p<0.01*.

C

% Cells with indicated BCR and LAMP-1 distribution

WT
KO

**
**

+   -   +/-

Colocalization between BCR and LAMP-1

D

Colocalization of BCR with LAMP1

*

0   30   0   30 (min)
WT   Abp1-/-

43
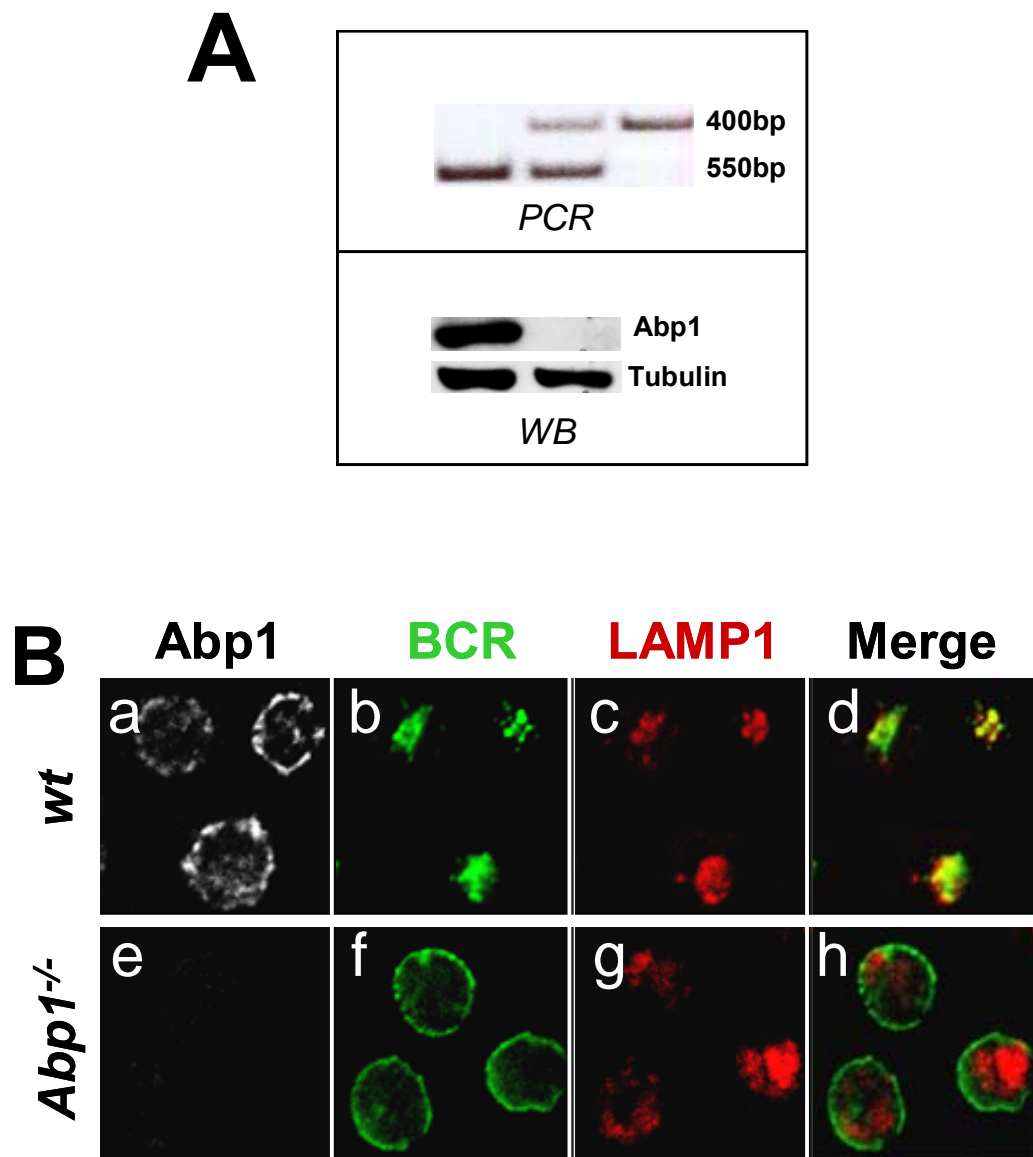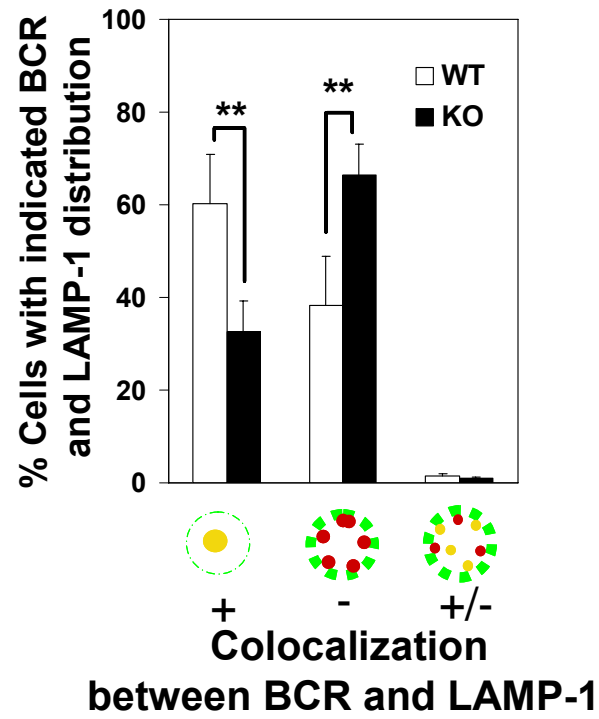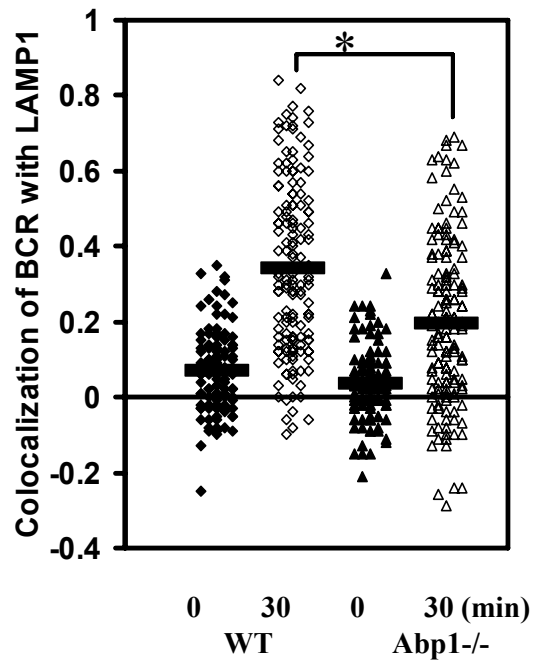
E
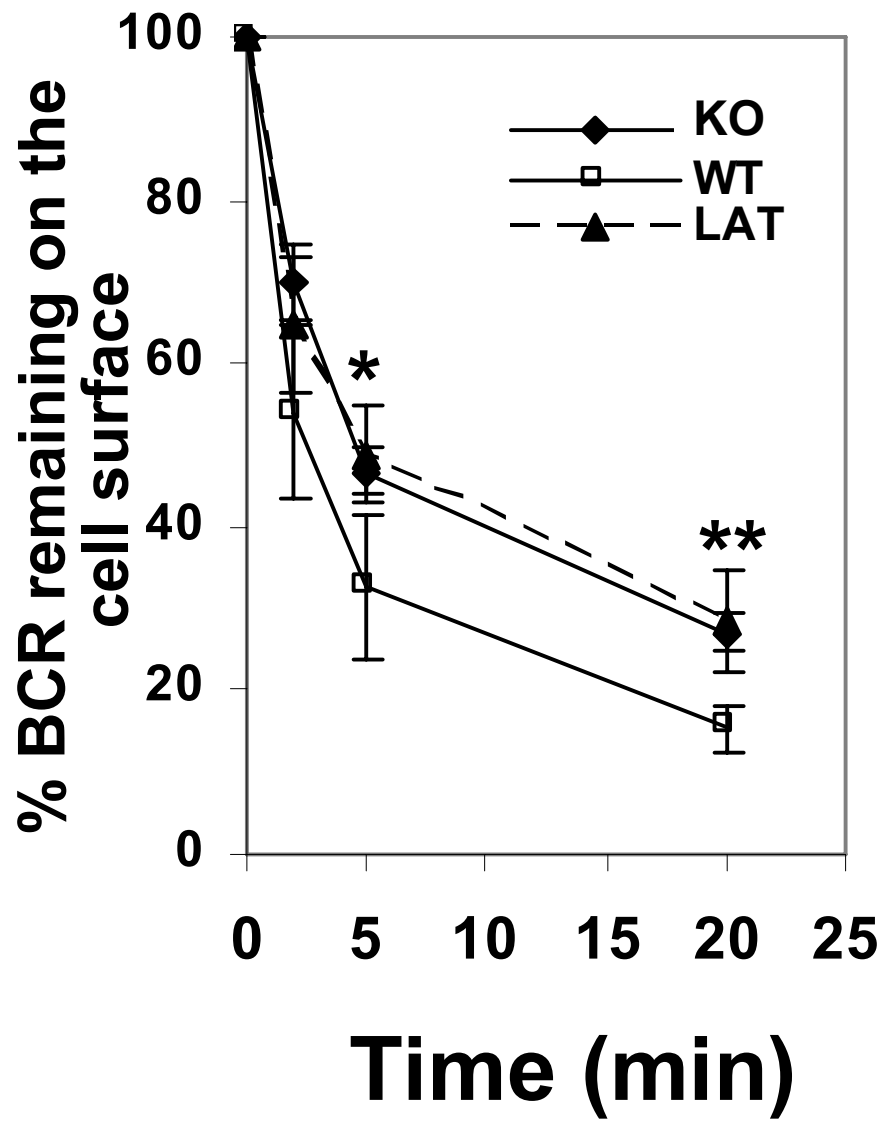
FIGURE 2.2. The effect of over expression of Abp1 domains and mutants on the movement of the BCR from the cell surface to late endosomes.

 (*A*) B cell lymphoma A20 cells were transiently transfected with myc tagged full-length protein of Abp1 (Abp1), Abp1 with its two tyrosine phosphorylation sites mutated (Y337FY347F), actin-binding domains (ABDs), PRD and/or SH3 domains (PRD-SH3 and SH3) of Abp1. Twenty-four hours after transfection, cells were labeled with AF488 goat anti-mouse IgG for 20 min at 4°C and chased for 30 min at 37°C. Cells were fixed, permeabilized, and labeled with anti-myc antibody for myc-Abp1 and anti-LAMP-1 mAb for late endosomes. Arrows indicate cells expressing transfected proteins. Bar, 10 μm. (*B*) Cells were categorized by visual inspection into three different categories as described in Fig. 2.1. Shown are the average percentages (±S.D.) of cells in each of the three categories from three independent experiments. **, *p<0.01*

B

% Cells with indicated BCR and LAMP-1 distribution

Colocalization between BCR and LAMP-1

Legend:
- Untransfected
- WT
- ABD
- Y337FY347F
- PRD-SH3
- SH3

+ − +/−
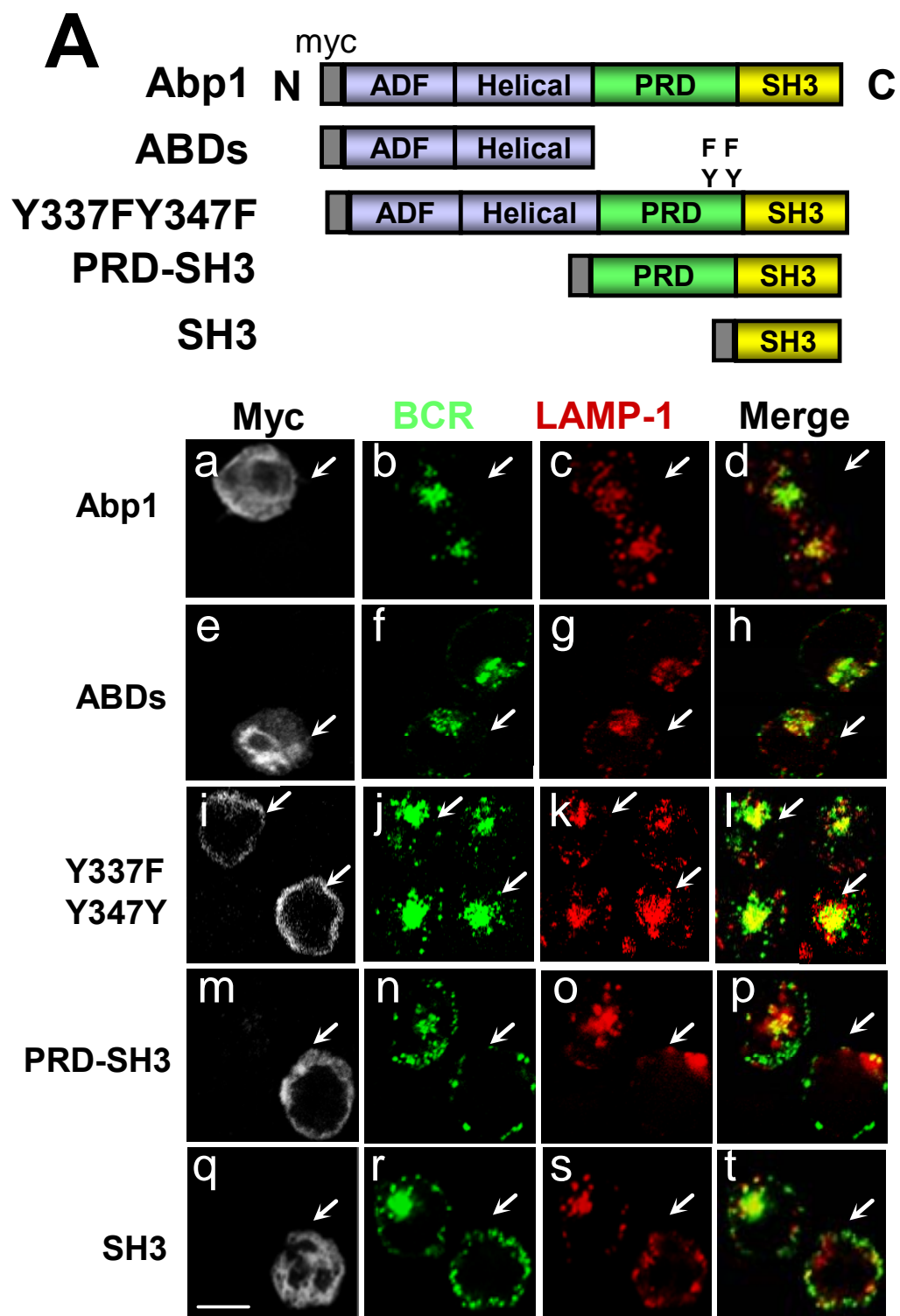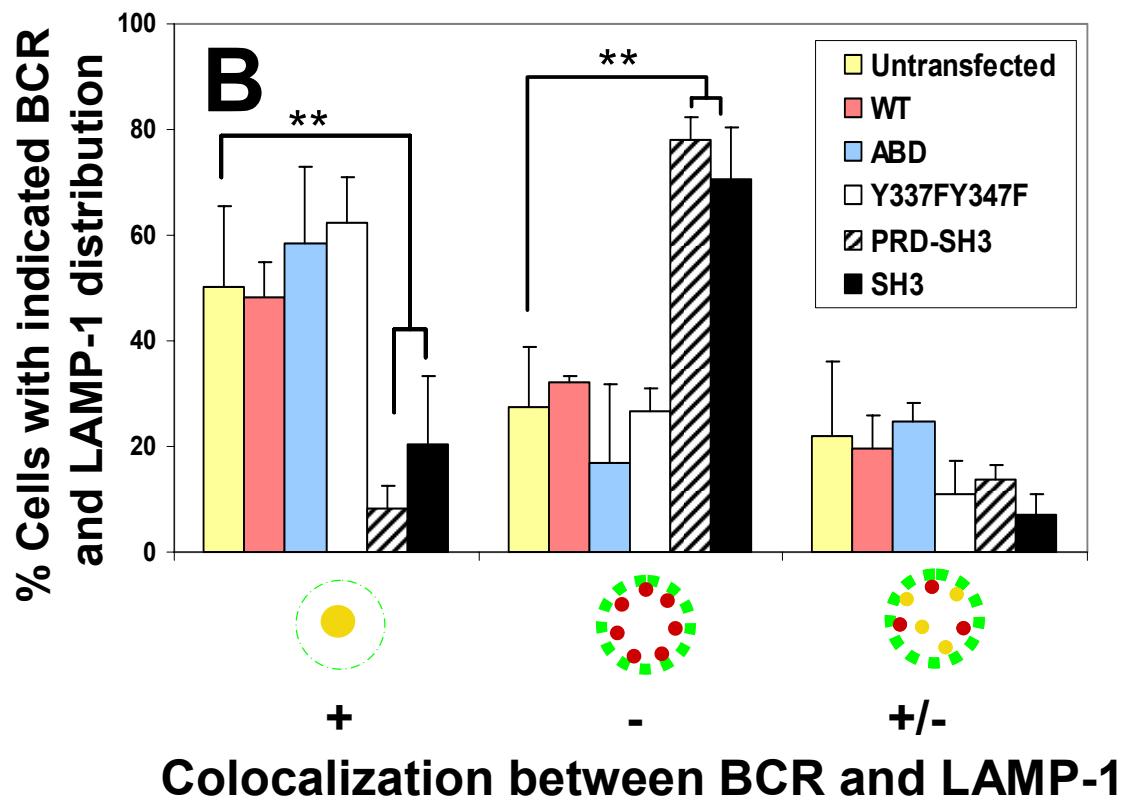
2.4.2 B cells with Abp1-deficiency are defective in BCR-mediated antigen

presentation

The inhibitory effect of Abp1 deficiency on BCR internalization and transport to late

endosomes suggests a reduced efficiency of antigen processing and presentation in Abp1^{-}

[/-] splenic B cells. To test this hypothesis, we determined the antigen processing and

presentation efficiency of mouse splenic B cells using an Eα peptide (a.a. 52-68)-red

fluorescent protein (EαRFP) chimera as the antigen. To follow BCR-mediated antigen

processing and presentation, we targeted EαRFP to the BCR using an antibody complex.

The specific internalization and delivery of EαRFP by the BCR to late endosomes was

confirmed by flow cytometry and immunofluorescence microscopy respectively (data not

shown). Eα peptide-loaded MHC class II I-A^b complexes (Eα-I-A^b) were detected using

Y-Ae mAb (150, 151), indicating levels of antigen presentation. Splenic B cells were

incubated with different concentrations of EαRFP alone for pinocytosis-mediated antigen

processing or EαRFP plus the antibody complex for BCR-mediated antigen processing at

37°C for 10 min to allow antigen internalization, and then washed and incubated at 37°C

for 14 h. The surface Eα-I-A^b staining levels were quantified by flow cytometry. The

surface Eα-I-A^b level of wt splenic B cells incubated with the antigen-antibody complex

was significantly higher than those incubated with EαRFP alone (Fig. 2.3A-B), indicating

a higher efficiency of BCR-mediated antigen processing and presentation than that of

non-specific mechanisms. In addition, the level of surface Eα-I-A^b on wt splenic B cells

that were incubated with the antigen-antibody complex increased with the concentration

of the complex (Fig. 2.3B). In comparison with wt splenic B cells, the surface Eα-I-A^b

level in Abp1$^{-/-}$ B cells was significantly lower and did not increase with the

concentration of the antigen-antibody complex (Fig. 2.3A-B). BCR crosslinking

increased the surface levels of MHC class II in both wt and Abp1$^{-/-}$ splenic B cells, and

Abp1$^{-/-}$ B cells showed MHC class II expression levels similar to wt B cells before and

after BCR crosslinking by the antigen-antibody complex (Fig. 2.3C). These results

indicate that the decrease in surface Eα-I-A^b levels was not the result of decreased I-A^b

expression. These data indicate that Abp1-deficiency decreases the efficiency of BCR-

mediated antigen processing and presentation.

2.4.3 BCR activation induces recruitment of Abp1 to the plasma membrane and

the internalizing BCR

To examine the relationship of Abp1 with BCR signaling pathway, we analyzed the

cellular redistribution of Abp1 in response to BCR activation in both A20 and splenic B

cells using immunofluorescence microscopy. B cells were activated via incubation with

crosslinking antibody for 2 and 10 min at 37°C, fixed, permeabilized, and labeled with an

antibody specific for Abp1. In the absence of BCR crosslinking, Abp1 was primarily

located in the cytoplasm (Fig. 2.4Aa-b, and 2.4Ca). BCR crosslinking for 2 min led to a

redistribution of Abp1 to the cell surface (Fig. 2.4Af-g, and 2.4Ce). Nearly 55% of the

A20 B cells showed this redistribution after 2-min of activation, compared to only 10%

of the unstimulated cells (Fig. 2.4B). By 10 min, Abp1 began returning to the cytoplasm

(Fig. 2.4Ak-l, and 2.4Ci). Such redistribution from the cytoplasm to plasma membrane in

response to BCR activation was observed with both transfected myc-Abp1 and

endogenous Abp1 in A20 and splenic B cells (Fig. 2.4A-C), showing that transfected

myc-Abp1 behaved in a similar manner to the endogenous Abp1.

FIGURE 2.3. B cells from Abp1 knockout mice are defective in Antigen processing and presentation.

(*A*) Splenic B cells from wt (filled) and Abp1$^{-/-}$ (dotted) mice were incubated at 37°C for 10 min with EαRFP (1 µg/ml) alone (gray line) or with the antibody complex that targets EαRFP to the BCR. Cells were washed and incubated at 37°C for 14 h. Eα peptide-loaded MHC class II I-A^{b} complexes on the cell surface were detected using anti-Y-Ae mAb and quantified using flow cytometry. (*B*) Splenic B cells from wt (open squares) and Abp1$^{-/-}$ (closed squares) mice were incubated with different concentrations of EαRFP alone or EαRFP-antibody complexes as described in A. Splenic B cells from wt mice were incubated with different concentrations of EαRFP alone (triangles). Cells were stained and analyzed as described above. Shown are the averages (±S.D.) of mean fluorescence intensity of Y-Ae staining from three independent experiments. **, *p<0.01*. (*C*) The expression levels of MHC class II I-A^{b} of splenic B cells from wt (solid line) and Abp1$^{-/-}$ (dotted lines) mice before (-XL) and after (+XL) exposure to EαRFP-antibody complexes were measured using flow cytometry.
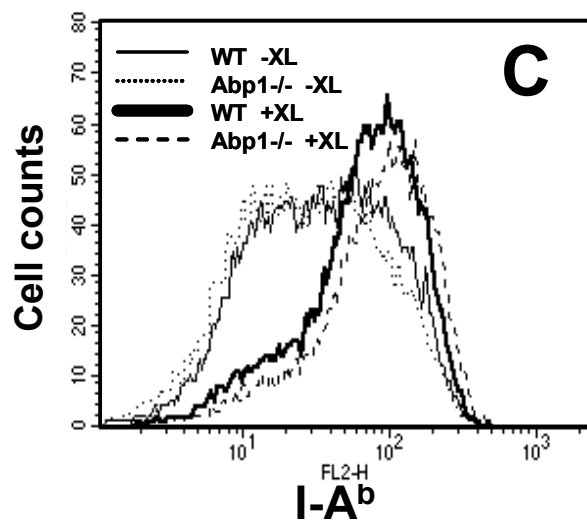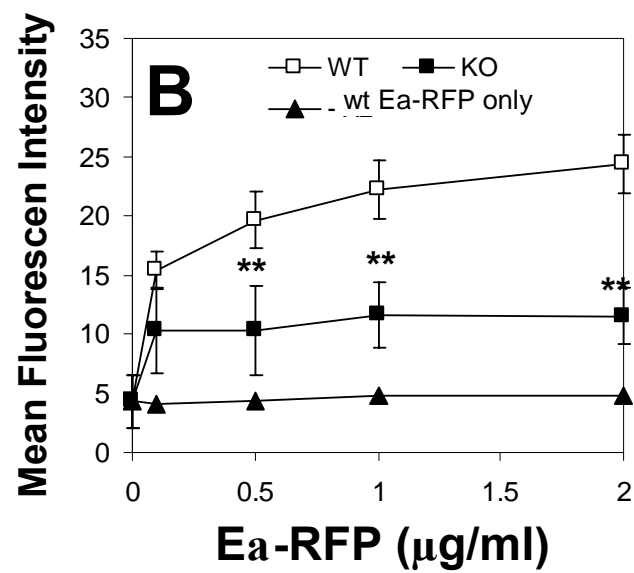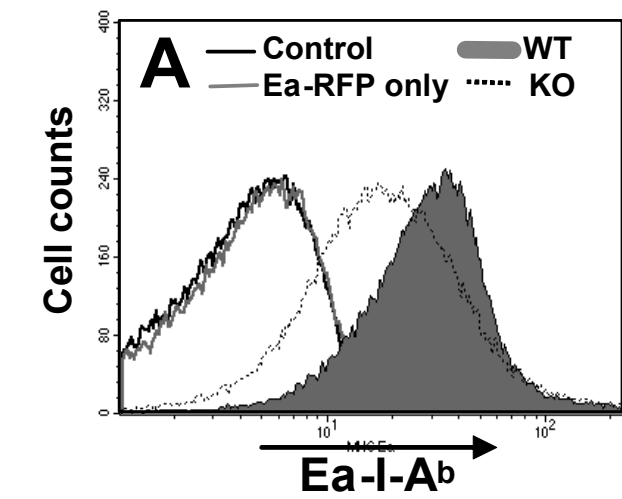
To examine the cellular distribution of Abp1 relative to the antigen bound BCR, the surface BCR in both A20 and splenic B cells were labeled and cross-linked at 4°C and the cells were warmed up to 37°C for 2 and 10 min. After fixation and permeabilization, myc-Abp1 and endogenous Abp1 was labeled with anti-myc mAb and anti-Abp1 antibody, respectively. Before the cells were warmed to 37°C, Abp1 was primarily localized in the cytoplasm, and there was little colocalization of the cytoplasmic Abp1 with the surface BCR observed (Fig. 2.4Ab-e and 2.4Ca-d). After 2-min incubation at 37°C, we observed a dramatic increase in the colocalization of Abp1 with the BCR at the cell surface (Fig. 2.4Ag-j mad 2.4Ce-h), suggesting that Abp1 was recruited to the surface BCR in response to the stimulation. By 10 min, when some of the BCR had been internalized and moved to late endosomes, Abp1 began moving back to the cytoplasm, however, some remained colocalized with the intracellular BCR (Fig. 2.4Al-o and 2.4Ci-l). The correlation analysis of splenic B cells showed an increase in the colocalization of Abp1 with the BCR at 2 min after BCR crosslinking, and this colocalization declined at later time points (Fig. 2.4D). These results showed that BCR stimulation induced the recruitment of Abp1 to the surface and internalizing BCR.

FIGURE 2.4. BCR activation induces the redistribution of Abp1 to the plasma membrane.

(*A and C*) A20 (*A*) and splenic B cells (*C*) were treated (+XL) or untreated (-XL) with either goat anti-mouse IgG (*Aa, Af, and Ak*) or AF488 goat-anti-mouse IgG (*Ab-e, Ag-j, Al-o and C*) at 4°C and chased at 37°C for indicated times. After fixation and permeabilization, cells were labeled with anti-Abp1 antibody for the endogenously expressed Abp1 (*Aa, Af, Ak, and C*) or anti-myc antibody for myc-Abp1 (*Ab-e, g-j, and l-o*). Cells were analyzed using a confocal fluorescence microscope. Shown are representative images from three independent experiments. Bar, 5 μm. (*B*) The redistribution of endogenous Abp1 and myc-Abp1 from the cytoplasm to the cell surface after BCR crosslinking for 2 min was quantified by visual inspection. Over 100 cells from five randomly selected fields of each experiment were analyzed, and the numbers of cells showing Abp1 concentrated on the cell surface were plotted as percentages of the total number of cells inspected. Shown are averages (±S.D.) from three independent experiments. * $p<0.05$, ** $p<0.01$. (*D*) The correlation coefficients of endogenous Abp1 with the BCR in splenic B cells before and after BCR crosslinking was determined using the LSM510 software. Shown are the data generated from >100 cells of two independent experiments. Black bars indicate the mean. ** $p<0.01$, paired two tailed student t test of mean correlation coefficient of 6 fields of view from two independent experiments.

A20 B cells

A

Abp1	myc-Abp1	BCR	Merge	Colocalized
a	b	c	d	e
f	g	h	i	j
k	l	m	n	o

-XL (row a–e)
2 min (row f–j)
10 min (row k–o)

B

% Cells with redistribution of Abp1

□ - XL
■ + XL

*
**

Endogenous   myc

Abp1

Splenic B cells

C

	Abp1	BCR	Merge	Colocalized
-XL	a	b	c	d
2 min	e	f	g	h
10 min	i	j	k	l

D

Abp1:BCR Correlation coefficient

Time (min)
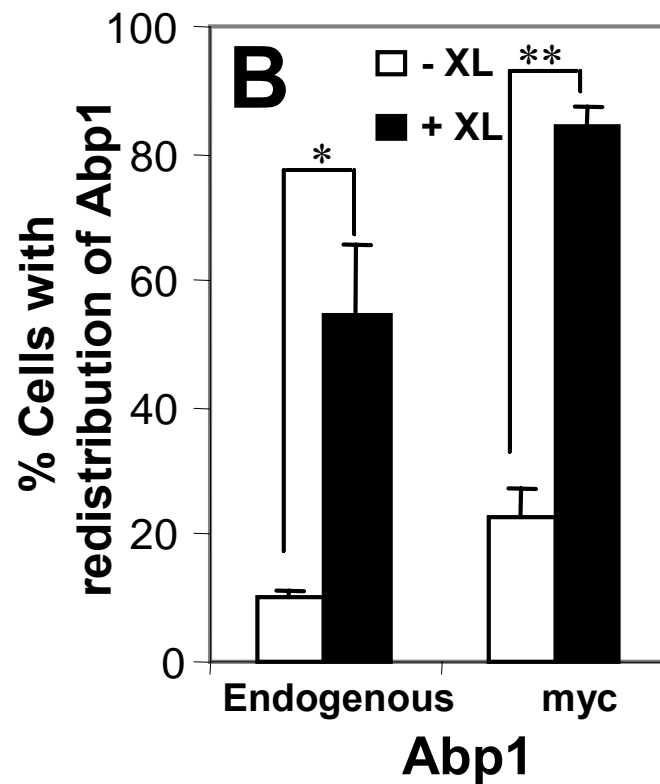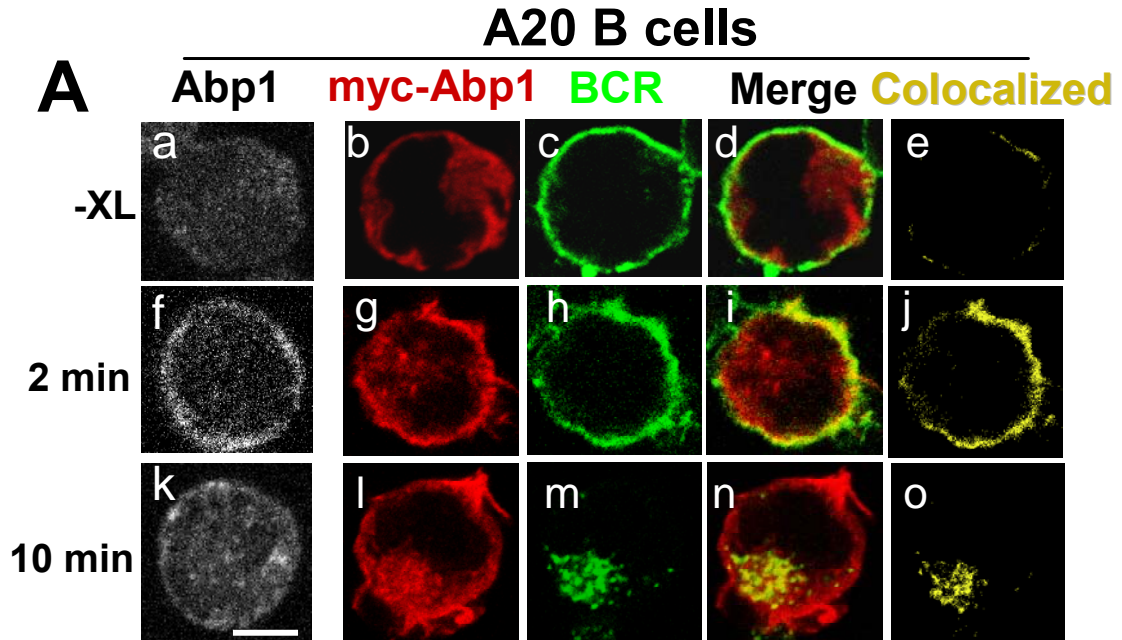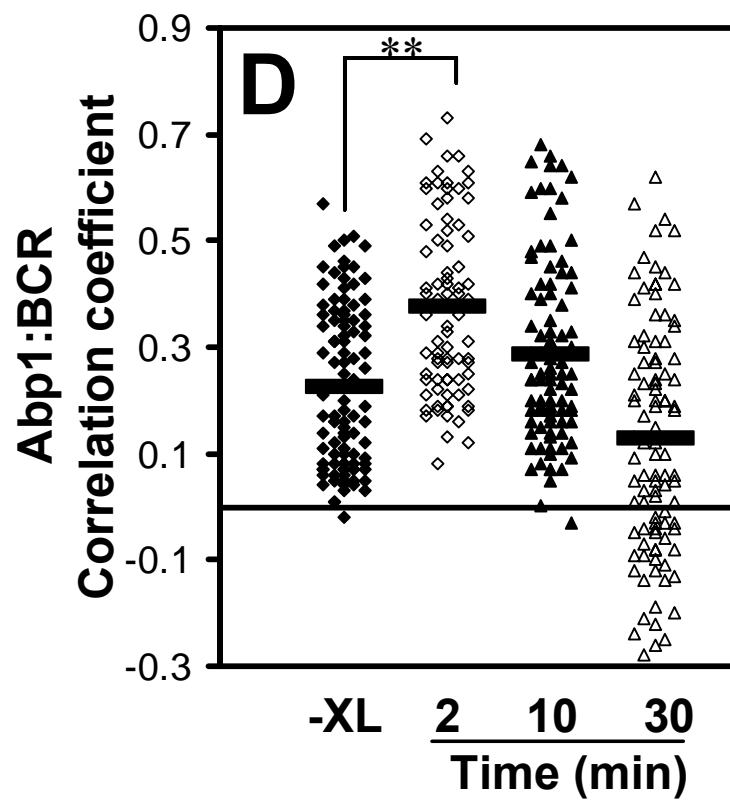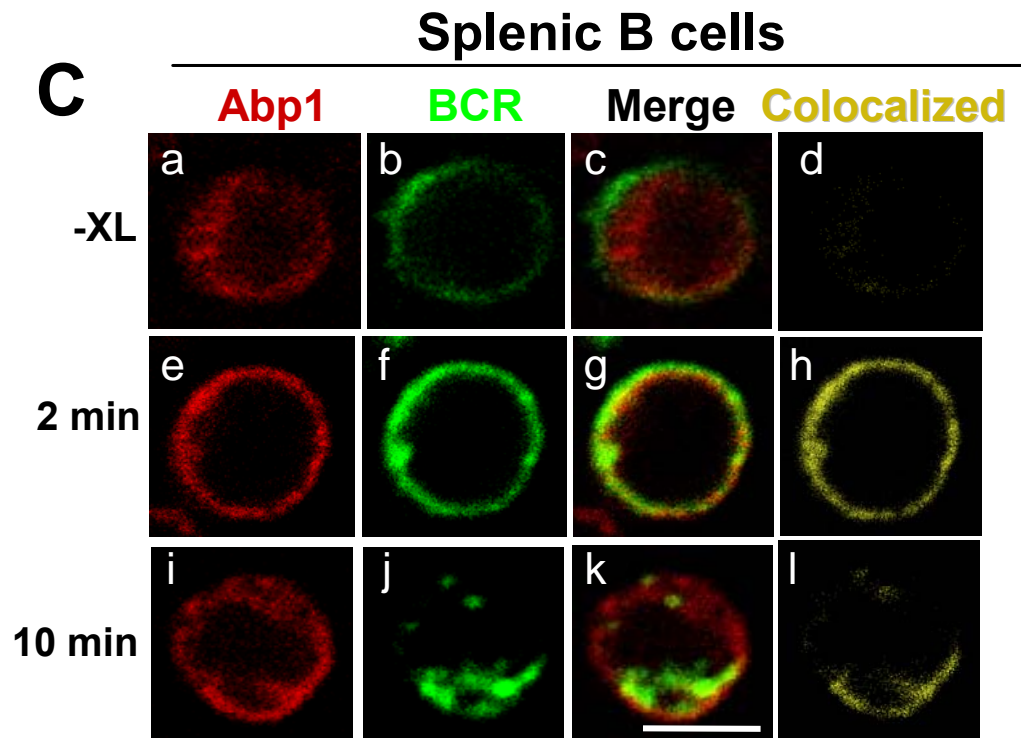
2.4.4 BCR-induced redistribution of Abp1 depends on BCR-induced tyrosine

phosphorylation of Abp1

Abp1 has been reported to undergo tyrosine phosphorylation at Y337 and Y347 in

response to BCR stimulation (114). To test whether BCR-induced tyrosine

phosphorylation of Abp1 is related to its cellular distribution, we followed the time

course of the tyrosine phosphorylation of endogenous Abp1 and transfected myc-Abp1.

Untransfected A20 B cells and A20 B cells that were transfected with full length myc-

Abp1 were activated for indicated times by crosslinking the BCR. Cells were lysed, and

the cell lysates were subjected to immunoprecipitation using anti-Abp1 antibody for

endogenous Abp1 or anti-myc mAb for transfected myc-Abp1. The immunoprecipitates

were analyzed by SDS-PAGE and Western blot, probing for phospho-tyrosine. As

shown in Fig. 2.5A, crosslinking of the BCR increased the tyrosine phosphorylation of

endogenous Abp1 as early as 2 min and this increase appeared to be sustained at least for

30 min (Fig. 2.5A, *top panels*). Similarly, BCR crosslinking induced the tyrosine

phosphorylation of myc-Abp1. This phosphorylation peaked at 2 min, but rapidly

decreased to undetectable levels by 10 min (Fig. 2.5A, *bottom panels*). These results

show that BCR crosslinking increases tyrosine phosphorylation of Abp1 at a time

corresponding to BCR-induced redistribution of Abp1.

To test whether BCR-induced tyrosine phosphorylation of Abp1 is important for its

cellular redistribution, we determined the effect of mutations of Abp1 tyrosines 337 and

347 into phenylalanines (Abp1 Y337F/Y347F) on the cellular redistribution of Abp1.

The DNA construct of myc-Abp1 Y337FY347F was introduced into A20 B cells by

FIGURE 2.5. BCR-induced cellular redistribution of Abp1 is dependent on BCR-induced tyrosine phosphorylation of Abp1.

(*A*) Untransfected A20 B cells (top) and A20 B cells transfected with myc-Abp1 (bottom) were treated (+XL) or untreated (-XL) with goat-anti-mouse IgG for varying lengths of time to activate the BCR. Then cells were lysed, and endogenous Abp1 and myc-Abp1 were purified from cell lysates by immunoprecipitation using anti-Abp1 and anti-myc antibodies, respectively. The immunoprecipitates were analyzed by SDS-PAGE and Western blot, probing with anti-phosphotyrosine mAb (4G10). The blots were stripped and reblotted with anti-Abp1 or anti-myc antibody. Shown are representative blots of three independent experiments. (*B*) A20 cells transiently transfected with wt myc-Abp1 (*Ba-Bb*) and myc-Abp1 Y337F/Y347F (*Bc-Bd*) were treated (+XL) and untreated (-XL) with goat anti-mouse IgG for 2 min, and then fixed, permeabilized, and labeled with Cy3-anti-myc mAb for myc-Abp1. Cells were analyzed using a confocal fluorescence microscopy. Shown are representative images of three independent experiments. Bar, 10 μm. (*C*) Cells in images were quantified as described in Fig. 2.4B. Over 100 cells from three independent experiments were analyzed. Shown are the averages (±S.E.) from three independent experiments. **, *p<0.01*.

B

| | **-XL** | **+XL** |

myc mAbp1

wt

a   b

Y337FY347F

c   d

C

% Cells with redistribution of Abp1

□ - XL
■ +XL

**

myc-Abp1   Y337F/Y347F
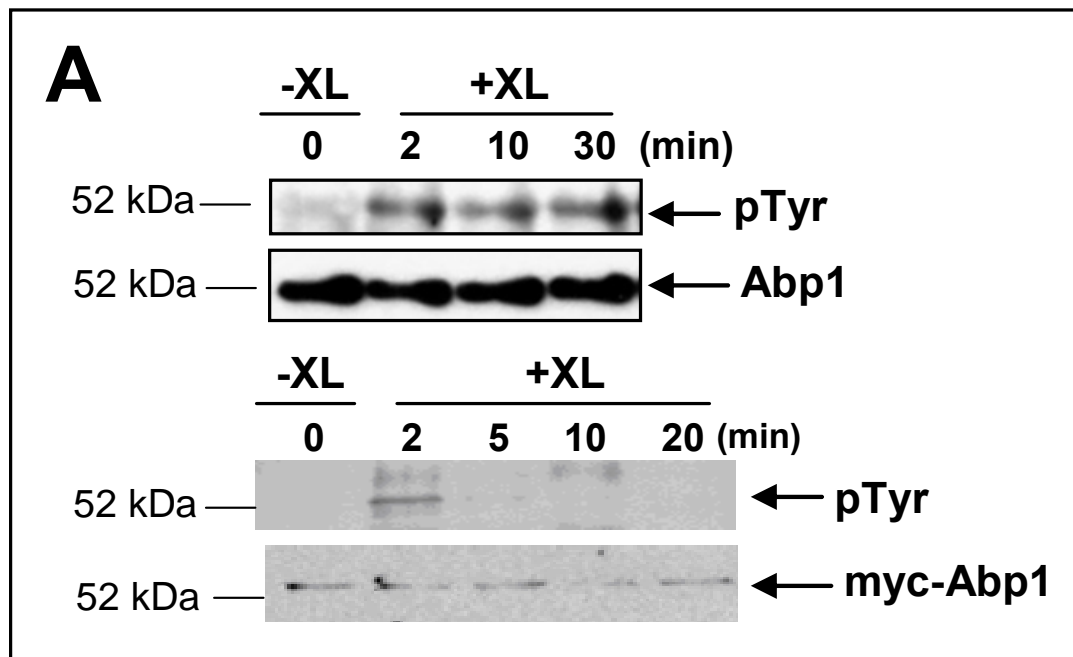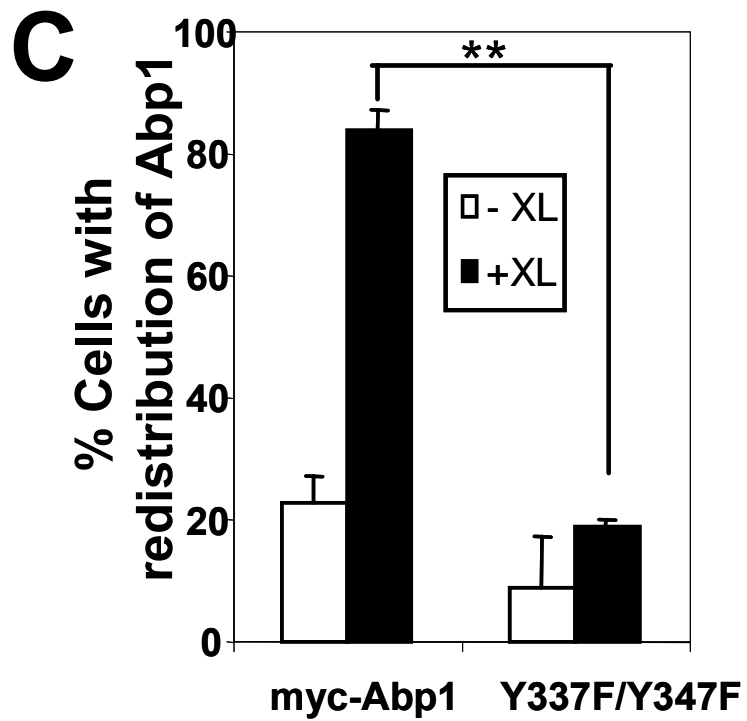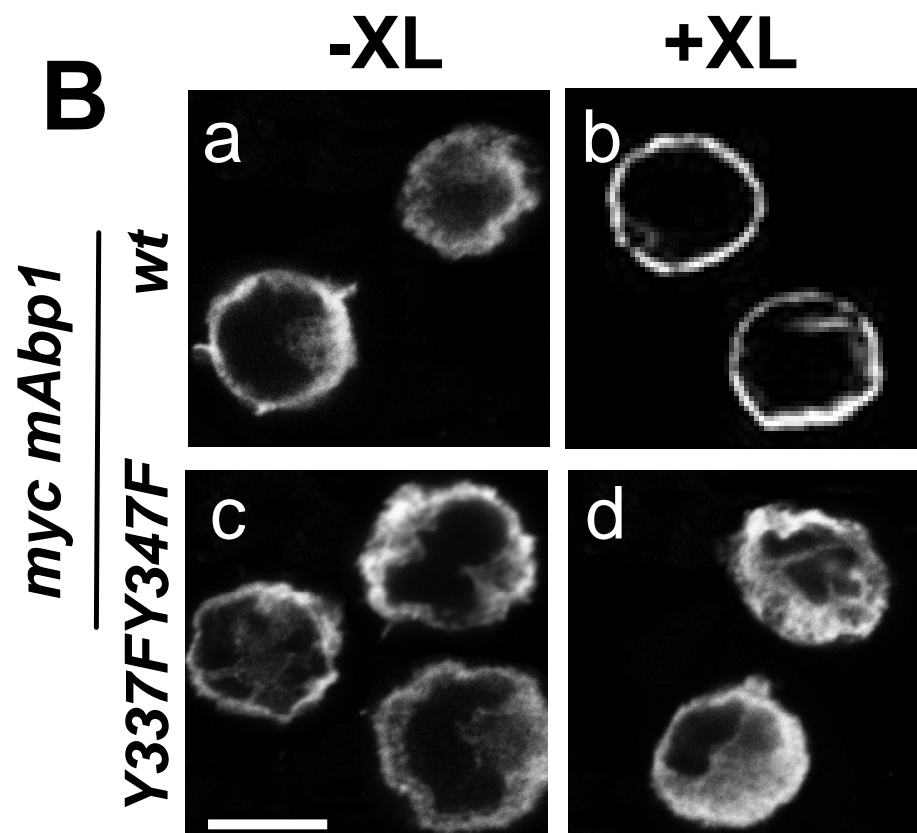
transfection and the cellular redistribution of expressed protein was analyzed by immunofluorescence microscopy in comparison to wt myc-Abp1 (Fig. 2.5Ba-Bd). The percentage of wt myc-Abp1-expressing cells showing cell surface redistribution of Abp1 increased from 20% to 80% after BCR crosslinking for 2 min (Fig. 2.5Ba-Bb and 2.5C). In contrast, there was no significant increase in the percentage of Abp1 Y337F/Y347F-expressing cells showing the redistribution (Fig. 2.5Bc-Bd and 2.5C). This indicates that the Abp1 redistribution depends on BCR-induced tyrosine phosphorylation of Abp1.

2.4.5 BCR-induced Abp1 redistribution depends on the actin cytoskeleton

BCR activation induces reorganization of the actin cytoskeleton (35, 87). The presence of two ABDs in Abp1 implies that its cellular redistribution may rely on BCR-induced actin cytoskeleton reorganization. To test this hypothesis, we analyzed the cellular distribution of Abp1 relative to F-actin and tested the effect of a G-actin sequestering agent latrunculin B on BCR-induced redistribution of Abp1. In A20 B cells, myc-Abp1 was colocalized with F-actin extensively with and without BCR activation (Fig. 2.6A). Both F-actin and endogenous Abp1 were located at the cell periphery and cytoplasm in unstimulated cells (Fig. 2.6Ba) and rapidly moved to the plasma membrane upon BCR activation (Fig. 2.6Bc). In latrunculin-treated cells, Abp1 was primarily located in the cytoplasm with (Fig. 2.6Bd) or without BCR activation (Fig. 2.6Bb). Latrunculin B treatment significantly decreased the number of cells showing BCR-induced redistribution of Abp1 (Fig. 2.6B-C). These data indicate that BCR-induced Abp1 redistribution is dependent on the actin cytoskeleton.

FIGURE 2.6. BCR-induced Abp1 redistribution depends on the actin cytoskeleton.

(*A*) A20 cells transiently transected with myc-Abp1 were incubated with (+XL) or

without (-XL) goat anti-mouse IgG for 1 min. After fixation and permeabilization, cells

were labeled with AF488-phalloidin for F-actin and Cy3-anti-myc mAb for myc-Abp1.

(*B*) A20 cells were pretreated with or without latrunculin (Lat) and activated in the

presence or absence of Lat for 1 min. Cells were labeled with anti-Abp1 antibody and

AF488-conjugated secondary antibody and analyzed using a confocal fluorescence

microscopy. Shown are representative images of three independent experiments. Bar, 10

μM. (*C*) Cells in images quantified as described in Fig. 2.4B, and shown are averages

(±S.D.) from three independent experiments. **, *p<0.01*

A

| phalloidin | myc | overlay |

-XL

+XL
1 min

B

| | - Lat | + Lat |

-XL

a   b

+XL

c   d

endogenous Abp1

C

□ -XL
■ +XL

**

% Cells with
redistribution of Abp1

-LAT   +LAT
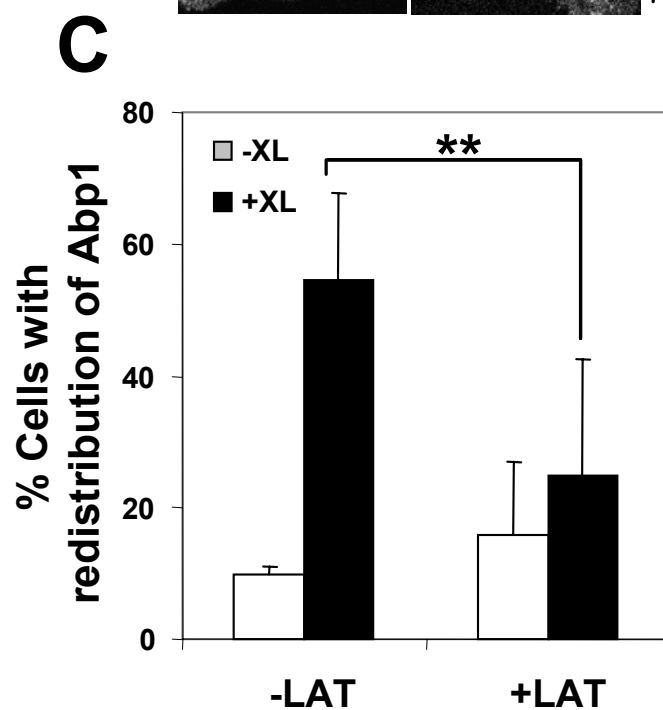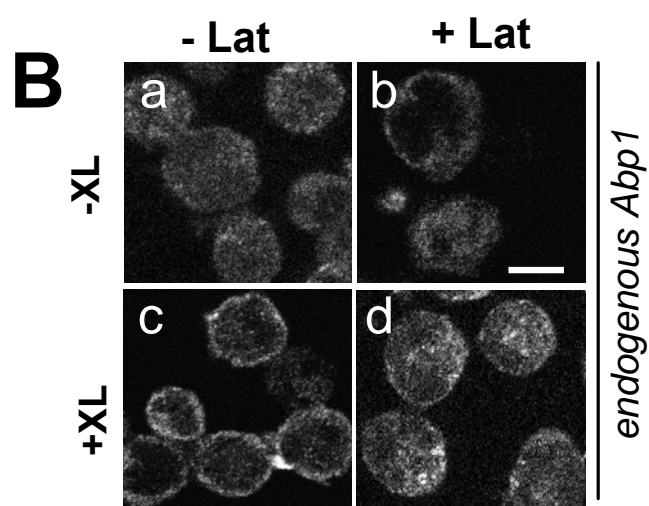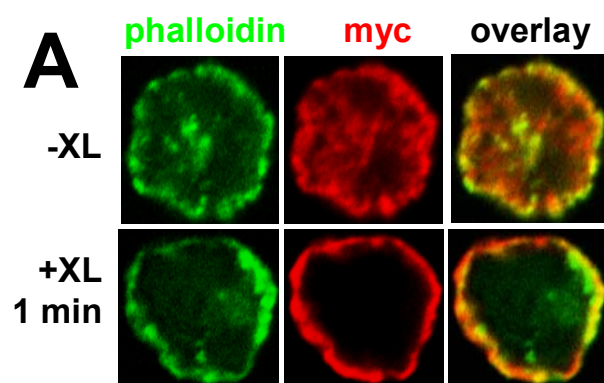
2.4.6 The interaction of Abp1 with dynamin 2

The interaction of dynamin and Abp1 through their PRD and SH3 domains has been

shown to be important for Tf internalization (70). To examine the relationship between

Abp1 and dynamin 2 in B cells, we followed the interaction of these two proteins by

immunofluorescence microscopy, co-immunoprecipitation, and co-precipitation.

Immunofluorescence microscopy studies showed that similar to Abp1, dynamin 2 was

primarily distributed in the cytoplasm in the absence of stimulation. In response to BCR

crosslinking, dynamin 2 was recruited from the cytoplasm to plasma membrane where it

colocalized with Abp1 (Fig. 2.7A). The co-recruitment of dynamin 2 with Abp1 upon

BCR activation suggests a potential interaction between Abp1 and dynamin 2. To further

test whether the interaction between the two proteins depends on their SH3 and PRDs, we

co-transfected A20 B cells with myc-Abp1 and GFP-dynamin 2 (GFP-Dyn, Fig. 2.7Ba-l)

or GFP-dynamin 2 with its PRD deleted (GFP-ΔPRD, Fig. 2.7Bm-x) and followed the

cellular distribution of these proteins by immunofluorescence microscopy. Similar to

endogenous dynamin 2, GFP-Dyn showed a cytoplasmic distribution in the absence of

stimulation (Fig. 2.7Bc) and was recruited to the cell surface where it colocalized with

myc-Abp1 and the BCR after crosslinking of the BCR for 5 min (Fig. 2.7Be-h). By 30

min, both proteins accumulated in the perinuclear region with the BCR (Fig. 2.7Bi-l). In

cells co-expressing myc-Abp1 and GFP-ΔPRD, both proteins appeared to be in the

cytoplasm without BCR activation (Fig. 2.7Bm-p). Upon BCR crosslinking, both GFP-

ΔPRD and Abp1 showed punctuate staining patterns (Fig. 2.7Br-t and 2.7Bv-x). In

contrast to what was observed in cells co-expressing GFP-Dyn and myc-Abp1, GFP-

ΔPRD and Abp1 were neither recruited to the cell surface, nor colocalized with each

other (Fig. 2.7Bq-x). The correlation analysis further showed that the colocalization coefficients between GFP-Dyn and myc-Abp1 were increased in response to BCR activation (Fig. 2.7C). In contrast, there was no significant increase in the colocalization coefficients between GFP-ΔPRD and myc-Abp1 following BCR activation (Fig. 2.7C). These results suggest that dynamin 2 interacts with Abp1 through its PRD.

We further examined the interaction between dynamin 2 and Abp1 using co-immunoprecipitation and GST-fusion protein co-precipitation. The cell lysates from unstimulated and stimulated A20 B cells were subjected to immunoprecipitation using a polyclonal antibody specific for Abp1. The presence of dynamin 2 in the Abp1 immunoprecipitates was detected by a dynamin 2-specific antibody. As shown in Fig. 2.7D, dynamin 2 was detected in the Abp1 immunoprecipitates in the presence or absence of BCR activation, and dynamin 2 was absent only when anti-Abp1 antibody was omitted. This result indicates a constitutive interaction between dynamin 2 and Abp1. To further confirm that this interaction is mediated through the PRD of dynamin 2 and the SH3 domain of Abp1, we used a GST fusion protein of dynamin 2 PRD (GST-Dyn-PRD) to precipitate Abp1 from the lysates of cells expressing full length myc-Abp1 and myc-Abp1 ABDs. Similar to the co-immunoprecipitation of endogenous dynamin 2 with Abp1, GST-Dyn-PRD co-precipitated similar amounts of myc-Abp1 from the lysate of A20 cells that were treated or untreated with BCR crosslinking antibodies (Fig. 2.7E), confirming the constitutive interaction of dynamin 2 with Abp1. In contrast, GST-dynamin-PRD failed to precipitate myc-Abp1 ABDs (Fig. 2.7E), indicating that the PRD and SH3 domains of dynamin and Abp1 are essential for their interaction. These results

FIGURE 2.7. Interaction of Abp1 with Dynamin 2.

(*A*) A20 cells were incubated with (+XL) and without (-XL) goat anti-mouse IgG for indicated times, and then fixed, permeabilized, and labeled with anti-Abp1 and anti-dynamin 2 antibodies and corresponding secondary antibodies. The cells were analyzed using a confocal fluorescence microscope. Shown are representative images from three independent experiments. Bar, 10 μm. (*B*) A20 cells were co-transected with myc-Abp1 and GFP-dynamin 2 (GFP-Dyn) (a-l) or GFP-dynamin 2 with PRD deletion (GFP-ΔPRD) (m-x). The cells were incubated with (+XL) (e-l, q-x) and without (-XL) (a-d, m-p) goat anti-mouse IgG for indicated times, and then fixed, permeabilized, and labeled with Cy3-anti-myc antibody to label myc-Abp1. The cells were analyzed by confocal fluorescence microscopy. Shown are representative images from three independent experiments. Bar, 10 μm. (*C*) Shown are the correlation coefficients between myc-Abp1 and GFP-Dyn or GFP-ΔPRD in the cell surface area of >30 cells from three independent experiments. Black bars represent the mean correlation coefficient. * *p<0.05,* paired two tailed student t test of mean correlation coefficient from three independent experiments. (*D*) A20 cells were treated or untreated (-XL) with BCR crosslinking antibody for indicated times. The cells were lysed, and the cell lysates were subjected to immunoprecipitation using goat anti-Abp1 antibody. The cell lysates and immunoprecipitates were analyzed using SDS-PAGE and Western blot, probing for dynamin 2. The blots were stripped and reblotted with anti-Abp1 antibody as loading controls. Shown are representative blots from three independent experiments. (*E*) A20 cells transiently transfected with myc-Abp1 or myc-Abp1 ADF-H were activated by crosslinking the BCR with goat anti-mouse IgG for indicated times and then lysed. The cell lysates were incubated with GST-fusion of

dynamin PRD bound beads. The cell lysates and precipitates were subjected to SDS-

PAGE and Western blot, probing for myc-Abp1. The blots were stripped and reblotted

with anti-GST antibody. Shown are representative blots from two independent

experiments.

A

Dynamin   Abp1   Merge

B

BCR   myc-Abp1   GFP-Dyn   Merge

-XL
a   b   c   d

5 min
e   f   g   h

30 min
i   j   k   l

BCR   myc-Abp1   GFP-ΔPRD   Merge

-XL
m   n   o   p

5 min
q   r   s   t

30 min
u   v   w   x

C

Colocalization of Abp1 and dynamin

*

-XL   +XL   -XL   +XL

Dyn   Dyn ΔPRD

67

D

Coimmunoprecipitation

| Time (min) | -XL | 2 | 10 | 30 | control |

◀Dynamin

◀Abp1

◀Dynamin
Lysate control

E

Coprecipitation

| | Abp1 | | ABDs | | | Lysate |

| Time (min) | -xl | 2 | 30 | -xl | 2 | 30 | |

52 kDa ▶

◀myc-Abp1

◀myc-ABDs

anti-GST blot
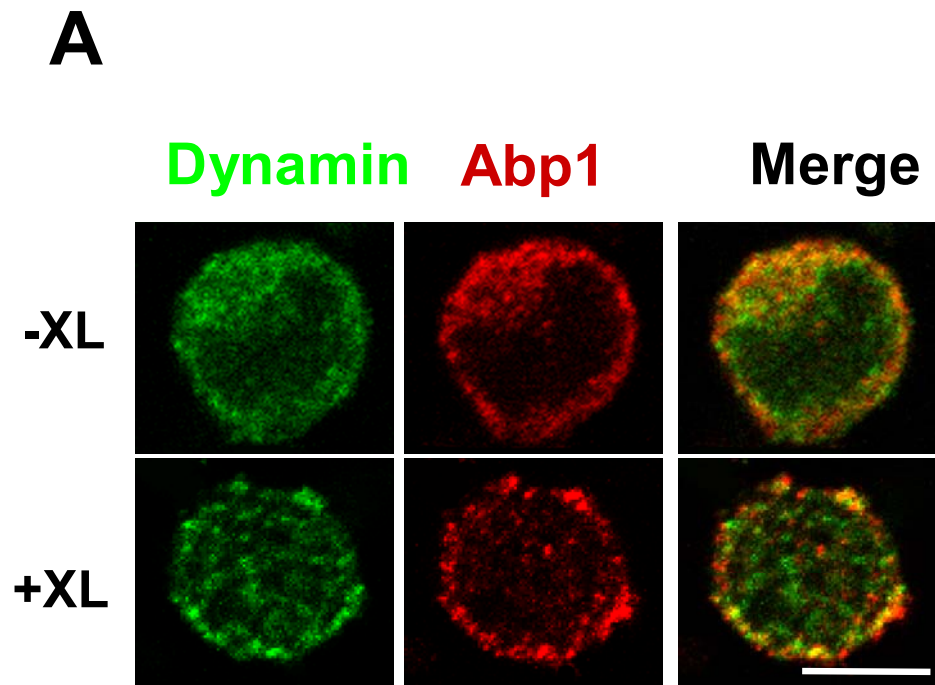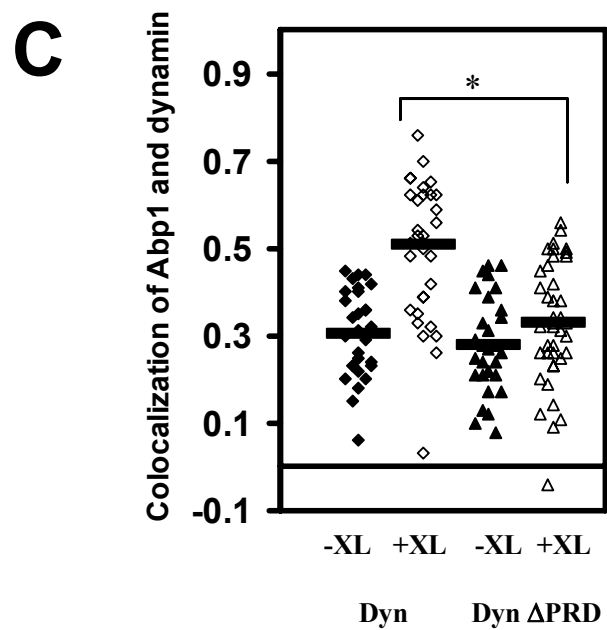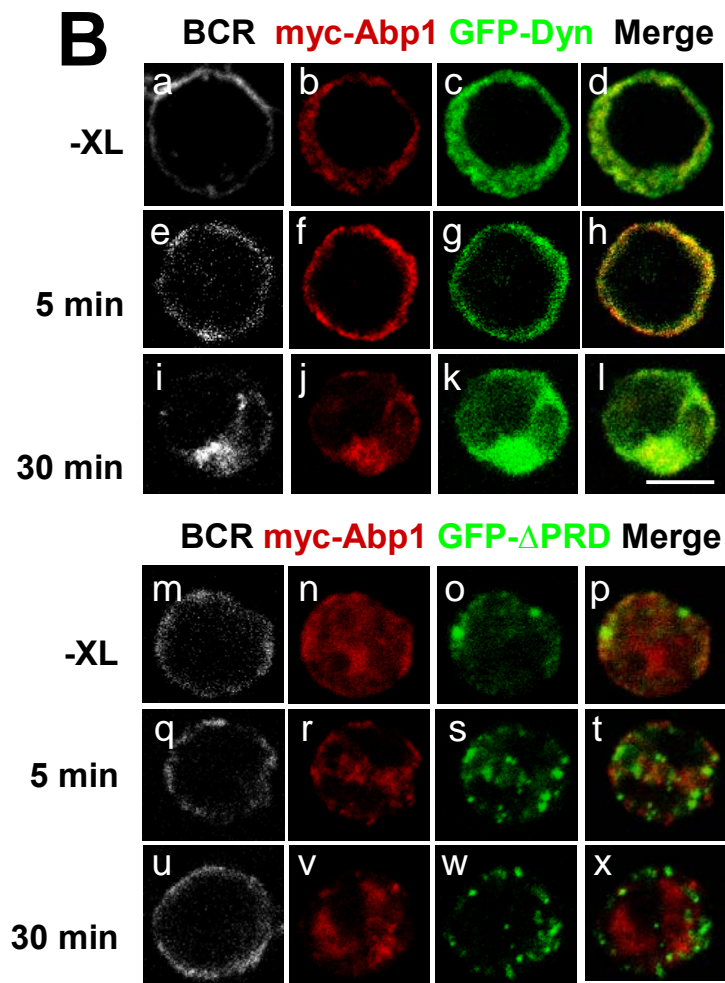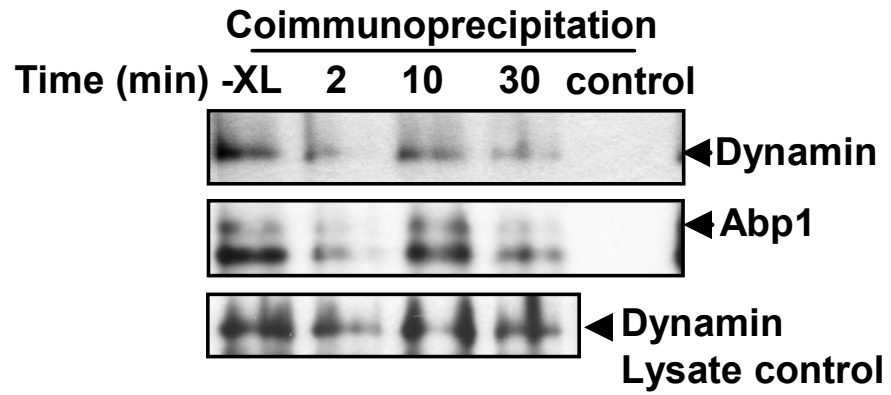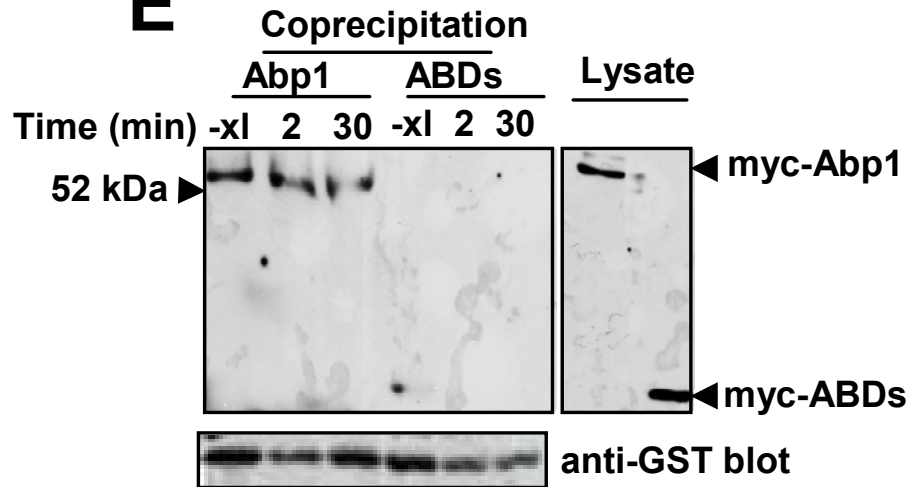
further confirm that Abp1 and dynamin 2 may constitutively interact with each other through their SH3 and PRDs.

2.5 Discussion

This study revealed a critical role for Abp1 in BCR-mediated antigen processing and presentation. Abp1 gene knockout and overexpression of the Abp1 SH3 domain reduced the rates of BCR-mediated antigen uptake, consequently reducing the rate of antigen transport to the antigen processing compartment and the efficiency of antigen processing and presentation. B cells process and present antigens to acquire T cell help, which is essential for the induction of isotype switching, somatic hypermutation, and affinity maturation in B cells. The BCR increases B cell antigen processing and presentation efficiency by binding to antigens with high specificity and affinity and by initiating rapid internalization and transport of antigens to antigen processing compartments. This allows B cells to present specific antigens even when antigen concentrations are extremely low. A previous study showed defective antibody responses to T cell-dependent antigens and reduced T cell receptor-mediated signaling and T cell activation in Abp1$^{-/-}$ mice (123). Here, we discovered defects in antigen processing and presentation of Abp1$^{-/-}$ B cells. Lowering the efficiency of B cells to process and present antigens to T cells would decrease the sensitivity of T cell-dependent B cell activation, contributing to defective T cell-dependent antibody responses in Abp1$^{-/-}$ mice. Abp1 contains two independent F actin-binding domains in the N-terminus, and a SH3 domain and a PRD or flexible domain in its C-terminus (114, 115, 118). Its multiple protein-protein interacting domains enable Abp1 to interconnect different cellular apparatuses. Previous studies

implicate Abp1 as an actin adaptor protein connecting the actin cytoskeleton to endocytosis machinery. The null mutation of the yeast homologue of Abp1, Abp1p, resulted in defects similar to those seen in Rvs167/amphiphysin mutation, including sporulation and reduced viability under certain suboptimal growth conditions. Double mutations in ABP1 and RVS167/amphiphysin genes or one of the genes encoding other cytoskeletal components were genetic lethal (152). In mammalian cells, Abp1 has been shown to be essential for Tf receptor internalization (70, 148) and synaptic vesicle recycling (147). Here, we showed that Abp1 was required for efficient BCR-mediated antigen internalization, further demonstrating an essential role for Abp1 in endocytosis. Previous studies have shown that Abp1 interacts directly with proteins of the endocytic machinery, including rvs167/amphiphysin in yeast (152) and dynamin in mammalian cells (70). Both amphiphysin and dynamin are important for the membrane fission step of endocytosis (91, 153). Furthermore, Abp1p was recruited to the endocytosis sites along with Arp2/3 and actin in yeast (146), suggesting a role for Abp1 in endocytic vesicle formation from the plasma membrane. However, a recent study using Abp1$^{-/-}$ mice placed Abp1 function downstream of vesicle fission in synaptic vesicle recycling in hippocampal neurons (147). Our previous studies showed that the dynamic properties of the actin cytoskeleton were required for BCR internalization at the fission step of clathrin-coated vesicle formation (35). Here we found that Abp1-deficiency and actin depolymerization inhibited BCR internalization to a similar extent. Furthermore, Abp1, F-actin, and dynamin 2 were recruited to the BCR at the plasma membrane at the same time and appear to interact with each other. These findings further support a role for Abp1 in clathrin-coated vesicle fission.

Unlike the constitutive internalization of Tf receptor, BCR internalization is triggered by antigen binding and is dependent on BCR-mediated signaling (40, 127-129). Previously, our lab showed that BCR activation induced the recruitment of clathrin to the BCR and phosphorylation of clathrin within lipid rafts, both of which were required for BCR internalization (140). In this study, we found that BCR activation induced the recruitment of Abp1 to the plasma membrane and to the internalizing BCR, suggesting that BCR signaling regulates the function of Abp1. Indeed, our data revealed that BCR activation induced tyrosine phosphorylation of Abp1 and that this phosphorylation was required for the recruitment of Abp1 to the cell surface and BCR. Abp1 colocalized with cortical F-actin, and BCR-induced recruitment of Abp1 to the cell surface depended on the actin cytoskeleton. These data indicate that BCR signaling can regulate the subcellular location of Abp1 through the tyrosine phosphorylation of Abp1 and the reorganization of the actin cytoskeleton. This is in line with a previous study showing the translocation of Abp1p from the perinuclear region to the leading edge of cells in a pattern that overlaps with Arp2/3 complex localization in response to activation of the GTPase Rac (115). The finding of a synchronized cellular reorganization of the actin cytoskeleton, Abp1 and dynamin 2 in response to BCR stimulation suggests that BCR signaling regulates the interaction of Abp1 with the actin cytoskeleton and dynamin 2. The interaction of Abp1 with dynamin during clathrin-mediated endocytosis has been previously reported (70). Here, we demonstrate this interaction in B cells by co-immunoprecipitation and GST fusion protein co-precipitation. While the interaction between Abp1 and dynamin 2 appeared to be constitutive, our observation of co-

recruitment of Abp1 and dynamin 2 to the plasma membrane following BCR activation indicates that BCR signaling regulates the subcellular location where Abp1 and dynamin 2 interact.

Another possible mechanism through which Abp1 functions in endocytosis is by regulating the dynamics of the actin cytoskeleton. In yeast, Abp1p recruits Arp2/3 complexes to the sites of actin filaments and is required for Arp2/3 complex activation *in vitro* (116). Over expression of Abp1p in yeast causes severe defects in cellular actin organization (144). Pinyol *et al*. (145) recently showed that Abp1 directly interacted with N-WASP, an activator of Arp 2/3 complex, and activated N-WASP in cooperation with Cdc42, suggesting that Abp1 may regulate the actin cytoskeleton indirectly through N-WASP. However, obvious defects in the actin cytoskeleton have not been observed in Abp1$^{-/-}$ B cells and B cells over expressing Abp1 and its dominant negative mutants (data not shown).

Abp1 has been shown to serve as a signaling regulator in T cells. Abp1 is recruited to the immunological synapse formed between T cells and antigen-presenting cells (122) and regulates the distal signaling of the TCR, including the activation of HPK1, the MAP kinase JNK, and the transcription factor NFAT (118, 119, 123). It is possible that Abp1 plays a similar role in regulation of BCR signaling events, which may provide feedback signals from the actin cytoskeleton and endocytic machinery to the BCR-mediated signaling pathway.

This study demonstrates the role of Abp1 in coupling BCR signaling and antigen processing/presentation functions by interacting with BCR signaling, endocytic, and actin cytoskeletal apparatuses. BCR activation induced Abp1 tyrosine phosphorylation and actin cytoskeleton reorganization, both of which are required for the recruitment of Abp1 to BCR internalization sites. Upon being recruited to the plasma membrane, the interaction of Abp1 with the actin cytoskeleton and endocytic proteins, such as dynamin 2, drives BCR internalization. Future studies will further examine the molecular mechanisms for interactions of Abp1 with BCR signaling, endocytic, and actin cytoskeletal apparatuses and regulatory mechanisms for these interactions.

Chapter 3: Dynamin is regulated by BCR signaling and is required for BCR internalization

3.1 Abstract

The binding of multi-valent antigens to the B cell antigen receptor (BCR) initiates signaling cascades, actin cytoskeleton reorganization and rapid internalization of the BCR-antigen complex for antigen processing. BCR internalization has been shown to primarily take place via clathrin-mediated endocytosis. Previous studies have demonstrated links among the actin cytoskeleton, BCR-mediated signaling and antigen uptake, however the molecular nature of such links has not been fully elucidated. Here, I studied the role of dynamin 2, a GTPase required for the fission of clathrin-coated vesicles from the plasma membrane, in coupling BCR signaling, actin cytoskeleton, and BCR internalization. It was found that BCR signaling induced the recruitment of dynamin 2 to the plasma membrane where it colocalized with the BCR and the actin cytoskeleton during BCR internalization. A PRD deletion mutation abrogated this recruitment. The recruitment of dynamin 2 to the plasma membrane was sensitive to PP2, a Src kinase inhibitor, but not latrunculin B which depolymerizes F-actin. While overexpression of dynamin 2 with a deletion of PRD did not significantly alter the organization of the actin cytoskeleton, it blocked BCR internalization and reduced the colocalization of the actin cytoskeleton with the BCR and dynamin 2. These results indicate that BCR-triggered signaling regulates the subcellular location of dynamin 2, and dynamin 2 is essential for BCR-mediated antigen uptake.

3.2 Introduction.

In naïve B cells, prior to crosslinking with antigen, the BCR is constitutively internalized via clathrin mediated endocytosis (CME) and recycles through the early endosome back to the cell surface (130). When the BCR encounters an antigen, oligomerization of the BCR by antigens leads to tyrosine phosphorylation of the Ig α/Ig β heterodimer, the signaling component of the BCR complex that is non-covalently bound to membrane immunoglobulin (6). The BCR then initiates signaling cascades that lead to the transcription and translation of genes required for B cell activation. In addition, BCR-triggered signaling facilitates the internalization of the BCR-antigen complex and the targeting of the complex to the antigen-processing compartment also called the MIIC compartment (154). In the MIIC compartment, antigens are fragmented and loaded unto the MHC class II complex and eventually presented on the surface of B cells to engage cognate T cells (14).

Previous studies from our lab and others have demonstrated that crosslinking of the BCR increases the antigen processing and presentation efficiency of B cells by increasing the kinetics and specificity of antigen uptake and transport to the MIIC. This allows B cells to effectively present an antigen even at very low concentrations of antigen and during a very short antigen exposure (127, 154). BCR signaling and antigen processing have been shown to be interrelated. Tyrosine kinase inhibitors that block BCR signaling were shown to inhibit accelerated antigen transport and also reduce the antigen-presenting efficiency of B cells (40, 129). We showed previously that crosslinking the BCR induced

the recruitment of clathrin to the cell surface, where it colocalized with BCR-containing vesicles, and the tyrosine phosphorylation of clathrin in lipid rafts (33).

The actin cytoskeleton has long been suspected of having a role in the generation of CME. It has been observed that F-actin was actively recruited to newly formed clathrin-coated vesicles just at the point of detachment from the plasma membrane, which raises the possibility that the actin cytoskeleton may have a role in the membrane fission step of CME (55, 155, 156). However, the use of various actin-disrupting agents has given conflicting results. In some systems, disrupting the actin cytoskeleton actually enhanced endocytosis (157). This is probably due to a barrier activity of cortical actin at the plasma membrane which inhibits the inward movement of clathrin-coated vesicles. Research by our lab and others is beginning to reveal a role for the actin cytoskeleton in BCR-mediated activation of B cells. Previous studies showed that antigen binding induced the translocation of the BCR and tyrosine kinases Lyn and Syk to the detergent-insoluble cytoskeletal fractions (133-135), reorganization of the actin cytoskeleton (90, 136-139), and transient increases in F-actin levels in B cells (35, 87). We have previously shown that the dynamic property of the actin cytoskeleton was required for signal-stimulated BCR internalization. BCR endocytosis is blocked at the pinching-off step during clathrin-coated vesicle formation in the absence of a functional actin cytoskeleton. Allam *et al*. (35) has revealed a role for the B-lymphocyte associated adaptor protein (Bam 32) in BCR-meditated actin reorganization. Bam32 was recruited to the BCR and F-actin rich areas of cells in response to BCR activation and regulated BCR-triggered actin polymerization through interactions with the Rho-family GTPase

Rac1 (85, 86, 88). Although these recent studies have provided evidence for the involvement of the actin cytoskeleton in BCR endocytosis, the molecular link between the actin cytoskeleton and BCR internalization pathway remains to be defined. Previous studies have revealed a number of proteins that might play a role in connecting the actin cytoskeleton to CME. Some of the proteins that are capable of binding both F-actin and proteins involved in CME include Huntington-interacting protein-1 (71), cortactin, (69, 108, 156), actin binding protein 1 (Abp1) (70, 158), and Myosin VI (159).

Dynamin is a GTPase that is involved in pinching off vesicles in a number of membrane processes (91). Dynamin is composed of multiple domains, including a PH domain that binds to PI(4,5)P on the plasma membrane, a GTPase domain that provides a signal for membrane fission, and a PRD that binds to an ever expanding list of SH3 domain-containing proteins (63). As a GTPase, dynamin provides mechanical force that constricts the neck of nascent endocytic vesicles (160). Recently dynamin has also been shown to be involved in connecting the actin cytoskeleton to the clathrin-mediated endocytosis pathway (63, 161). Although there is no evidence for a direct interaction between dynamin and F-actin, dynamin has been shown to interact with a number of proteins that interact with the actin cytoskeleton (63). In T cells, dynamin has been shown to be important for actin reorganization at the immunological synapse that is formed between a T cell and an antigen-presenting cell. Dynamin knockdown inhibited T cell antigen receptor-triggered signal transduction, probably due to the failure of formation of the immunological synapse (100). Unsworth *et al.* showed that dynamin is required for the formation of actin-based pedestals in enteropathogenic *E.coli*-infected epithelial cells

(112), implying a role in regulating the actin cytoskeleton, in addition to linking the actin cytoskeleton to the endocytic machinery.

Here I studied the role of dynamin in BCR-mediated antigen uptake and showed that BCR internalization requires dynamin. In response to BCR activation, dynamin 2 was recruited to the plasma membrane, the internalizing BCR, and the actin cytoskeleton, and this recruitment was dependent on the PRD of dynamin and BCR-triggered signaling, but not the actin cytoskeleton.

3.3 Materials and Methods

3.3.1 DNA constructs and transfection.

B cell lymphoma A20 IIA1.6 cells (H-2^d, IgG$_{2a}^+$, FcγRIIB$^-$) were cultured at 37^oC in DMEM supplemented with 10% FBS. The DNA constructs for GFP fusion protein of dynamin 2aa (GFP-Dyn) and dynamin 2aa mutant with its PRD deleted (GFP-Dyn ΔPRD) were kindly provided by Dr. Mark McNiven, (Mayo Clinic, Rochester), and the DNA construct of GFP fusion protein of actin (GFP-actin) was obtained from Clonetech. DNA constructs were introduced into A20 B cells by electroporation using Nucleofection kit (Amaxa, Gaithersburg, MD).

3.3.2 Analysis of the movement of the BCR from the cell surface to late endosomes.

A20 B cells, untransfected or transfected with either GFP-Dyn or GFP-Dyn ΔPRD, were incubated with Alexa Fluor (AF) 546-conjugated F(ab')$_2$-goat anti-mouse IgG (Invitrogen, Carlsbad, CA) for 20 min at 4°C to label the surface BCR. Cells were washed and then adhered to poly-lysine-coated slides (Sigma-Aldrich) for 20 min at 4°C and then chased at 37°C for varying lengths of time to allow for the internalization of the BCR. At each time point, cells were fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin in DMEM, 10%FBS, 10mM Hepes and 10mM glycine, and incubated with a mAb specific for lysosome-associated membrane protein 1 (LAMP-1) (ID4B, ATCC, Manassas, VA) and an AF633-conjugated secondary antibody. Cells were mounted with gel mount (Biomeda, Foster City, CA) and analyzed using a laser-scanning confocal microscope (LSM 510; Zeiss, Oberkochen, Germany). For quantitative analysis of images, the cellular distribution of the BCR was divided into two different categories: the BCR mainly distributed on the cell surface without colocalization with LAMP-1 or extensively colocalized with LAMP-1 at the perinuclear region of cells. Cells were categorized by visual inspection to determine the localization pattern of the BCR. Over 100 cells from three independent experiments were analyzed for each time point.

3.3.3 Analysis of BCR internalization.

A20 B cells, untransfected or transfected with either GFP-Dyn or GFP-Dyn ΔPRD, were incubated with biotinylated goat anti-mouse IgG (20 µg/ml; Jackson ImmunoResearch) for 30 min at 4°C to label the surface BCR. After washing off unbound antibodies, cells were

chased at 37°C for varying lengths of time. The chase was terminated by adding ice-cold

DMEM containing 6 mg/ml BSA (DMEM-BSA). The biotinylated antibodies remaining on

the cell surface after the chase were stained with PE-streptavidin (5 µg/ml; Qiagen, Valencia,

CA) at 4°C. The cells were then fixed with 2% paraformaldehyde and analyzed using a flow

cytometer (FACSCalibur, BD Bioscience, San Jose, CA). GFP-expressing cells were gated

for analysis. The data was plotted as a percentage of the mean fluorescence intensity of cell-

surface PE-streptavidin at time 0.

3.3.4 Analysis of cellular distributions of BCR, F-actin, and dynamin 2.

Untransfected A20 B cells or A20 cells transfected with either GFP-Dyn or GFP-Dyn ΔPRD

were incubated with Cy3 (Jackson ImmunoResearch) or AF 633 (Invitrogen)-conjugated Fab

fragment of goat anti-mouse IgG (5 µg/ml) for 10 min at 4°C to label the surface BCR,

followed by rabbit anti-mouse IgG (20 µg/ml, Jackson ImmunoResearch) for indicated times

at 37°C to crosslink the BCR. Cells were fixed with 4% paraformaldehyde, permeabilized

and stained with anti-dynamin antibody (BD biosciences) followed by AF488-anti-mouse

IgG$_1$ as a secondary antibody for endogenous dynamin 2. AF 555-phalloidin (Invitrogen) was

used to stain for F-actin in GFP-Dyn transfected cells. Cells were analyzed using a confocal

fluorescence microscope.

3.3.5 Live cell imaging.

GFP-actin, GFP-Dyn, or GFP-Dyn ΔPRD were introduced into A20 B cells by

electroporation using Nucleofection kit (Amaxa). 24 hours after transfection, cells were

placed into chambered polylysine (10 µM)-coated cover glasses (Nalge Nunc Int. Rochester, NY) and incubated at 37°C, 5% CO_2 for 30 min. Cells were then incubated with Cy3-Fab-goat anti mouse IgG (5 µg/ml; Jackson ImmunoResearch) at room temperature for 10 min, washed with 1%FBS in PBS. Cells were activated by crosslinking the BCR with rabbit-anti-mouse IgG (20 µg/ml; Jackson ImmunoResearch) and images were acquired every 3 seconds. Some of the images were acquired using the Zeiss LiveDUO confocal microscope while other images were obtained using the Zeiss LSM510.

3.4 Results

3.4.1 BCR crosslinking induces the recruitment of Dynamin 2 to the plasma membrane and to the BCR.

BCR crosslinking has been shown to increase the phosphorylation of clathrin and induce its recruitment to the cell surface where it colocalizes with the BCR (33). Dynamin 2 has also been shown to recruit to nascent clathrin-coated vesicles prior to the scission event during CME (55). To test whether dynamin 2 is regulated by BCR activation, we monitored the cellular distribution of dynamin 2 after BCR crosslinking using immunofluorescence microscopy. The surface BCR of A20 B cells were labeled with Cy3-conjugated Fab fragment of goat anti-mouse IgG and cross-linked by rabbit-anti-mouse IgG for varying lengths of time. After fixation and permeabilization, cells were stained for dynamin 2. Prior to BCR crosslinking, dynamin 2 was distributed mainly in the cytoplasm (Fig. 3.1Aa-c). Ten minutes after BCR crosslinking, dynamin 2 had redistributed from the cytoplasm to the cell periphery where it colocalized with the BCR

(Fig. 3.1Ad-f). 30 min after BCR crosslinking we observed that dynamin 2 still remained colocalized with the clustered BCR in the perinuclear region of the cell (Fig. 3.1Ag-i). Using A20 cells expressing GFP-Dyn or GFP-Dyn ΔPRD mutant, I examined BCR-induced dynamin 2 redistribution in live cells. The BCR on transfected cells was labeled with Cy3-Fab-anti-mouse IgG and cross-linked with anti-mouse IgG. Cells were imaged before and after BCR crosslinking. Similar to what was seen with the endogenous dynamin in fixed cells, prior to BCR crosslinking, GFP-Dyn 2 was evenly distributed in the cytoplasm (Fig. 3.1Ba), 2 min after BCR crosslinking GFP-Dyn was colocalized with the BCR in discrete spots near the plasma membrane (Fig. 3.1Bb). Surprisingly, while the surface labeled BCR underwent characteristic clustering during its internalization, dynamin co-clustered with the BCR (Fig. 3.1Bc) and remained with the BCR while it moved inwards. This suggests that dynamin 2 remains with nascent endocytic vesicles following the internalization of the cargo, which has not been reported before.   By 15 min GFP-Dyn still remained clustered with the BCR and seemed to accumulate with the BCR in the perinuclear region of the cell (Fig.3.1Bd-Be). In contrast, GFP-Dyn ΔPRD mutant failed to redistribute to the cell surface in response to BCR crosslinking (Fig. 3.1Bf – BJ). These results show that dynamin 2 is recruited to the cell surface following BCR activation where it colocalizes with the BCR during BCR internalization and that the PRD of dynamin is required for its recruitment to the cell surface.

FIGURE 3.1. Dynamin colocalizes with the BCR following BCR crosslinking.

(A) A20 B cells were incubated with Cy3-conjugated Fab-goat anti mouse IgG to label the BCR and rabbit-anti-mouse IgG to cross-link the BCR for indicated time points. Cells were fixed, permeabilized, and labeled with anti-dynamin mAb and a fluorochrome-conjugated secondary antibody. Cells were analyzed using a confocal fluorescence microscope. Shown are representative images of three independent experiments. Bar, 10 μm. (B) A20 B cells were transiently transfected with wt GFP-dynamin 2aa (GFP-Dyn) or GFP-dynamin 2aa ΔPRD mutant (GFP-Dyn-ΔPRD). Twenty-four hours after transfection, the surface BCR was labeled with Cy3 Fab-goat anti-mouse IgG on chambered cover glasses and activated by crosslinking the BCR with rabbit anti-mouse IgG. Images were acquired every 3 sec with a confocal fluorescence microscope (Bar, 10 μm). Shown are representative images of three independent experiments.

A

	Dynamin	BCR	Merge
Unactivated	a	b	c
10 min	d	e	f
30 min	g	h	i

B

	0 min	2 min	5 min	15 min	30 min
wt Dyn	a	b	c	d	e
Dyn-ΔPRD	f	g	h	i	j

BCR/GFP-Dyn
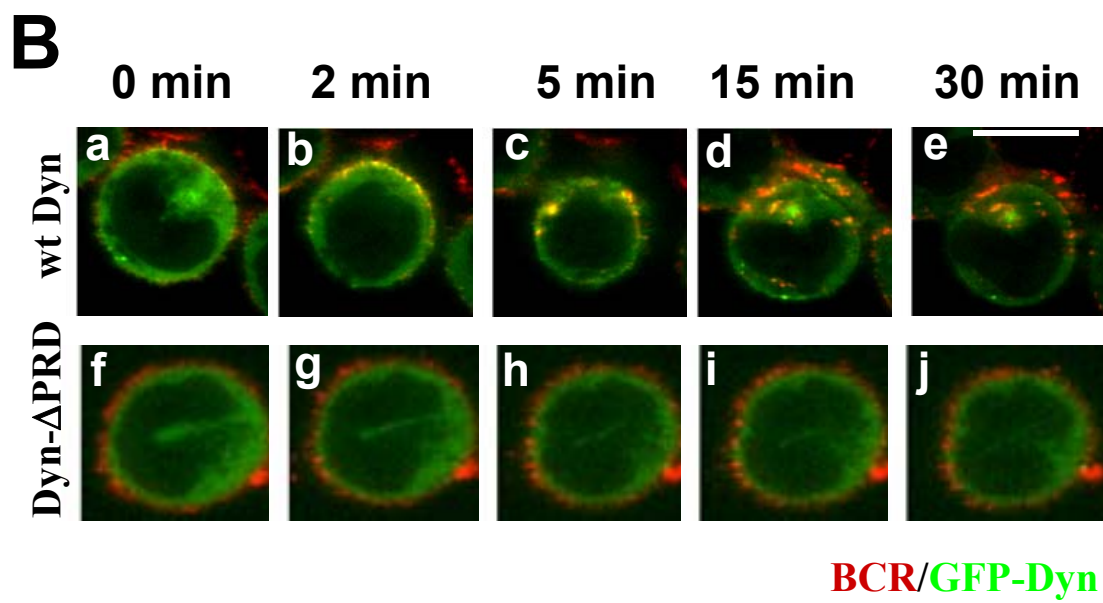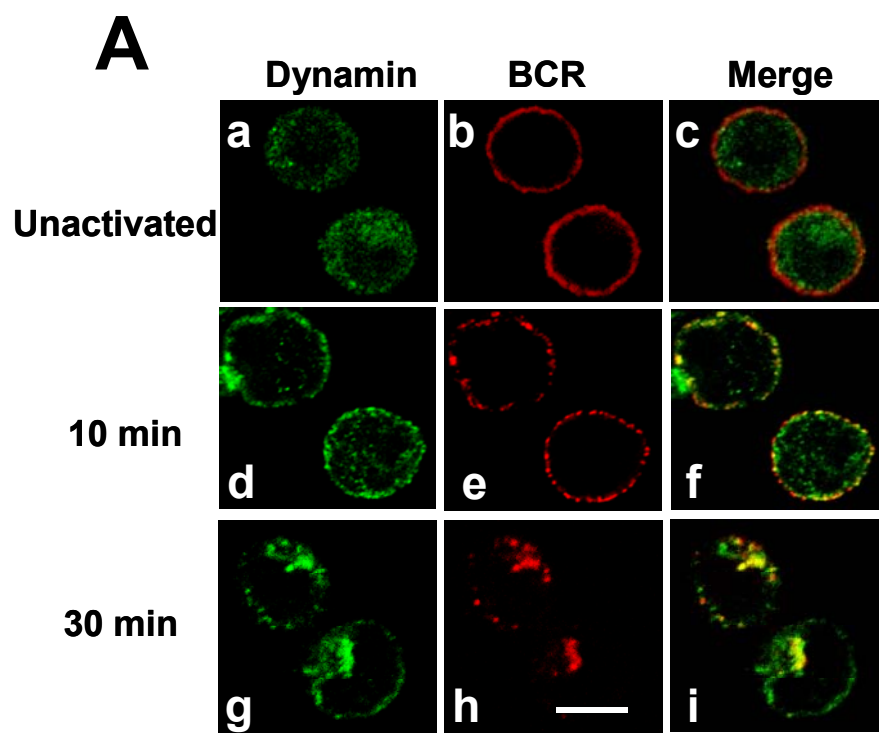
3.4.2 Dynamin 2 is important for BCR internalization.

The role of dynamin in membrane fission events has been well established. The GTPase mutation (K44A) and the PH deletion mutation of dynamin 2 have been shown to disrupt CME of transferrin (162), EGFR (163) and the AT1 angiotensin receptor (164, 165). To investigate the role of dynamin in BCR internalization, we transiently transfected GFP tagged wt and the PRD-deleted mutant of Dynamin 2 into A20 B cells. The BCR of transfected B cells were labeled with Cy3-Fab-goat-anti-mouse IgG and crosslinked with rabbit-anti-mouse IgG. The internalization of the BCR was monitored using live cell imaging. Similar to untransfected cells, in cells that were transiently transfected with wt GFP-Dyn, the BCR clearly moved into the cell right after the recruitment of dynamin 2 to the cell surface (Fig. 3.1Ba-e). However, the BCR failed to internalize in cells transfected with dynamin ΔPRD mutant (Fig.3.2Aa-d). Next, we further quantified BCR internalization in A20 cells transfected with wt GFP-dynamin or its ΔPRD mutant using flow cytometry. The surface BCR of A20 B cells were labeled with biotin-conjugated anti-mouse IgG at 4°C and chased for 0, 5 and 20 min at 37°C. Biotin-anti-mouse IgG remaining at the cell surface after the chase was detected with PE-streptavidin and quantified using flow cytometry. As shown in Fig. 3.2B, overexpression of GFP-Dyn ΔPRD significantly decreased the kinetics of BCR internalization while overexpression of wt GFP-Dynamin 2 had no significant effect on BCR internalization. This data demonstrates that dynamin 2 is required for BCR internalization, and the PRD of dynamin 2 likely plays an important role in BCR internalization although the non specific effect of overexpressing a mutant protein cannot be ruled out.

The movement of the BCR from the cell surface to the antigen-processing compartment was followed by immunofluorescence microscopy. The surface BCR was labeled with Cy3-Fab-anti-mouse IgG at 4°C and activated with anti-mouse IgG at 37°C for varying lengths of time. After fixation and permeabilization, the antigen-processing compartment was marked with anti-LAMP1 mAb. Cells were analyzed by a confocal fluorescence microscope. Based on the cellular distribution pattern of the BCR and LAMP-1, cells were categorized into two groups, BCR colocalizing with LAMP-1 extensively in the perinuclear region and BCR remaining on the cell surface and periphery with no significant colocalization with LAMP-1. The numbers of cells in each category were plotted as percentages of the total number of cells in the field (Fig. 3.2D). After a 30 min chase at 37°C, the surface labeled BCR was extensively colocalized with the LAMP-1 in over 60% of cells expressing wt GFP-Dyn, compared to just 40% of cells expressing GFP-Dyn ΔPRD (Fig. 3.2C-D). These results indicate that dynamin 2 is required for the movement of the BCR from the cell surface to the antigen processing compartments and that the PRD of dynamin 2 may be important for this function.

3.4.3 Dynamin regulates F-actin and BCR interaction following BCR crosslinking.

To examine the relationship between the actin cytoskeleton and dynamin during BCR internalization, we monitored actin dynamics in live cells. GFP-actin was transiently transfected into A20 B cells. GFP-actin-expressing cells were stained with Cy3-Fab-anti-

FIGURE 3.2. The PRD of Dynamin is important for BCR internalization.

 (A) The experiments are presented as freeze frames of selected time points from movies taken of B cell lymphoma A20 cells transiently transfected with Dynamin 2aa Δ PRD GFP. Twenty-four hours after transfection, surface BCR was labeled with Cy3-conjugated goat anti-mouse IgG on 4-well chambered cover glasses and activated by crosslinking the BCR with rabbit anti-mouse IgG. Images were obtained every 3 seconds with a Zeiss confocal microscope. (B) A20 cells were transiently transfected with GFP-Dyn or GFP-Dyn ΔPRD. Twenty-four hours later the surface BCR was labeled with biotin-F(ab')$_2$-goat anti-mouse IgG at 4°C and chased at 37°C for indicated times. Biotin-anti-mouse IgG left on the surface was detected by PE-streptavidin and quantified using a FACS Calibur. The data was plotted as the percentage of the labeled BCR remaining at the cell surface. Shown are the averages ($\pm$S.D.) of three independent experiments. ** $p<$ 0.01. (C) A20 cells were transiently transfected with GFP-Dyn or GFP-Dyn ΔPRD. Twenty-four hours after transfection, cells were labeled with AF546-conjugated goat anti-mouse IgG for 20 min at 4°C and chased for 30 min at 37°C. Immediately following the chase, cells were fixed, permeabilized, and labeled with anti-LAMP-1 mAb for late endosomes. Arrows indicate cells expressing transfected proteins (Bar, 10 μm). (D) Quantification of the effect of over expression of GFP-Dyn or GFP-Dyn ΔPRD on the cellular distribution of the BCR. Cells were divided into two different categories: cells showing extensive colocalization between the BCR and LAMP-1 and those showing no colocalization. Cells were categorized by visual inspection. Shown are the averages ($\pm$S.D.) of over 100 cells randomly selected from three independent experiments and plotted as percentages of the total number of counted cells. $p<0.01$.

A

Time (min)

0

5

10

30

BCR/**GFP-Dyn ΔPRD**

B

C

	GFP	BCR	LAMP-1	Merge
Dynamin	a	b	c	d
ΔPRD-dynamin	e	f	g	h
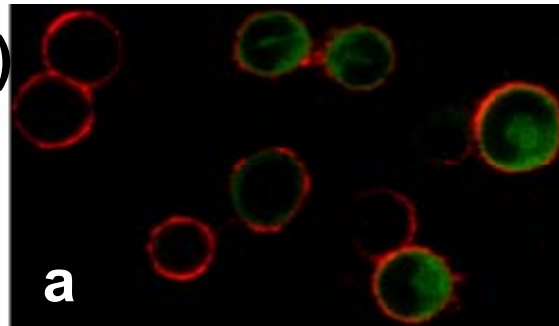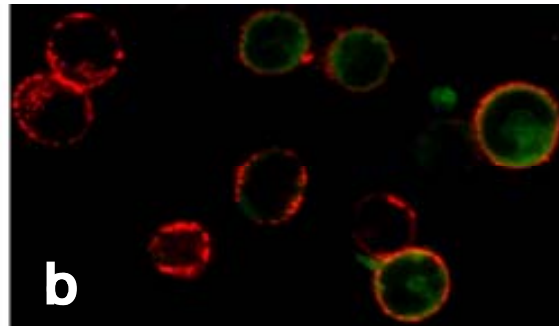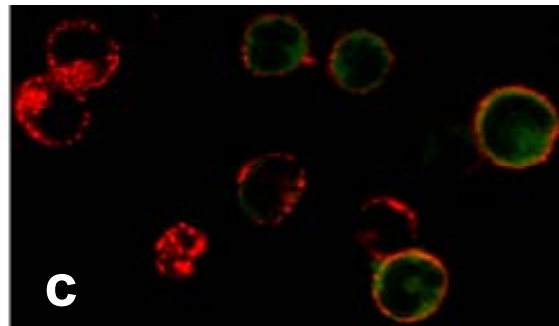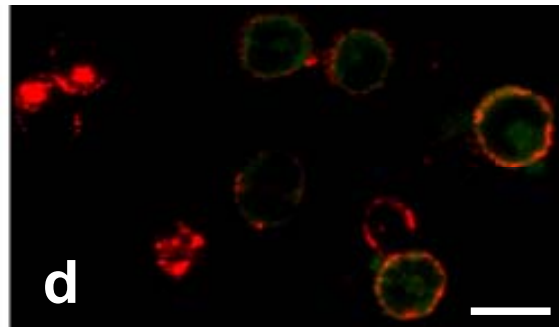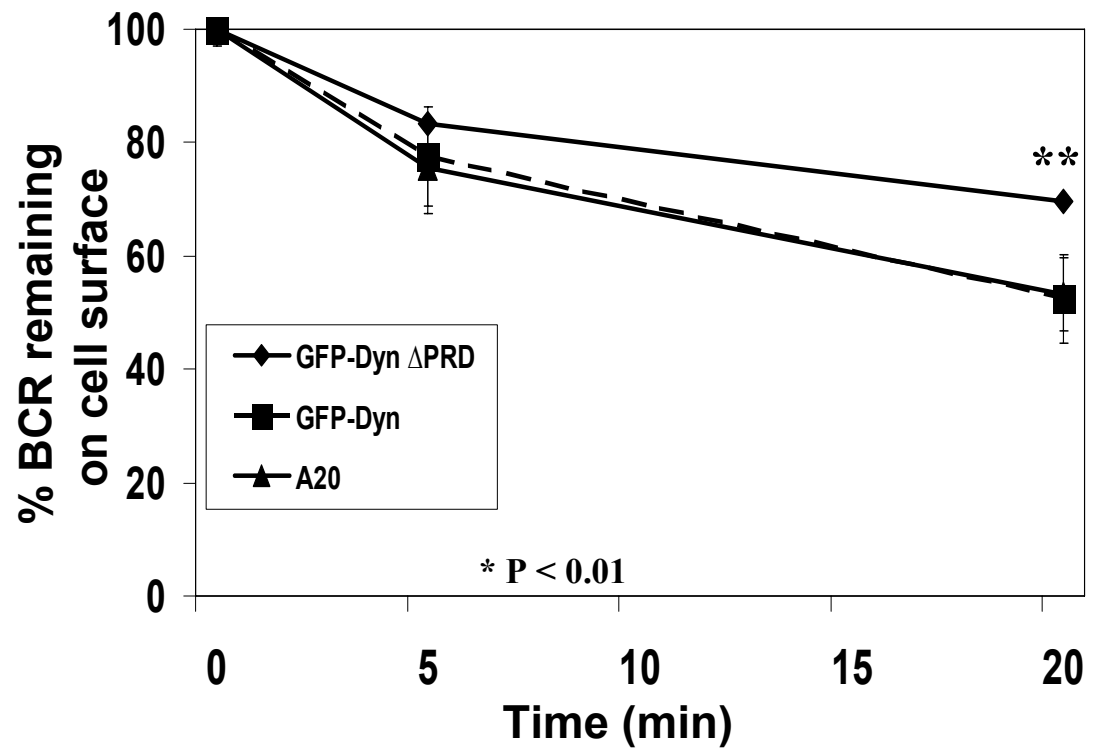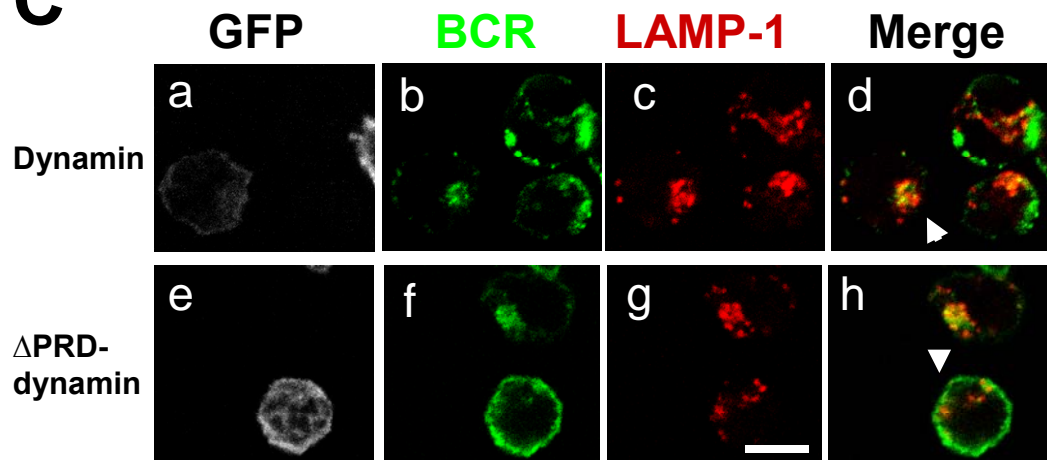
D
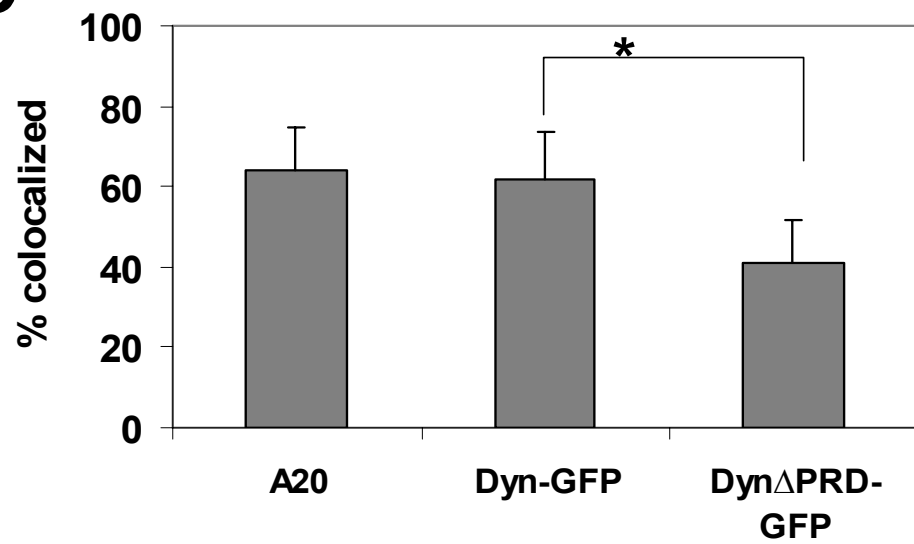
mouse IgG to label the BCR and treated with anti-mouse-IgG whole antibody to crosslink

the BCR. The cellular distribution of GFP-actin and BCR was monitored in real time

using a confocal fluorescence microscope (Fig. 3.3A-H). Prior to BCR crosslinking,

GFP-actin was evenly distributed around the cytoplasm and showed some colocalization

with the BCR (Fig. 3.3Aa). Upon BCR crosslinking, GFP-actin co-clustered with the

BCR at a particular pole of the B cell (Fig. 3.3Ab). The GFP-actin and BCR co-cluster

increased in size over time and was sustained for about 20 min. This was followed by the

BCR moving into the cell (Fig. 3.3Ac-h). Furthermore, GFP-actin remained colocalized

with the BCR or located in the vicinity of the BCR when the BCR moved to the

perinuclear location (Fig. 3.3Af-h). This revealed that BCR crosslinking mobilizes the

actin cytoskeleton from the cytoplasm to the surface BCR, and the reorganized actin

accompanies the BCR during its internalization and movement to the antigen processing

compartments. We next examined the cellular distribution of dynamin 2 relative to GFP-

actin and the BCR. A20 B cells expressing either wt GFP-Dyn or its ΔPRD mutant were

incubated with AF633-anti-mouse IgG for 10 min to label and cross-link the BCR. After

fixation and permeabilization, cells were stained with AF555-phalloidin for F-actin. It

was observed that the both F-actin (red) and wt GFP-dynamin (blue) co-clustered with

BCR (green) at the cell membrane (Fig. 3.3Ba-d). However, the co-clustering of F-actin

and dynamin with the BCR was reduced in cells expressing GFP-Dyn ΔPRD (Fig. 3.3Be-

h). This result suggests that dynamin has the potential to play a role in directing the

reorganization of the actin cytoskeleton to the BCR during BCR internalization. This

hypothesis, however, remains to be tested fully.

FIGURE 3.3. Dynamin regulates F-actin and BCR interaction following BCR crosslinking.

(A) B-cell lymphoma A20 cells were transiently transfected with GFP-Actin. Twenty-four hours after transfection, surface BCR was labeled with cy3 conjugated goat anti-mouse IgG on 4 well chambered cover glasses and activated by crosslinking the BCR with rabbit anti-mouse IgG. Images were obtained every 3 seconds with a Zeiss confocal microscope, (bar, 10μm). Image shows freeze frames of selected time points and is representative of three independent experiments.

(B). A20 cells transiently transfected with GFP-Dyn (a - d) or GFP-Dyn Δ PRD (e - h) were activated with goat anti-mouse IgG for 10 min, and then fixed, permeabilized, and labeled with AF 555 Phalloidin to stain F-actin Cells were analyzed using confocal fluorescence microscopy. Shown are representative images of three independent experiments (Bar, 10 μm).

A

0 min   2 min   5 min   10 min

a   b   c   d

15 min   20 min   25 min   30 min

e   f   g   h

BCR/GFP-Actin

B

Dynamin   BCR   F-ACTIN   MERGE

a   b   c   d   WT

e   f   g   h   ΔPRD

10 min after BCR crosslinking

93
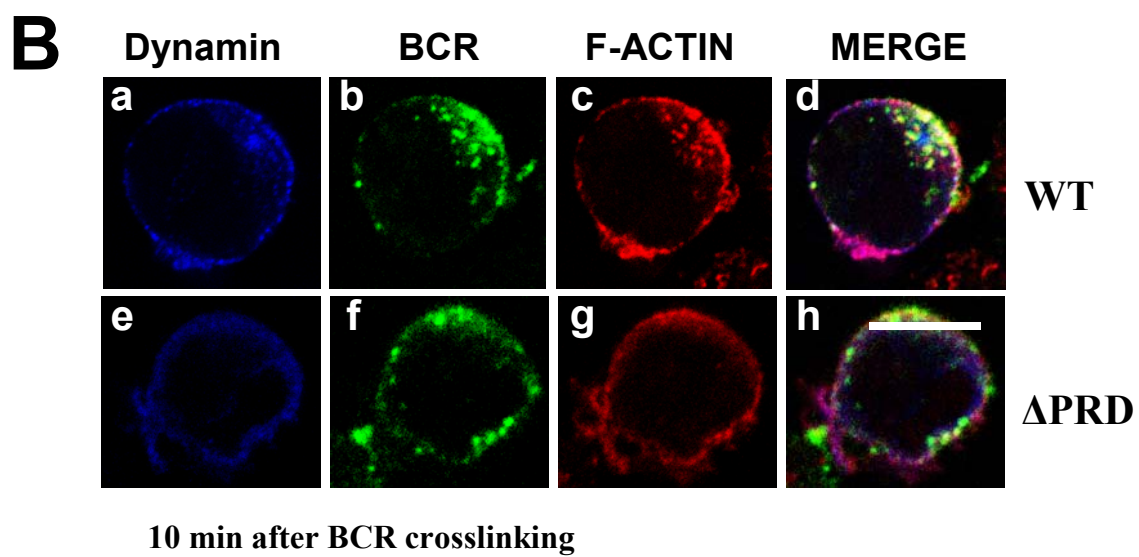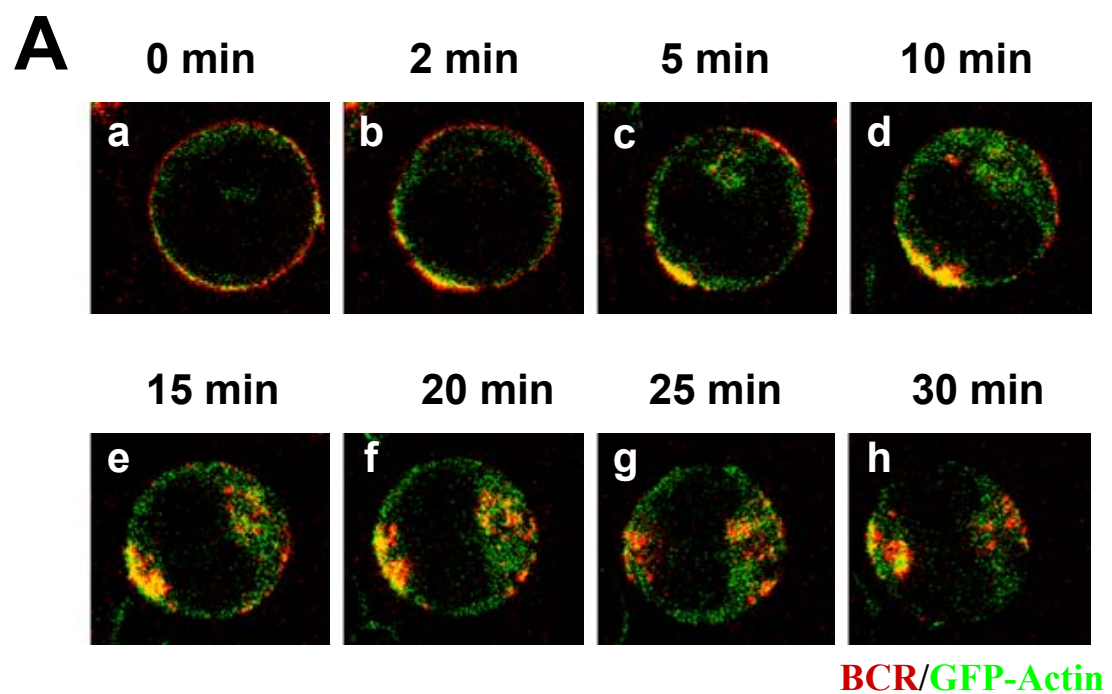
3.5 Discussion

Here I show that dynamin 2 is recruited to the cell surface in a BCR signaling-dependent manner and that this recruitment depends on the PRD of dynamin 2. I also show that deletion of the PRD of dynamin 2 has a dominant negative effect on the internalization of the BCR as well as disrupting the colocalization of F-actin with the BCR on the plasma membrane.

The role of dynamin in CME has been clearly elucidated (55, 91, 166). Dynamin acts as a GTPase that is recruited to the neck of nascent clathrin-coated vesicles and helps to generate the force that pinches of the clathrin-coated vesicles (91, 92). Studies have shown that mutations in the GTPase domain (167) as well as the PH domain (94) of dynamin can disrupt CME. The PRD of dynamin is required for the recruitment of dynamin to the cell surface by interacting with amphiphysin and mutations of this domain don't seem to have any effect on endocytosis (91, 94). On the contrary, mutations in the PRD of dynamin inhibited the dominant negative effect of a PH domain deletion dynamin mutant on endocytosis in Cos-7 cells (94). My data shows that in B cells, deletion of the PRD had a dominant negative effect on BCR internalization as well as trafficking to the late endosomes (Fig 3.2). This was relatively surprising considering previous data to the contrary in other systems like the findings from the previously stated Cos-7 cells. It is possible that overexpressing the ΔPRD mutant might disrupt BCR internalization by inhibiting the interaction between the actin cytoskeleton and the endocytic machinery of the B-cell. The PRD of dynamin is required for interacting with the actin cytoskeleton by binding a number of proteins that interact with the actin

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cytoskeleton (59, 63, 77). Overexpressing the SH3 domain of some of these proteins had a dominant negative effect on CME. These proteins include amphiphysin (53), syndapin (76), intersectin (96) and Abp1 (70). Work in our lab by Bruce Brown has also shown an involvement of the actin cytoskeleton in BCR trafficking (35). In the presence of cytochalasin D, an agent that disrupts the actin cytoskeleton, there was a preponderance of deeply invaginated long necked clathrin-coated pits reminiscent of the expression of dynamin mutants that block endocytosis (168). Interestingly Bruce showed that constitutive internalization of the BCR as well as the internalization of transferrin (both via CME) did not seem to be affected by the disruption of the actin cytoskeleton, implying that an intact actin cytoskeleton is not required for this form of CME. This implies that the actin cytoskeleton is required for driving the rapid internalization of the BCR once it is crosslinked but is not required for the constitutive internalization of the BCR. This might help explain the obvious discrepancy between our data and that of others, that showed a lack of involvement of the PRD of dynamin in other cell systems (91, 94). In addition, I observed that actin co-localized with the BCR and stayed with the BCR during BCR internalization (Fig 3.3a and b). I also noticed that F-actin, BCR and GFP-Dynamin 2 colocalized at the cell surface during BCR internalization. When I expressed GFP-Dyn ΔPRD there was a marked reduction in the extent of colocalization between these proteins (Fig 3.3b), implicating the PRD of dynamin in the regulation of the interaction between actin and the internalizing BCR. These observations provide further evidence that the observed defect in BCR internalization in the presence of the GFP-Dyn ΔPRD might be due to a disruption in the recruitment of the actin cytoskeleton to the nascent BCR containing vesicles.

Another possibility is that the ΔPRD mutant might have an effect on the GTPase function of dynamin in generating scission at the plasma membrane. This event is dependent on its ability to oligomerize at the plasma membrane during CME (91, 92). The oligomerization of dynamin has some dependency on its PRD (169), although this is a primary function of the GED. It is, therefore, possible that overexpression of a PRD deletion mutant may competitively inhibit dynamin from forming spirals at the sites of endocytosis and thus inhibit its GTPase activity. GFP-Dynamin 2 ΔPRD may also be interfering with the interaction of dynamin oligomers at nascent CCVs with other SH3 domain containing proteins that link dynamin to the endocytic machinery during BCR internalization (55, 166). Examples include amphiphysin(170) and endophilin (51, 171).

It is important to point out that, unlike GFP-Dyn 2, we did not observe the recruitment of GFP-Dyn ΔPRD to the cell surface (Fig 3.1) during the internalization of the BCR, which implies that the inhibition of dynamin 2 may be occurring at sites other than the plasma membrane. One example is the Golgi where interactions between dynamin 2 and cortactin link the actin cytoskeleton to Golgi transport and is required for the transport of newly formed proteins from the trans-Golgi network (172).

The recruitment of dynamin 2 to nascent CCVs has been shown to be dependent on the interaction of its PRD with the SH3 domain of amphiphysin (53) as well as the interaction of the PH domain with membrane phospholipids, notably PI(4,5)P (94, 95). Studies on sonicated plasma membranes of Cos-7 cells show that dynamin accumulates with CCPs, while a mutant with a disrupted PRD did not (101). The recruitment of

dynamin 2 in response to PDGF stimulation was shown by Mark McNiven to be

dependent on the PRD of dynamin 2 via an interaction with cortactin, an activator of the

actin cytoskeleton (173). Therefore the inhibition of dynamin recruitment in the absence

of the PRD domain could be as a result of its inability to interact with amphiphysin or

some other protein at the cell surface. This scenario is, however, yet to be tested and

should be investigated in the future. It will also be interesting to test what role

amphiphysin plays in BCR trafficking.

The recruitment of dynamin could also be dependent on BCR signaling. Work in our lab

in collaboration with the Brodsky lab had shown that clathrin is recruited to the cell

surface upon BCR crosslinking and that this recruitment is dependent on BCR signaling-

mediated phosphorylation of clathrin (33). In addition, previous work in our lab had

shown that the Src kinase inhibitor PP2 inhibited the BCR crosslinking mediated

recruitment of dynamin to the cell surface, implying that dynamin recruitment may also

be signaling-dependent (Brown, B.K. unpublished observations). Dynamin 2 is tyrosine

phosphorylated at Y231 and Y597 in rat endothelial cell lines in response to albumin

binding to its receptor gp60, where it colocalized with caveolin, and the phosphorylation

was Src kinase-mediated (174). The recruitment of dynamin 2 to the plasma membrane

has also been observed at the immunological synapse formed between a T cell and an

antigen-presenting cell (100). In this study, the researchers did not find any evidence of

dynamin phosphorylation before or after T cell stimulation and suggested that the

recruitment of dynamin 2 to the immunological synapse is not dependent on dynamin

phosphorylation(100). Whether dynamin 2 is phosphorylated in response to BCR

crosslinking and accounts for its recruitment to the plasma membrane is not known and would be a study to be considered in the future.

BCR signaling could also direct dynamin recruitment via activation of Btk. BCR crosslinking leads to the activation of Btk. Btk is involved in the synthesis of PIP2, the precursor of PIP3, by recruiting phosphatidylinositol-4-phosphate 5-kinase (PIP5K) which synthesizes PIP2 (16). The binding of the PH domain of dynamin to PIP2 is one of the mechanisms that recruits dynamin to the cell surface (94, 175). It will be interesting to test this hypothesis first by observing the role of the PH domain of dynamin during BCR crosslinking and also testing whether Btk plays a role in dynamin recruitment.

One intriguing observation we made was the colocalization of dynamin with the BCR for a sustained period of time during endocytosis. Dynamin 2 not only colocalized with the surface BCR, but also colocalized with BCR-positive vesicles moving inward (Fig.3.1). Recent reports by Merrifield *et al*. pointed to a transient involvement of dynamin in CME (55, 156). The significance of our observation is not yet known. One possibility is that dynamin remains associated with the newly formed vesicle in order to direct F-actin reorganization at or near the neck of the nascent CCV and thus position the vesicle for trafficking towards the cell interior as against a more random movement if actin was polymerizing all over the vesicle. A similar hypothesis for actin directing the inward movement of the CCV had been proposed by Drubin *et al.* (157).

The results presented here demonstrate that dynamin plays an essential role in the internalization of the BCR and may be regulated by BCR crosslinking-mediated signaling events. Future work will focus on the effect of other domains of dynamin on BCR signaling as well as deciphering exactly how BCR signaling regulates dynamin function.

Chapter 4: Abp1, an actin adapter protein, is important for optimal activation of JNK and ERK in response to BCR activation

4.1 Abstract

BCR crosslinking leads to the activation of signaling cascades that turn on transcription factors and the expression of genes required for B cell activation. Abp1 is an actin-binding adaptor protein that has been shown to be important in both clathrin-mediated endocytosis and signal transduction. In T cells, it regulates the activity of the MAP kinase, JNK, by interacting with HPK1, a MAP4K. Abp1 knockout mice have defective T-dependent antibody responses. In Chapter 2, we demonstrated a role for Abp1 in BCR-mediated antigen processing and presentation. Here, we have studied the role of Abp1 in BCR signaling using Abp1 knockout and knock down approaches. In response to BCR crosslinking by antigen, splenic B cells from Abp1 knock out mice and A20 B cells transfected with Abp1 shRNA had higher levels of protein tyrosine phosphorylation than the wild type splenic B cells and untransfected A20 B cells. While BCR-triggered ERK phosphorylation in Abp1-deficient B cells occurred sooner and for a much shorter duration than the wild type B cells, both Abp1 knockout and knockdown significantly reduced BCR-induced phosphorylation of JNK. These data demonstrate a role for Abp1 in BCR-induced activation of the MAP kinases, ERK and JNK.

4.2 Introduction

Crosslinking of the BCR by antigen leads to activation of signal transduction pathways that lead to the transcription and translation of proteins required for B cell proliferation (176). The initial event of activation is the phosphorylation of tyrosine residues in the ITAM of Igα and Igβ by Src-family kinases (6). The phosphorylated ITAMs recruit and activate tyrosine kinases, Syk, and adaptor proteins (14). The kinases and adaptor proteins activate downstream effectors that include phospholipase Cγ2, phosphatidylinositol-3-kinase, and Ras, which leads to calcium mobilization and activation of the MAP kinases, including ERK, JNK and p38. The MAP kinases activate a host of transcription factors (14).

Recent studies have provided evidence for links between the actin cytoskeleton and signal transduction pathway. In B cells, BCR-mediated signaling regulates the actin cytoskeleton. BCR crosslinking by antigen induces polymerization and subsequent depolymerization of F-actin (35). The binding of antigen to the BCR triggers the phosphorylation and activation of Vav, a guanidine nucleotide exchange factor for a Rho-family GTPase, Cdc42, which regulates the actin cytoskeleton (177). The actin cytoskeleton has been shown to influence the signal transduction events in B cells. Our lab previously showed that perturbing the actin cytoskeleton with cytochalasin D did not inhibit BCR-induced protein tyrosine phosphorylation, but extended the duration of the phosphorylation. Hao *et al.* recently showed that F-actin depolymerization by latrunculin alone led to ERK activation in CH27 B cell lines (87). This indicates a regulatory relationship between the actin cytoskeleton and signal transduction pathway and implies a possible role for actin adaptor proteins in linking the actin cytoskeleton to the BCR signal transduction pathway.

Abp1 is a 55 kDa adaptor protein composed of various protein-protein interaction domains including two N-terminal actin binding domains, a PRD or flexible domain, and a C-terminal SH3 domain (115). Its PRD contains two tyrosine phosphorylation sites, Y337 and Y347 (114). Mammalian Abp1 is a homologue of the Abp1 protein first found in yeast, and yeast Abp1 has an important role in yeast endocytosis (178). Similarly, Abp1 has been shown to be important for transferrin endocytosis in mammalian cells (70). Yeast Abp1 can directly regulate the actin cytoskeleton by interacting with N-WASP. However, a similar function for mammalian Abp1 has not been observed. In Chapter 2 of this thesis, we have shown a role for Abp1 in BCR-mediated antigen internalization and antigen presentation. Larbolette *et al* showed that Abp1 was phosphorylated at Y337 and Y347 by Src kinases Lyn and Blk and Syk in vitro (114). In Chapter 2, we showed that this phosphorylation was important for the recruitment of Abp1 to the surface of the B cells, suggesting that BCR signaling can regulate the cellular location of Abp1.

Groups led by Tse-Hua Tan and Marcel Deckert have shown a role for Abp1 in T cell receptor-mediated signal transduction (119, 122, 123). Abp1 binds to hematopoetic progenitor kinase 1 (HPK1), a serine/threonine protein kinase (118) that negatively regulates T-cell signaling (121). HPK1 is a MAP4K that activates the JNK pathway in T cells (120, 179, 180). Both HPK1 and JNK phosphorylation was attenuated in two T cell lines treated with Abp1 siRNA (119) and primary T cells from Abp1 knockout mice (123). Using RNA interference, Le Bras *et al*. found Abp1 to be a negative regulator of NFAT in Jurkat T cells (122), and Han *et al*. revealed defects in TCR- induced proliferation of T cells, IL-2 production, and reduced levels of T-cell activation markers, notably CD69, in Abp1^{-/-}. In

response to TCR signaling, these defects in Abp1$^{-/-}$ were upstream of protein kinase C and calcium influx, because the response of the T cells to phorbol 12-myristate 13 acetate and ionomycin (which bypasses TCR signaling-induced activation of protein kinase C) was similar to that of wildtype cells (123). Furthermore, Abp1$^{-/-}$ had reduced IgG1 production during both the primary and secondary immune response in Abp1$^{-/-}$ mice when immunized with specific antigens. There was also reduced T-cell proliferation and IL-2 production in response to the same antigen (123), implying that Abp1$^{-/-}$ may play a role in antigen-specific immune responses in T cells. Abp1$^{-/-}$ had reduced JNK and HPK1 activation, as well as a reduction in the phosphorylation of PLCγ1 and LAT in response to TCR stimulation. The role of Abp1 in BCR-mediated signaling pathways has not been examined. As an actin adaptor protein, Abp1 has the potential to simultaneously interact with both F-actin and a signaling protein. Its multiple domains for protein-protein interaction and F-actin binding, and its role in TCR-mediated signal transduction, suggest a role for Abp1 in the interaction of the actin cytoskeleton and signal transduction pathway in B cells.

Using a mouse knockout model, we have started to examine the role of Abp1 in BCR-mediated signaling. We found that both splenic B cells from Abp1 knockout mice (Abp1 $^{-/-}$), and B cells treated with Abp1-specific siRNA, had higher levels of protein tyrosine phosphorylation than wt splenic B cells in response to BCR crosslinking. Both Abp1 knockout and knockdown reduced BCR-triggered phosphorylation of JNK. In addition, the activation kinetics of ERK was altered in Abp1-deficient splenic B cells. These demonstrate a role for Abp1 in BCR signaling.

4.3 Materials and method

4.3.1 Mice, cells and cell culture.

B cell lymphoma A20 IIA1.6 cells (H-2^d, IgG$_{2a}$$^+$, FcγRIIB$^-$) were cultured at 37^{o}C in DMEM supplemented with 10% FBS. C57BL6 mice that were 6-8 weeks old were purchased from Taconic (Hudson, NY). Abp1 knockout mice (Abp1$^{-/-}$) in a C57BL6 background were established and kindly provided by Dr. Tse-Hua Tan at Baylor Medical College (Houston, TX). To isolate splenic B cells, single-cell suspensions of splenocytes were subjected to density-gradient centrifugation (2300 x g) in Ficoll (Sigma-Aldrich, St Louis, MO) to obtain mononuclear cells, treated with anti-Thy1.2 mAb (BD biosciences, San Jose, CA) and guinea pig complement (Rockland Immunobiochemicals, Gilbertsville PA) to remove T cells, and panned for 2 h to remove monocytes.

4.3.2 Developing of Abp1 shRNA.

Sequences with the potential to target mouse ABP1 mRNA were designed using software provided by the Qiagen siRNA website. Four sequences were designed and ligated into a lentiviral vector pLL3.7 GFP (181) (this process was done with the assistance of Dr. Wenxia Song) and introduced into 293T cells with the packaging vectors (Invitrogen) using Lipofectamine to generate virus-infected 293T cells. Virus-containing supernatants were collected 36 h after infection. A20 cells were infected with the virus for 5 days. GFP-expressing A20 cells were sorted for GFP expression by BD Aria and cloned by limiting dilution. Clones with Abp1 knockdown were screened by immunofluorescence microscopy. Cells were washed and adhered to poly-lysine-coated slides (Sigma-Aldrich) for 40 min at

$4^{\circ}C$ and then fixed with 4% paraformaldehyde. Cells were permeabilized with a permeabilization buffer (0.05% saponin, 10mM HEPES, 10mM Glycine and 10% Fetal bovine serum in DMEM) and stained for Abp1 using rabbit anti-Abp1 (70) and AF 633 conjugated anti-rabbit mAb. Cells were mounted with gel mount (Biomeda, Foster City, CA) and analyzed using a laser-scanning confocal microscope (LSM 510; Zeiss, Oberkochen, Germany). Abp1 knockdown was confirmed by western blot. The shRNA sequence corresponding to AAGGATAACTTCTGGGCCAAA was shown to be effective in knocking down Abp1 expression and the sequence corresponding to AGCACCTCCTTCCAGGATGT showed no knockdown of Abp1, which will be used as control.

4.3.3 Analysis of phosphorylation.

Splenic B cells from wildtype C57BL6 and Abp1 knockout mice, as well as A20 B cells that express Abp1 shRNA, were activated by crosslinking the BCR with goat anti-mouse IgG+M (20 µg/ml, Jackson ImmunoResearch), or goat anti-mouse IgG (20 µg/ml, Jackson ImmunoResearch) for A20 B cells, for the indicated times and lysed in a lysis buffer containing 0.5% Triton X100, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_3VO_4, and protease inhibitors (Roche, Basel Switzerland). To test the effect of actin depolymerization on BCR signaling, splenic B cells were incubated with 2 µM latrunculin for 30 min before activating the cells. Cell lysates were analyzed using SDS-PAGE and Western blotting, probing with anti-phospho-tyrosine mAb (4G10, Upstate Biotech, Lake Placid, NY), rabbit anti-phosphorylated JNK (pJNK), rabbit anti-phosphorylated ERK (pERK) (Cell signaling), and mouse anti-tubulin (IgG1 specific, Sigma)

and HRP-conjugated antibodies (Jackson ImmunoResearch). The blots were quantified using densitometry.

4.4 Results

4.4.1 Abp1 deficiency alters BCR-induced protein tyrosine phosphorylation.

In order to study the role of Abp1 in BCR signaling, we attempted to knockdown Abp1 by the approach of siRNA. Abp1 knockdown had previously been reported in Jurkat T cells (119) and human embryonic kidney 293T (148). Due to low transfection efficiency of B-cell lines, we interfered with Abp1 expression by introducing shRNA using a lentiviral system. A short hairpin sequence that targeted the mRNA of Abp1 was inserted into a previously described vector (181) that was engineered to include a GFP cDNA for detection of shRNA-positive cells. A20 B cells expressing four potential shRNA sequences were sorted for GFP expression. One of the sequences revealed a substantial inhibition of Abp1 expression by western blot analyses (Fig. 4.1A). Immunofluorescence microscopy analyses showed a decreased Abp1 staining in GFP-expressing cells (Fig. 4.1B). The cells were subsequently cloned and cells expressing the shRNA were selected. The expression level of Abp1 in A20 cells that stably express Abp1 shRNA was less than 20% of wt cells (Fig. 4.1D). This shows that Abp1 can be significantly knocked down in A20 B cells by stably expressing Abp1 shRNA.

Using the A20 cell line in which Abp1 is stably knocked down, we examined the role of Abp1 in BCR signaling. A20 B cells with or without Abp1 knockdown, were incubated with

anti-mouse IgG to cross-link the BCR. The cells were lysed, and the cell lysates were subjected to SDS-PAGE and Western blot, probing with antibodies specific for phosphotyrosine. A20 B cells expressing Abp1 shRNA had a significant increase in the level of protein tyrosine phosphorylation when compared with wt A20 B cells (Fig. 4.1C). To further confirm the role of Abp1 knockdown in BCR signaling, a mouse knockout model of Abp1 knockdown was employed (123). The splenic B cells from both wt and Abp1$^{-/-}$ mice, on C57BL6 background, were incubated with anti mouse IgG + M for indicated times to activate the BCR. Cells were lysed, and the lysates were analyzed by SDS-PAGE and western blot. As shown in Fig. 4.1E, crosslinking of the BCR induced protein tyrosine phosphorylation in both wt and Abp1$^{-/-}$ splenic B cells with the level of protein tyrosine phosphorylation in Abp1$^{-/-}$ splenic B cells significantly higher than wt ones. Specifically, we observed increases in tyrosine phosphorylation for bands around 75KDa (Fig. 1F), 100KDa (Fig. 4.1G) and 120KDa (Fig. 4.1H), although the specific identity of these proteins is unknown. This result shows that Abp1 plays a role in BCR signaling by regulating BCR-triggered protein tyrosine phosphorylation.

FIGURE 4.1. Abp1-deficiency alters BCR-induced protein tyrosine phosphorylation.

(A) Putative Abp1 shRNA's were introduced into A20 B cells using the lentiviral system. shRNA-positive cells were sorted based on co-expressed GFP. Cells were lysed and the lysates were analyzed by SDS-PAGE and Western blot, probing for Abp1. (B) Mixture of A20 B cells expressing and not expressing Abp1 shRNA (Green) were fixed, permeabilized, and labeled with rabbit anti-Abp1 and an Alexa Fluor 546-conjugated secondary antibody. Shown are representative images of three independent experiments. (Bar, 10 μm). The protein tyrosine phosphorylation of Abp1 shRNA-expressing A20 B cells in response to BCR activation was compared with wt A20 B cells using western blot. Wt and Abp1 shRNA-expressing cells were incubated with goat-anti-mouse IgG for 5 min to activate the BCR. Then, cells were lysed, and the lysates were analyzed by SDS-PAGE and Western blot, probing with anti-phosphotyrosine mAb (4G10) (C). The blots were stripped and reblotted with either anti-Abp1 or actin antibody (C). (D) Shows Abp1 expression in both wt and Abp1 shRNA expressing cells, quantified using densitometry, and normalized to actin levels. (E) The protein tyrosine phosphorylation in response to BCR activation in splenic B cells from Abp1$^{-/-}$ mice was compared with that in wt splenic B cells using western blotting. Splenic B cells were incubated with goat-anti-mouse IgG + M for varying lengths of time to crosslink the BCR. Next the cells were lysed, and the lysates were analyzed by SDS-PAGE and Western blotting, probing with anti-phosphotyrosine mAb (4G10). Shown is a representative of three independent experiments. (F-G) Phosphorylation levels for selected bands were quantified using

densitometry and normalized to tubulin levels. Shown is a representative of three

independent experiments.

C

A20   shRNA

-XL +XL -XL +XL (+XL = 5 min)

Phospho-tyrosine blot

130 KDa

90 KDa

42 KDa

◄ Abp1

◄ Actin

D

Abp1 Levels (Arbitrary units)

80

60

40

20

0

A20   shRNA

E

WT   Abp1$^{-/-}$

-xl 2 10 30   -xl 2 10 30 Time (min)

Phospho-tyrosine blot

131 KDa

90 KDa

42 KDa

◀ **Tubulin**

F 75kDa

Legend:
- 0min
- 2min
- 10min
- 30min

G 100kDa

Legend:
- 0min
- 2min
- 10min
- 30min

H 120kDa

Legend:
- 0min
- 2min
- 10min
- 30min
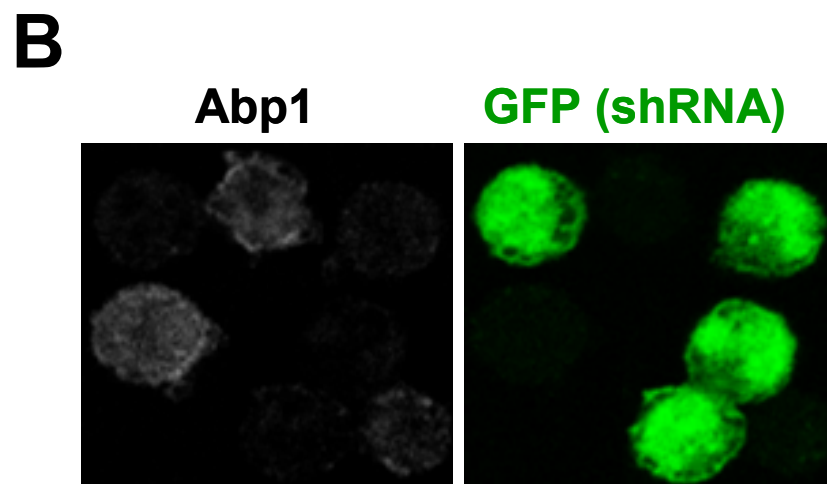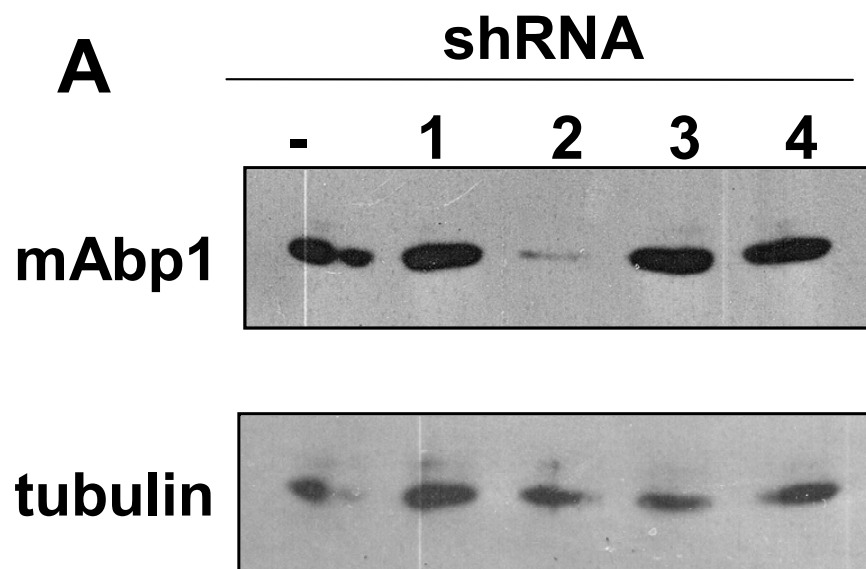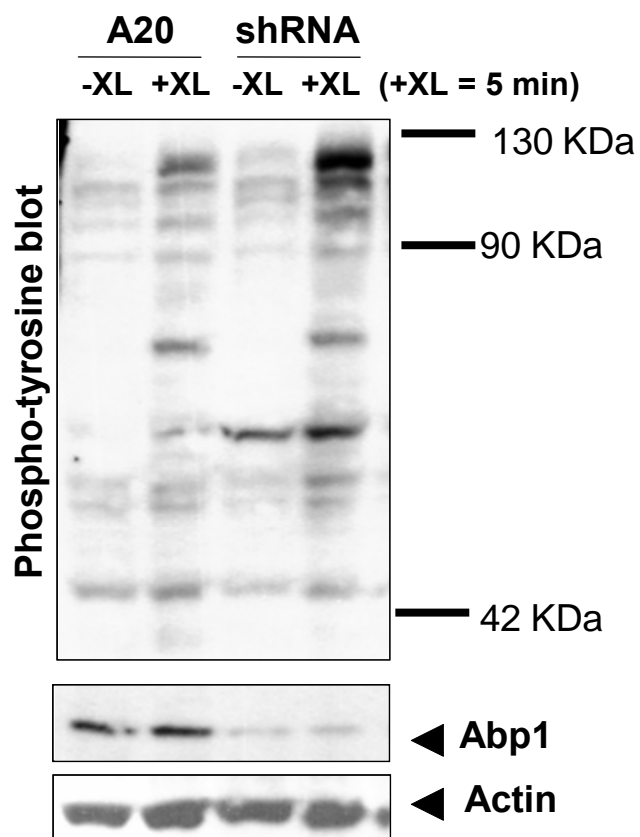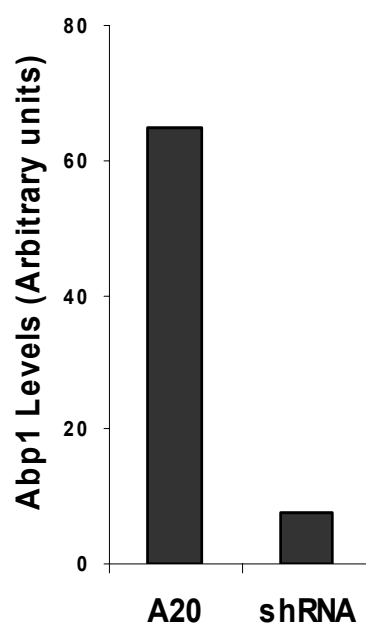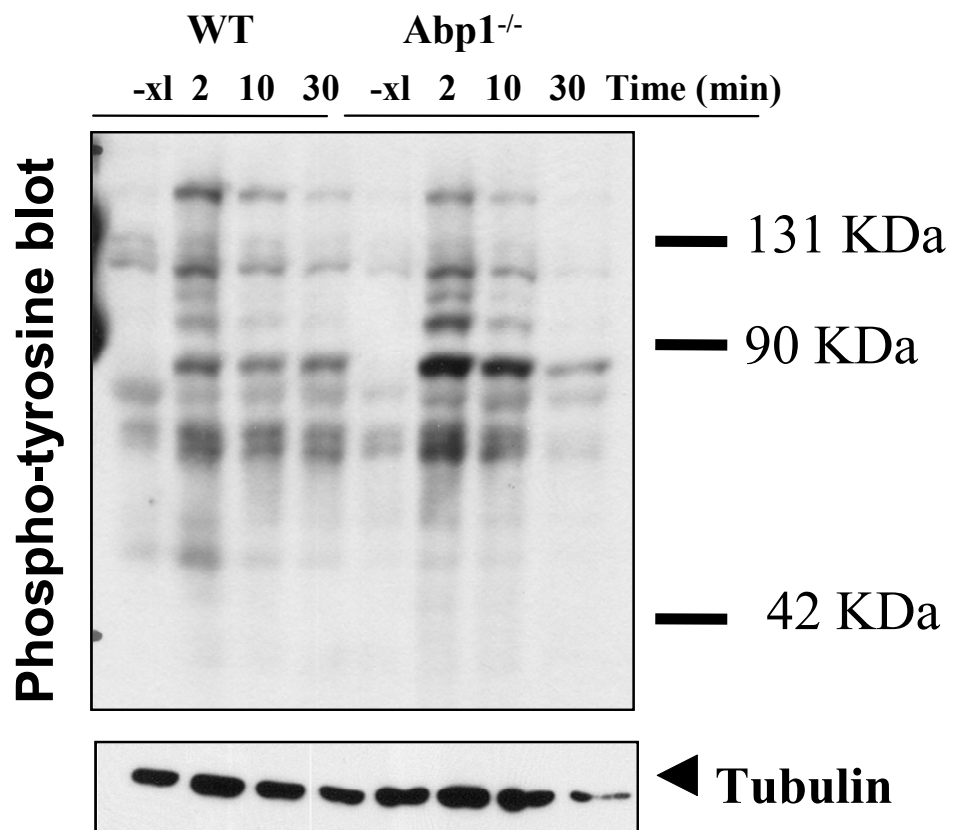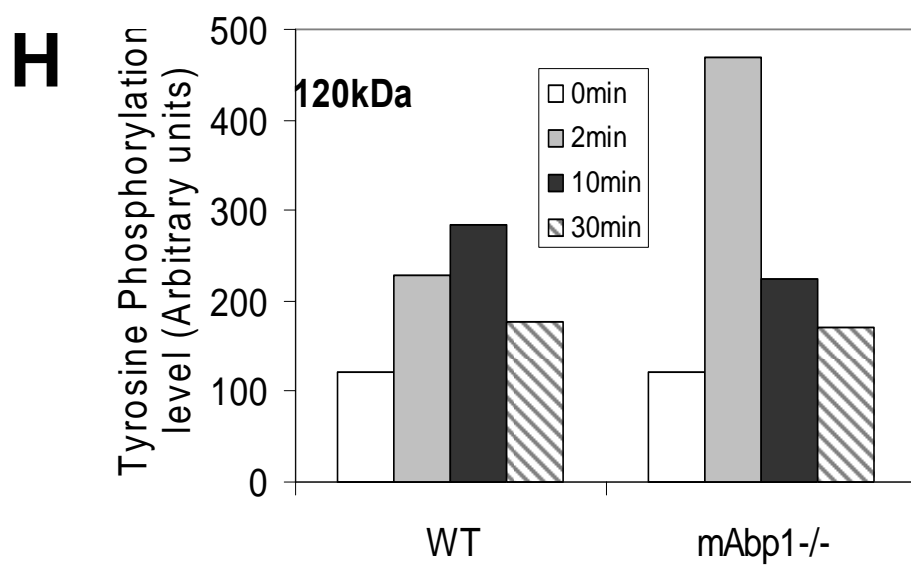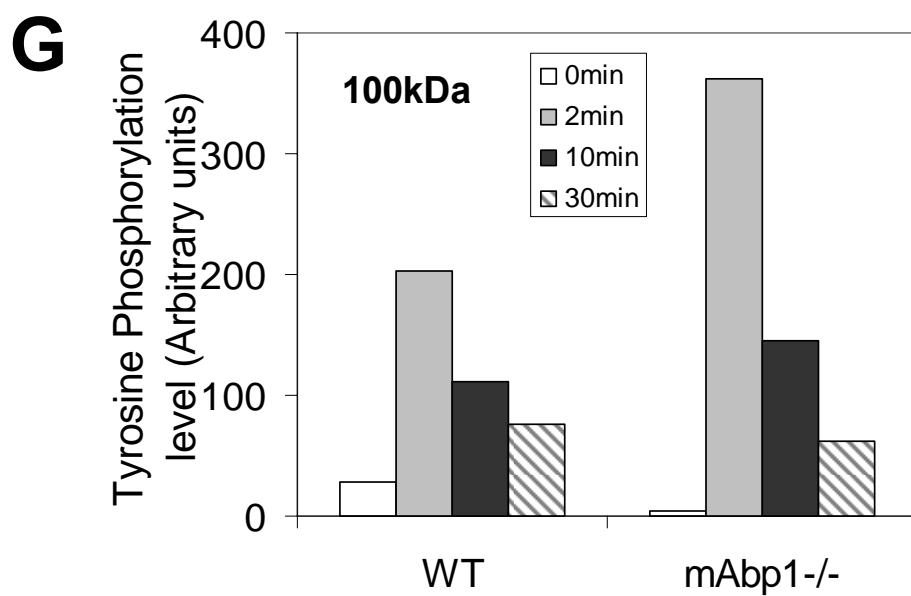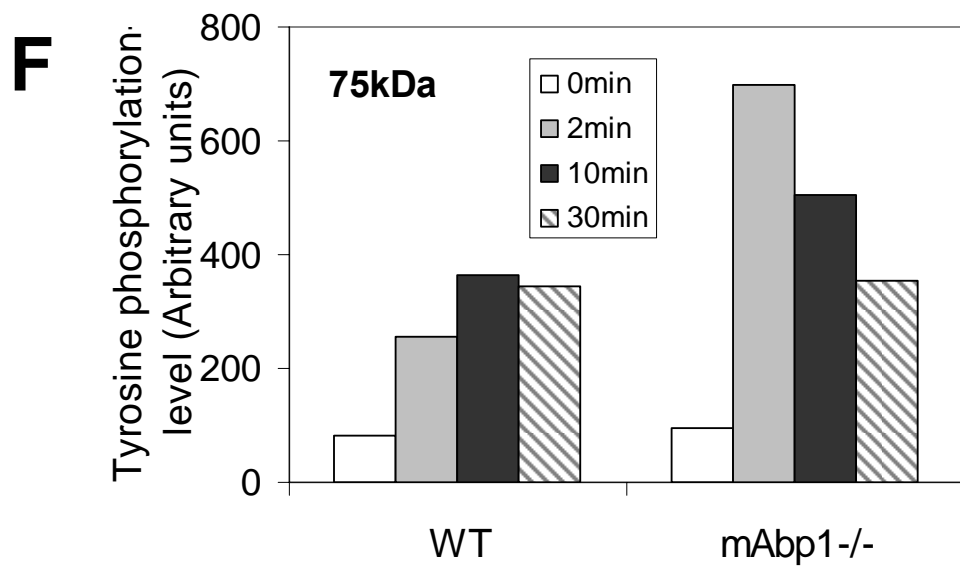
4.4.2 Abp1 deficiency alters BCR-induced phosphorylation of JNK and ERK.

Abp1 has previously been shown to play an indirect role in JNK activation by interacting

with HPK1, an upstream kinase of JNK in T cells (119, 123). To determine whether

Abp1 plays a role in BCR-triggered JNK activation in B cells, we compared BCR-

triggered phosphorylation of JNK in wt and Abp1$^{-/-}$ splenic B cells. Western blot analyses

using anti-phospho JNK antibodies showed that BCR crosslinking increased JNK

phosphorylation in wt A20 B cells and splenic B cells (Fig. 4.2A ,B and C,D

respectively), however, this increase in JNK phosphorylation was significantly reduced in

Abp1-knockdown A20 B cells and Abp1$^{-/-}$ B cells (Fig. 4.2A ,B and C,D respectively).

ERK phosphorylation resulting from BCR-mediated activation has been shown to be

important for the proliferation of the B cell and the upregulation of B-cell activation

markers (14). To study the role of Abp1 in B-cell signaling, we compared the

phosphorylation of ERK following BCR crosslinking in Abp1$^{-/-}$ and wt splenic B cells.

ERK phosphorylation increased to a similar level in Abp1$^{-/-}$ and wt splenic B cells 2 min

after BCR crosslinking. However, at 10 min, ERK phosphorylation in Abp1$^{-/-}$ B cells

declined, while ERK phosphorylation in wt B cells remained high (Fig. 4.2C and E). We

did not observe any obvious differences in BCR-induced phosphorylation of p38 between

Abp1$^{-/-}$ and wt splenic B cells (Fig. 4.2C).

FIGURE 4.2. Abp1-deficiency alters BCR-induced phosphorylation of JNK and ERK.

The phosphorylation of JNK in Abp- shRNA expressing A20 B cells in response to BCR activation was compared with wt A20 B cells using western blotting. Wt and Abp1 shRNA expressing cells were incubated with goat-anti-mouse IgG for 5 min to crosslink the BCR. Next the cells were lysed and the lysates were analyzed by SDS-PAGE and Western blot. Blots from Fig 4.1C were stripped and probed with anti-phosphorylated JNK (pJNK) mAb (A). The blots were stripped and re-probed with either anti-Abp1 or actin antibody. (B) Shows pJNK level in both wt and Abp1-shRNA expressing cells quantified using densitometry and normalized to actin levels. (C) The phosphorylation of JNK, ERK and p38 in response to BCR activation in splenic B cells from Abp1$^{-/-}$ mice was compared with that in wt splenic B cells using western blotting. Blots from Fig 4.1E were stripped and probed with anti-pJNK, anti pERK and anti p38 mAb. JNK (D) and ERK (E) phosphorylation levels in Abp1$^{-/-}$ and wt mice were quantified using densitometry and normalized to tubulin levels. Shown are averages (S.D.) of three independent experiments.

C

	WT				Abp1	-/-		
	-xl	2	10	30	-xl	2	10	30	Time (min)

◀ p-ERK

◀ p-JNK

◀ pp38

◀ Tubulin

D

E
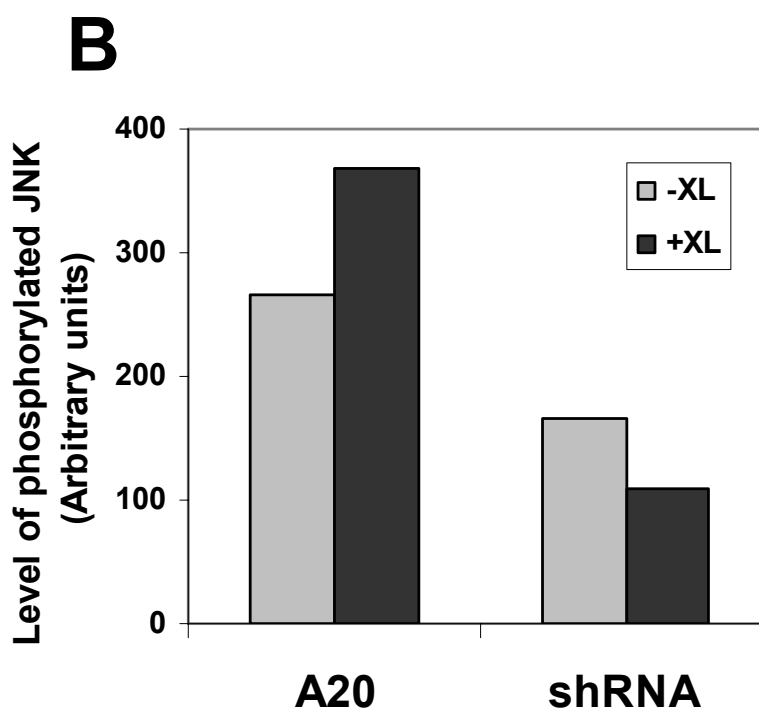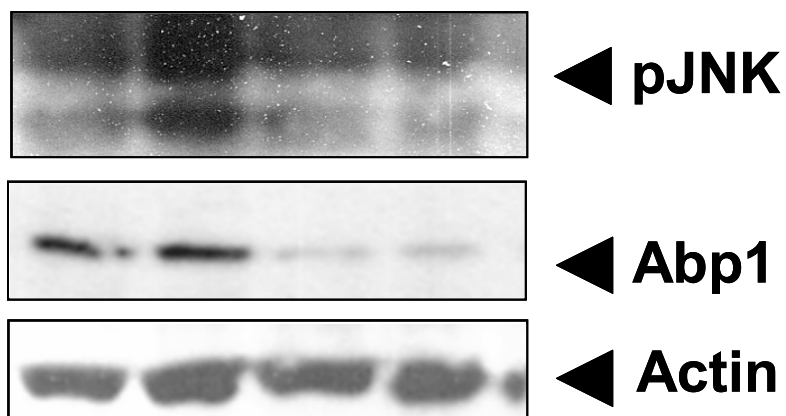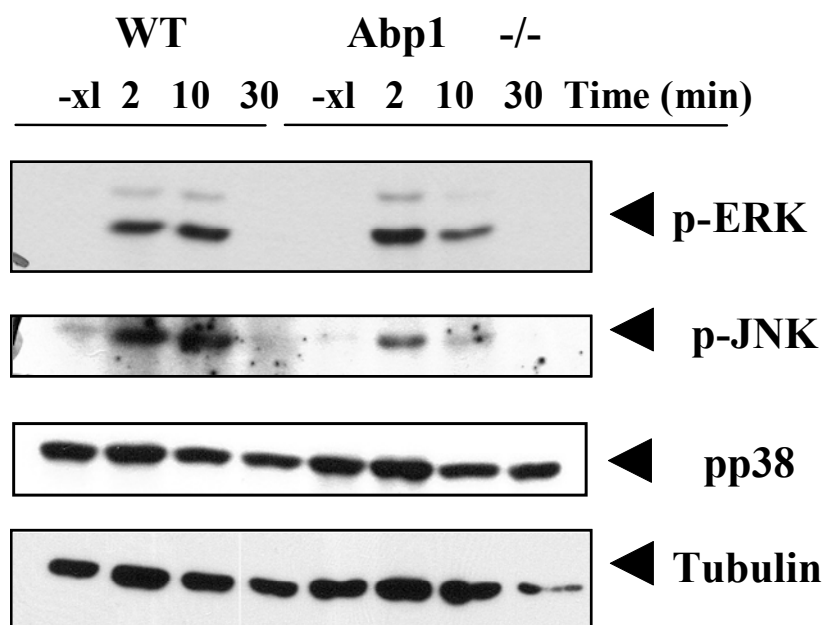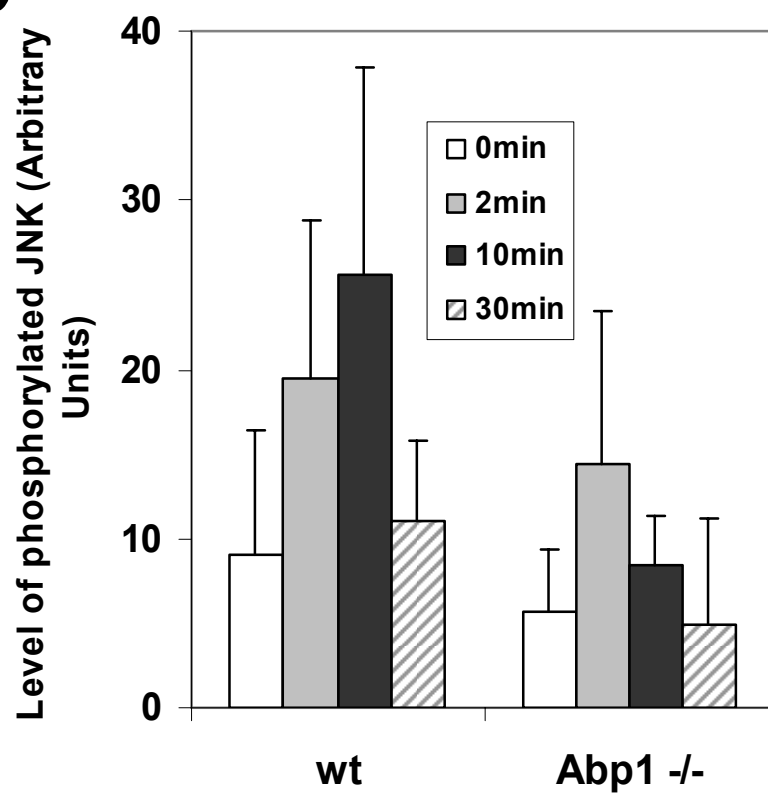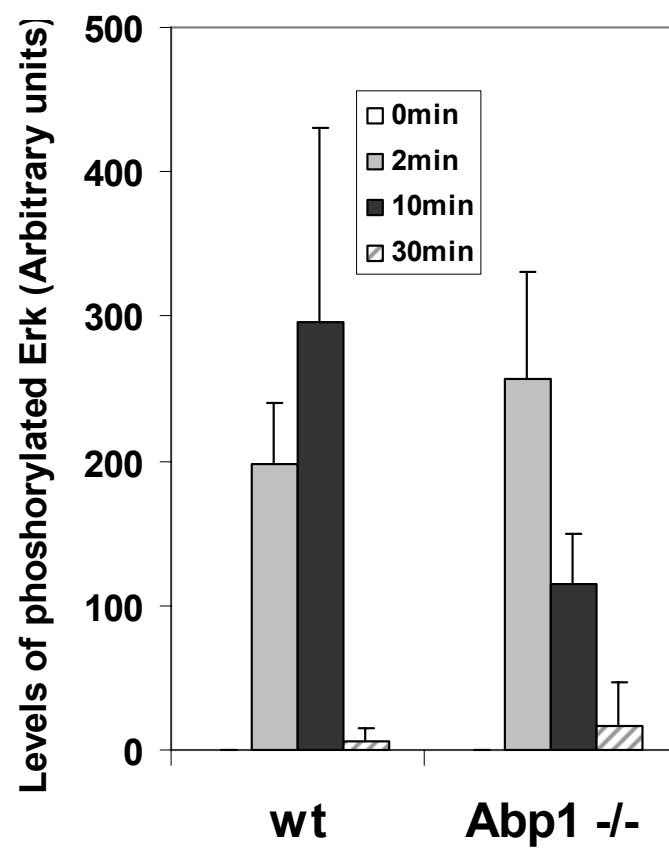
4.4.3 Alteration of BCR signaling in Abp1$^{-/-}$ B cells is not simply caused by reduced BCR internalization.

In the previous chapter, we demonstrated that the internalization rate of the BCR was reduced in Abp1$^{-/-}$ B cells. In order to test whether the increase in BCR-induced protein tyrosine phosphorylation is a result of reduced BCR internalization, we compared the protein tyrosine phosphorylation levels in Abp1$^{-/-}$

splenic B cells with wt splenic B cells treated with latrunculin, which blocks BCR internalization through actin depolymerization (35). We did not observe a similar increase in protein tyrosine phosphorylation in latrunculin-treated B cells as seen in Abp1$^{-/-}$ B cells (Fig. 4.3B). This result suggests that inhibition of BCR internalization is not the sole cause of upregulated protein tyrosine phosphorylation in Abp1$^{-/-}$ B cells. Furthermore, in latrunculin-treated splenic B cells, we observed that BCR-induced ERK phosphorylation was considerably higher than in cells that were not treated with latrunculin (Fig. 4.3A and B). This is in sharp contrast to Abp1$^{-/-}$ mice where there was a substantial attenuation of ERK phosphorylation (Fig. 4.2C and E). This result is in line with a previously published report showing that latrunculin-treatment enhances BCR-induced ERK phosphorylation (87). In addition, JNK phosphorylation was higher in latrunculin treated splenic B cells when compared with wt (Fig. 4.3A and B) contrasting with what was observed in Abp1$^{-/-}$ (Fig. 4.2C and E) where JNK activation was attenuated. This further supports the conclusion that the altered signaling profiles observed in Abp1$^{-/-}$ mice may not be connected to the observed defects in BCR internalization.

FIGURE 4.3. Defect in BCR internalization is not sufficient to explain signaling defect in Abp1$^{-/-}$.

(A) The protein tyrosine phosphorylation in response to BCR crosslinking in latrunculin-treated splenic B cells was compared with that in wt splenic B cells using western blotting. Splenic B cells were treated with or without 2μM latrunculin for 30 min and incubated with goat-anti-mouse IgG for varying lengths of time to crosslink the BCR. Then the cells were lysed, and the lysates analyzed by SDS-PAGE and Western blotting, probing with anti-phosphotyrosine, (4G10) anti-pJNK and anti-pERK mAb. Shown is a representative of two independent experiments. ERK (B) and JNK (C) phosphorylation levels in wt and latrunculin-treated wt splenocytes were quantified using densitometry and normalized to tubulin levels.

A

Phospho-tyrosine blot

| Control | Latrunculin |
| -xl 2 10 30 | -xl 2 10 30 Time (min) |

131 KDa

90 KDa

42 KDa

◄ p-ERK

◄ p-JNK

◄ Tubulin

B

Levels of phosphorylated JNK (Arbitrary units)

- 0min
- 2min
- 10min
- 30min

wt   Lat

C

Levels of phosphorylated ERK (Arbitrary units)

- 0min
- 2min
- 10min
- 30min

wt   Lat
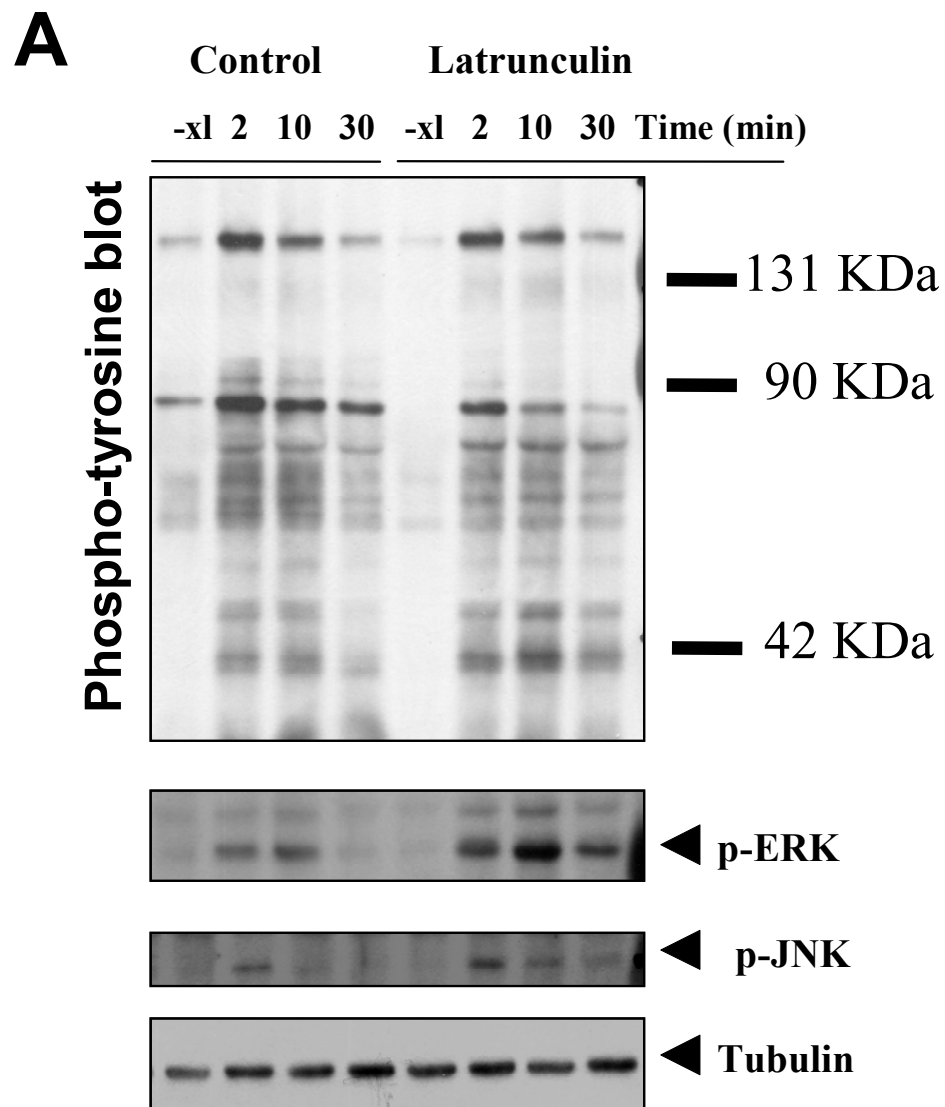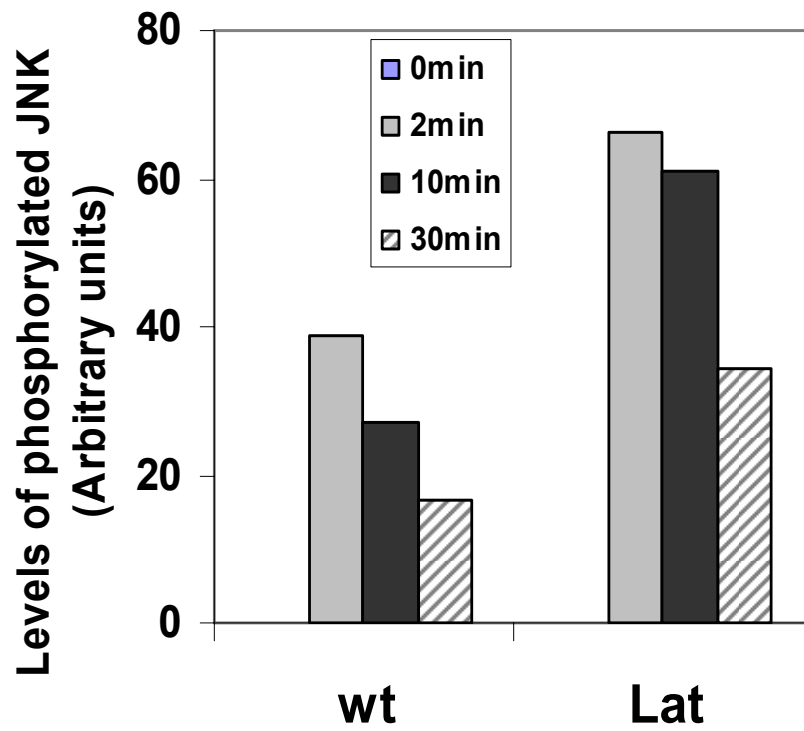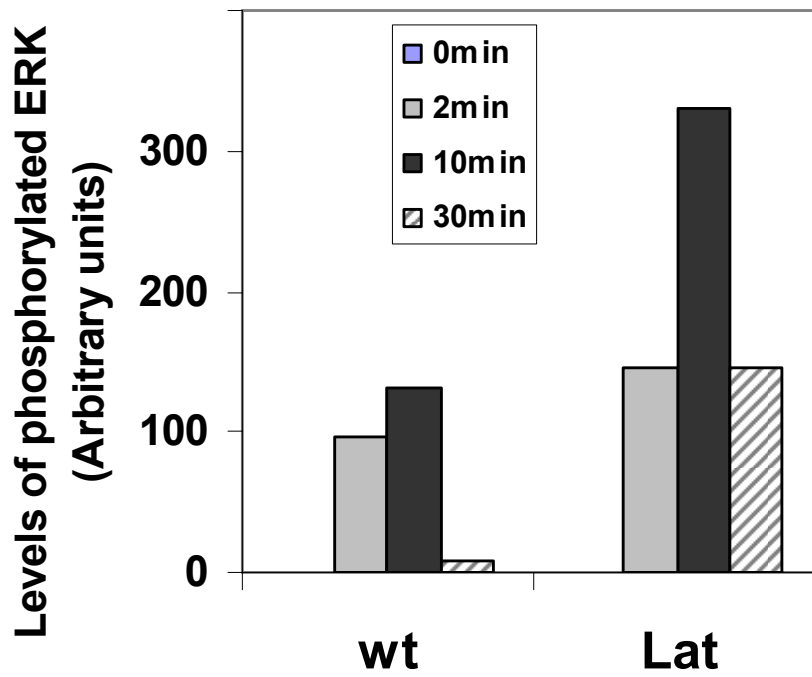
4.5 Discussion

Abp1, an actin adaptor protein, plays an important role in T cell signaling. In T cells, Abp1 regulates the function of the MAP kinase, JNK, via interactions with HPK1 (123). However, the role of Abp1 in B cell signaling has not yet been studied. In this study, we showed that both Abp1 knockdown and knockout enhanced BCR-induced protein tyrosine phosphorylation, and reduced BCR-induced phosphorylation of the MAP kinases JNK and ERK. These results demonstrate an important role for Abp1 in BCR-mediated signaling.

The attenuation of JNK activation observed in B cells was similar to previously published reports in Abp1$^{-/-}$ T cells (123). The same report showed a defect in p38 activation in Abp1$^{-/-}$ T cells. However the effect of Abp1-deficiency on TCR-induced ERK activation has not been reported. In B cells, ERK phosphorylation, but not p38 phosphorylation was altered by Abp1 deficiency, suggesting that Abp1 regulates BCR signaling by a mechanism different from TCR signaling. The regulatory role of Abp1 in TCR signaling has been attributed to the interaction of Abp1 with HPK1, a MAP4K. This interaction regulates the activation of HPK1 (119, 120, 122, 123) which activates JNK through MAP3K, e.g. MEKK1 (180, 182). Whether or not Abp1 uses a similar mechanism to regulate the activation of JNK in B cells remains to be tested.

Alteration of BCR-triggered tyrosine phosphorylation levels of multiple proteins was observed in Abp1 knockdown and knockout B cells, suggesting the involvement of Abp1 in the proximal signaling of the BCR. BCR crosslinking by antigen results in the translocation of the BCR and signaling molecules to lipid rafts. This process has been shown to be

important for BCR signaling (9, 33, 183, 184). Previously published data show that Abp1 is translocated into glycolipid-enriched microdomains (also described as lipid rafts) formed at the T-cell synapse following antibody stimulation of the TCR (119, 122). This recruitment is followed by the phosphorylation of Abp1 by ZAP70 (119). Previously published reports also show that the recruitment and phosphorylation of Abp1 at the T cell synapse occurs concurrently with HPK1 recruitment following TCR activation (122). Although Abp1 binds constitutively with HPK1 (119, 122), it is possible that the recruitment of Abp1 directs the recruitment of HPK1 in T cells as well. The observation that BCR-triggered protein tyrosine phosphorylation was upregulated in both Abp1$^{-/-}$ and Abp1 knockdown B cells suggests a negatively regulatory role for Abp1 in BCR signaling and this may be due to its interaction with HPK1. A recent report showed that HPK1 serves as a negative regulator of T-cell function (121). HPK1 deficiency resulted in the enhanced phosphorylation of SLP-76, PLC-γ1 and enhanced calcium influx (121). Abp1 has the potential to recruit HPK1 to the BCR-signaling microdomain, permitting HPK1 to regulate BCR signaling, and this could help explain the observed defect in BCR signaling dependent tyrosine phosphorylation in Abp1 deficient B cells.

In the second chapter my observations show that Abp1 knockout and knockdown can inhibit BCR internalization. This may prolong the lifetime of BCR surface signaling microdomains and enhance proximal signaling. However, inhibiting BCR internalization by disrupting the actin cytoskeleton with latrunculin gave a markedly different signaling profile, suggesting that enhanced BCR signaling due to defects in internalization cannot fully explain the observations. I was also able to observe a number of tyrosine-phosphorylated bands that

showed substantial increases in activation. This included bands that corresponded to approximately 75, 100 and 120 kDa. Speculating on the identity of these proteins based on molecular weight alone is difficult, since the accuracy of the markers used in SDS/PAGE is limited, and a long list of signaling proteins exist at this molecular weight range. Determining the identity of these proteins, by either mass spectrometry or some other method, should provide important clues about what stage in the BCR signaling cascade Abp1 might be exerting its influence.

Surface-signaling microdomains formed by coalescing of BCR and signaling molecules in the lipid rafts, are similar to the immunological synapses between T cells and APCs. A role for the actin cytoskeleton has been described in the successful formation of the synapse in T cells and one of the major defects observed in WASP (a regulator of the actin cytoskeleton) knockout mice was an inability to form a synapse between the APC and the T-cell (111). Vav-deficient mice (a GEF for Rac) have defects in synapse formation in T cells (100, 177). It has also been shown in B cells that actin plays a role in regulating the lipid-raft complex formed after BCR crosslinking. It was observed that disrupting the actin cytoskeleton led to a delay in the BCR crosslinking-induced internalization of the lipid rafts and may explain the sustained ERK signaling observed in the presence of actin-disrupting agents (87). It is possible that Abp1's activity might be connected to the actin cytoskeleton. It was observed that Abp1 recruitment to the cell surface was disrupted in the presence of latrunculin, an actin-disrupting agent, indicating that Abp1 function is dependent on an intact actin cytoskeleton (Unpublished observations). This allows us to envision a scenario where the BCR-induced recruitment of the actin cytoskeleton facilitates the translocation of Abp1 to the

plasma membrane, where it is phosphorylated by Src kinases and it acts as an adaptor protein that helps transduce signals generated by BCR crosslinking to downstream effectors. The identity of these downstream effectors is not known although obvious candidates include HPK1, which regulates JNK activity (180, 182). This may explain the attenuation I observed in JNK activation. A proteomic approach can be used to identify other proteins that might be regulated by Abp1.

Bam32, an actin adaptor protein, has recently been shown to be important for BCR-induced reorganization of the actin cytoskeleton via interactions with Rac1 (85, 86, 88). Splenic B cells, from Bam32-deficient mice and Bam32-deficient DT40 cells, shared similar JNK and ERK activation defects as Abp1^{-/-} B cells. Bam32 was shown to interact with HPK1, and Bam32^{-/-} mice had defective HPK1 activation in B cells (185), reminiscent of the phenotypes observed in T cells from Abp1^{-/-} mice (123). Furthermore, both Abp1 and Bam32 are recruited to the plasma membrane and tyrosine phosphorylated following BCR-mediated activation (114, 185). However, Bam32 is involved in regulating the actin cytoskeleton, while Abp1 function in mammalian cells seems to be dependent on the actin cytoskeleton. Although no functional relationship between Abp1 and Bam 32 has been established, similarities shared by Abp1 and Bam32 deficiency indicate the involvement of these two proteins in the same pathway. It is possible that both of these proteins are part of a signalsome complex that is maintained by the actin cytoskeleton and is required for optimal BCR signaling. A recent report by Batista's group showed the formation of signaling microclusters composed of antigen-crosslinked BCR, tyrosine phosphorylated proteins, and the non receptor tyrosine kinase, Syk (186). It is possible that the formation of these clusters

is also dependent on an intact actin cytoskeleton as a well as a number of actin binding proteins like Abp1 that might help with recruiting signaling proteins to these microclusters.

The data presented here clearly show a role for Abp1 in regulation of BCR-mediated signaling. However, the underlying mechanism for the regulatory role of Abp1 has not been well defined. Future work will focus on the impact of Abp1 knockout on HPK1 activity and downstream transcription factor activation in B cells as well as the functional and physical interactions between Abp1 and Bam32.

Chapter 5: General discussion

The main objective of my thesis was to study how BCR signaling regulates the interaction of the actin cytoskeleton with the endocytic machinery of the B cell leading to the rapid internalization of antigens and their successful processing and presentation.

The B cell is a major component of the adaptive immune response. It is capable of recognizing an incredibly diverse array of antigens with its clonally specific receptor, the BCR. Multivalent antigens that can crosslink these receptors trigger a series of signaling events that lead to signaling-dependent internalization and processing of the antigen in MHC class II-containing compartments for the eventual presentation of the processed antigens to T cells on the surface of the B cell. The mechanism by which BCR signaling upregulates its own internalization is still being worked out. Previous work in Dr. Song's laboratory showed that a dynamic actin cytoskeleton is required for the efficient internalization of the BCR following BCR crosslinking (35). Furthermore, we showed that BCR signaling regulates major proteins that are required for endocytosis of the BCR, notably clathrin (33). These studies and studies from other laboratories point to an intimate relationship between BCR signaling and the molecular events that direct the internalization of the BCR, and elucidating these mechanisms has been the primary focus of the lab.

In this thesis, I demonstrate that Abp1 is a linker that connects the actin cytoskeleton to the endocytic machinery of the BCR upon antigenic crosslinking of the BCR. Abp1

mediates this connection by interacting with both F-actin and dynamin, and these interactions are important for the efficiency of BCR-mediated antigen processing and presentation. The interaction between dynamin and Abp1 depends on the PRD of dynamin and the SH3 domain of Abp1, respectively, and this was confirmed by co-immunoprecipitation and GST-based precipitation. This was further supported by the inhibitory effect seen with overexpression of the SH3 domain of Abp1 or the PRD deletion mutant of dynamin on BCR internalization and movement to the antigen processing compartment. These results underscore the importance of both Abp1 and dynamin in antigen-induced BCR internalization and BCR-mediated antigen processing and presentation. Antigen-induced BCR internalization is primarily clathrin-mediated (33, 140), and the role of dynamin in pinching off nascent vesicle during CME has been well documented (55, 91). A role for dynamin in linking the endocytic vesicle to the actin cytoskeleton during CME has also begun to emerge (59, 63, 77). My data describes a critical link between the actin cytoskeleton and the internalizing BCR, mediated by the interaction between dynamin and Abp1.

The rapid internalization of the BCR following antigen crosslinking is accompanied by the efficient processing of the antigen in the MIIC compartment and the eventual presentation of the antigen in the context of the MHC (14, 187). The efficient delivery of antigen to the MIIC and its subsequent processing has been shown to be dependent on BCR-mediated signaling (42). The engagement of the BCR by antigen triggers the phosphorylation of ITAMs on Igα/Igβ, and the phosphorylation sites of the ITAM are also required for the accelerated targeting of antigens to the antigen processing

128

compartment or MIIC compartment (34, 42, 43, 132, 154). Following BCR crosslinking, the phosphorylated ITAMs on Igα/Igβ recruit the tyrosine kinase Syk, which activate a number of signaling and adaptor molecules (14). Overexpression of a Syk mutant that does not interact with the phosphorylated ITAMs on Igα leads to defects in antigen presentation (132). These further indicate that BCR-induced signaling regulates the antigen processing and presenting functions of the BCR. The reduction of the efficiency of BCR-mediated antigen processing and presentation in Abp1-knockout mice indicates the importance of Abp1 in this process. This defect can be explained by the reduced rates of BCR internalization and movement to the antigen-processing compartment in Abp1 knockout mice.

The interaction of Abp1 with dynamin provides a link between Abp1 and the endocytosis machinery. However, the link between Abp1 and the machinery responsible for targeting of antigens to the MIIC is not known. In this thesis, I show that dynamin was recruited to the BCR at the plasma membrane upon BCR activation and maintained its colocalization with the BCR when it was on its way to the late endosomes. This sustained colocalization and trafficking of dynamin with the internalized BCR is disrupted by deleting the PRD of dynamin, suggesting that interactions mediated by this domain are important for co-trafficking dynamin with the BCR to the antigen processing compartment. I hypothesize that dynamin recruits the actin cytoskeleton to BCR-containing vesicles, which provides not only the driving force for the intracellular movement but fusion of these vesicles to their targets. Previous studies have shown that actin comet tails provide a mechanism for pushing vesicles within the cell interior (67, 188), and in at least one study, dynamin was

shown to be a part of the actin comet (189). A working model can therefore be

envisioned whereby BCR activation recruits dynamin to BCR containing clathrin-coated

pits or vesicles, where it recruits F-actin by interacting with actin binding proteins, such

as Abp1 or induces localized actin polymerization by binding to actin regulating proteins.

The interaction of dynamin with Abp1 could provide actin nucleation sites in the vicinity

of the BCR for active actin polymerization and branching. The localized polymerization

of actin could then generate the force that moves BCR-containing clathrin-coated pits and

vesicles.

How does BCR signaling regulate the interaction between the actin cytoskeleton and

BCR endocytosis machinery? Tyrosine phosphorylation of Abp1 is induced by PTKs

Syk, Lyn and Blk in *in vitro* kinase assays (114). Here, I show that Abp1 is recruited to

the BCR in a phosphorylation-dependent manner, since mutating the tyrosine

phosphorylation sites to phenylalanine inhibited the recruitment. The particular kinase

responsible for phosphorylating Abp1 in response to BCR activation *in vivo* is not known

and should be a topic for future studies. Syk is a kinase that could be a likely candidate

for regulating Abp1. In addition to being the potential kinase for Abp1 phosphorylation,

Syk could regulate the subcellular location of Abp1 by modulating the actin cytoskeleton

organization. Syk-mediated regulation of the actin cytoskeleton has recently been

reported to be important for the endocytosis and intracellular trafficking of BCR–antigen

complexes (84). Using a Syk-deficient A20 cell line, Le Roux *et al*. showed that the

targeting of BCR–antigen complexes to the MIIC compartments as well as the formation

of the MHC II-peptide complexes is Syk-dependent (84). It was also observed that,

unlike wildtype A20 cells, where the actin cytoskeleton colocalized with the MIIC compartment, Syk deficient cells had a disorganized actin cytoskeleton. How Syk regulates the actin cytoskeleton has not been fully elucidated. Syk has been shown to activate Vavs, which are guanine exchange factors for Rho-family GTPases, Rac and Cdc42, both key regulators of the actin cytoskeleton (190, 191). Another possibility is that Syk might phosphorylate and thus recruit Abp1 to the BCR. This enables Abp1 to connect the BCR-antigen complex to the actin cytoskeleton and thus direct the complex to the late endosomes.

BCR signaling may also regulate dynamin function and potentially direct its interaction with Abp1. Collaborative work with the Brodsky lab has shown that clathrin is recruited to the cell surface upon BCR crosslinking and this recruitment is dependent on BCR signaling mediated phosphorylation of clathrin (33). In addition, previous work in our lab has shown that the Src kinase inhibitor PP2 inhibits BCR crosslinking-mediated recruitment of dynamin to the cell surface, implying that dynamin recruitment may also be signaling-dependent (Brown, B.K. unpublished observations). Dynamin 2 is phosphorylated in a Src-dependent manner at Y231 and Y597 in rat endothelial cell lines in response to albumin binding to its receptor gp60, where dynamin 2 colocalized with caveolin (174). The recruitment of dynamin 2 to the plasma membrane has also been observed at the immunological synapse formed between a T cell and an antigen presenting cell (100). The researchers, however, did not find any change in dynamin phosphorylation before and after T cell stimulation and suggested that the recruitment of dynamin 2 to the immunological synapse is not dependent on its phosphorylation (100).

As discussed previously, my data shows that dynamin 2 is recruited to the cell surface following BCR crosslinking where it colocalizes with the BCR. This could be a direct result of BCR signaling-dependent phosphorylation (or dephosphorylation) of dynamin. It is also possible that proteins responsible for recruiting dynamin are regulated by BCR signaling and, hence, indirectly recruit dynamin to the cell surface. My data supports this hypothesis by showing that deleting the PRD of dynamin inhibits the recruitment of dynamin to the cell surface. The PRD of dynamin is responsible for binding to a number of SH3 domain-containing proteins that are involved in the endocytic process, notably endophilin and amphiphysin (51, 54, 102, 171). These proteins could be directly regulated by BCR signaling, which then regulate dynamin indirectly. Dynamin binding to amphiphysin via its PRD has been shown to be required for its recruitment (91). So, it is plausible that BCR signaling might regulate amphiphysin, which, in turn, recruits dynamin to the cell surface. This hypothesis is further buttressed by the fact that amphiphysin is itself recruited to the plasma membrane by binding to the α-adaptin subunit of AP-2 (102), which is recruited with clathrin in a BCR signaling-dependent manner ((33) and Bruce Brown unpublished observations). Interestingly, amphiphysin phosphorylation prevented it from binding to AP-2, while dephosphorylation enhances its binding to AP-2 in synaptic vesicles (103). It will be interesting to study the regulation of these proteins during BCR signaling and whether they have any effect on dynamin regulation.

In addition to a role for Abp1 in endocytosis, studies in T cells using both siRNA (119, 122) and Abp1$^{-/-}$ mice have revealed a role for Abp1 in T cell signaling (123). Abp1

recruitment to the T cell side of the APC – T cell immune synapse is dependent on both the SH3 domain and the ADH domain of Abp1 (122). Abp1 knockdown using siRNA results in a reduced expression of the TCR, indicating a possible defect in the recycling of the TCR to the cell surface (122). In T cells, Abp1 interacts with the kinase HPK1 (118), and this interaction is required for the activation of HPK1, a MAP4K that acts in the JNK pathway (180). Furthermore, Abp1$^{-/-}$ mice have defects in the activation of LAT, PLCγ1, HPK1 and JNK in T cells, and these may contribute to defects in T cell-derived IL-2 production as well as proliferation. Thus, Abp1 may be involved in both proximal and downstream signaling from the TCR. We delineate a role for Abp1 in BCR-mediated signaling as demonstrated by enhanced tyrosine phosphorylation and altered JNK and ERK signaling in splenic B cells from Abp1$^{-/-}$ mice. However, the exact role of Abp1 in BCR signaling, though significant, remains undetermined. The enhanced tyrosine phosphorylation suggests that Abp1is a regulator for BCR signaling. Such a regulatory effect could be mediated by the interaction of Abp1 with HPK1. HPK1 has recently been shown to be a negative regulator of T cell signaling (121). T cells from HPK1 knockout mouse display hyper-responsiveness to TCR stimulation as indicated by enhanced phosphorylation of PLCγ1, ERK and SLP-76, increased proliferation, cytokine production and humoral response (121). Abp1-deficiency may cause defects in the recruitment to and phosphorylation of HPK1 at the plasma membrane, consequently failing to activate JNK. The interaction of Abp1 and HPK1 in B cells however, has not yet been reported. Recent reports have described a link between Bam32, a BCR adaptor protein, and HPK1 in B cells, where Bam32 was shown to directly interact with HPK1 and regulate JNK activation in response to BCR crosslinking (23, 85). This points to a

similar role for Abp1 and Bam32 in regulating JNK activation in B cells, indicating that these two proteins may have a functional relationship, this, however, remains to be elucidated.

My studies reveal an important function performed by Abp1 in linking, BCR signaling, antigen processing and presentation pathways, and the actin cytoskeleton. Abp1 facilitates BCR-mediated antigen internalization and subsequent processing and presentation in response to BCR activation by interacting with F-actin and dynamin. Additionally, Abp1-facilitated BCR internalization and the interaction of Abp1 with HPK1 potentially provide feedback signals for BCR-triggered signaling cascades.

5.1 .Future studies

While my research has revealed important roles for Abp1 and dynamin in BCR signal transduction, endocytosis and intracellular trafficking, a number of questions remain.

The coordination of the endocytic machinery with the actin cytoskeleton during BCR signaling and internalization is not well understood. Live imaging studies of the interaction between dynamin and Abp1 would help shed some light on its nature. Studies using Fluorescence Resonance Energy Transfer (FRET) between tagged proteins can provide critical visual data on the interaction between these two proteins during BCR signaling and internalization. The kinetics of the interaction between the endocytic machinery, Abp1, dynamin, and the actin cytoskeleton can also be studied at the plasma

membrane using total internal reflection microscopy (TIRF) to determine the timing of BCR movement from the plasma membrane, the involvement of the actin cytoskeleton as well as other proteins that may be connected to BCR endocytic machinery.

My research has revealed that dynamin and Abp1 seem to be regulated by BCR signaling. The precise nature of their regulation, however, is yet to be determined. It will be important to determine exactly how BCR signaling might be regulating both Abp1 and dynamin. Previous work in our lab by Bruce Brown has revealed disruption of dynamin recruitment in the presence of PP2, a Src kinase inhibitor. This raises the possibility that BCR signaling might directly regulate dynamin recruitment by phosphorylating dynamin. To test this, dynamin can be immunoprecipitated with antibodies, and the phosphorylation of dynamin can be detected with anti-phospho-tyrosine mAb using SDS/PAGE and western blotting. Furthermore, a dynamin mutant lacking its two tyrosine phosphorylation sites (Y231/597F) has recently been developed by Dr. McNiven (personal communications with Dr. Song). The effect of these mutations on dynamin recruitment and colocalization with the BCR following BCR crosslinking can be monitored. The regulation of dynamin by BCR signaling could also be indirect, and proteins that recruit dynamin to the endocytosis machinery may exert a regulatory effect on dynamin by their recruitment to the plasma membrane during BCR signaling. Amphiphysin is a protein that could potentially regulate dynamin activity during BCR signaling. Future work would test if amphiphysin is regulated by BCR signaling and whether it is responsible for recruiting dynamin to the B cell surface in response to BCR activation. Amphiphysin activity can be monitored by its cellular localization before and

after BCR activation and the effect of Src kinase inhibitors and/or phosphatase inhibitors on its cellular localization. It can also be tested whether the SH3 domain of amphiphysin, which potentially interacts with dynamin (53, 102), has a dominant negative effect on dynamin recruitment and BCR internalization.

Although BCR internalization has been shown to be dependent on the clathrin-mediated endocytic pathway (33, 140), the importance of dynamin in BCR internalization and trafficking has not been determined. The work in this thesis underscores the significance of dynamin in BCR internalization, since overexpressing a dynamin 2 mutant with a deletion of its PRD in A20 cells caused a significant reduction in BCR internalization and trafficking, implying that dynamin and its PRD are important for BCR internalization. A role for the PRD of dynamin in CME is, thus far, unreported, and a unique role for dynamin in BCR internalization is implied (91, 94). To determine how dynamin ΔPRD might disrupt BCR endocytosis, the effect of overexpressing a dynamin ΔPRD mutant on the recruitment of endogenous dynamin should be determined. Tests should also be conducted to determine the extent of oligomerization of endogenous dynamin with the ΔPRD mutant before and after BCR crosslinking by immunoprecipitation, which will provide answers to the question of whether disrupting dynamin oligomerization is the mechanism for defects caused by the ΔPRD mutant. The effect of other mutants of dynamin, which have been shown to inhibit CME, on the internalization and intracellular trafficking of the BCR, should also be tested: Notably, the mutant K44A that disrupts the GTPase function of dynamin (192) and mutations in the pleckstrin homology domain that inhibit binding to membrane lipids (91, 94).

The role of dynamin in signal transduction in lymphocytes is an emerging area of research. Studies reported by Gomez *et al*. (100), show that dynamin is important for regulating actin-dependent formation of synapses by recruiting Vav to the synapses. In addition, they suggested dynamin having a role in forming a signaling scaffold in T cells (100). Dynamin has also been known to bind to Grb2 (97, 193) and PLC-γ1 (194) two major signaling molecules in lymphocytes. Dynamin could, therefore, play a significant role in BCR signal transduction, and studies should be done to determine its role in modulating BCR signaling. The effect of dynamin on BCR signaling could be tested using shRNA-mediated knockdown of dynamin as well as over-expressing dynamin in cell lines, a system that has already been developed in our lab. Furthermore, co-immunoprecipitating dynamin or co-precipitation using GST pull down with the PRD of dynamin can be used to look for interacting signaling partners, following antigen crosslinking of the BCR.

The role of Abp1 in BCR signal transduction is yet to be elucidated. My research shows that Abp1-knockout mice have defects in the BCR-mediated activation of the ERK and JNK MAPK pathways. Further studies will shed light on how Abp1 might be regulating these pathways following BCR crosslinking. One likely mechanism is the interaction of Abp1 with HPK1 through their SH3 and PRDs respectively (119, 120, 180). Future studies should address the status of HPK1 activation following BCR crosslinking and the effects of knocking out Abp1 on the activation of HPK1. These experiments will address the functional link between Abp1 and HPK1 in B cells and the regulation of this link by BCR crosslinking. Future studies could also test the role of Abp1 in regulating kinases

upstream of JNK. The observation that Abp1-knockout mice and Bam32-knockout mice have similar defects in JNK and ERK activation as well as BCR internalization (23, 86) may provide an important link as to how Abp1 might be regulating JNK and ERK activation. Both Abp1 and Bam32 are recruited to the plasma membrane where they are tyrosine phosphorylated following BCR crosslinking (unpublished observations and (88)). Additionally, both of them interact directly with HPK1: Abp1 in T cells (119) and Bam32 in B cells (185). A known difference between the two is that Bam32, but not Abp1 is involved in regulating actin polymerization (85) (unpublished observations). Based on the similarities between Bam32- and Abp1-knockout mice, it is possible that Abp1 and Bam32 regulate the formation and stabilization of BCR signaling microdomains by modulating actin dynamics and organization, consequently influencing the activation of upstream kinases of the JNK and ERK pathways. Future studies should focus on the interactions between Bam32 and Abp1. Another interesting question is whether Bam32 regulates Abp1 function. To test this, initial studies will focus on the cellular location and phosphorylation status of Abp1 in B cells from Bam32-knockout mice before and after BCR crosslinking. The interaction between Abp1 and Bam32 can also be followed by the colocalization and FRET between these two proteins.

The JNK and ERK kinase pathways also play significant roles in antigen induced activation of the B-cell, leading to proliferation and upregulation of various activation markers (14). BCR signaling induced ERK activation leads to activation of cyclin D which is important for cell cycle progression (195), while activation of JNK activates the AP-1 transcription factor (26, 196). The role of BCR signaling in JNK activation is not

clear, but it seems to be anti-apoptotic (197) and may aid in proliferative responses to BCR signaling (185). It will be important to determine what effect Abp1 knockdown might have on B cell proliferation following BCR crosslinking.

Studies on yeast Abp1 point to a negative feedback role for Abp1 in yeast endocytosis (157), and there is a potential for this role to exist in mammals. *S. cerevisiae* has two actin-regulating kinases, Ark 1 (actin regulating kinase 1) and Prk1 (p53 regulating kinase), that are involved in shutting down actin polymerization at the sites of newly formed endocytic vesicles (198, 199). Abp1 recruits these proteins to endocytic sites in yeast (200). The mammalian homologues of these two kinases are AAK (adaptor protein complex 2 associated kinase 1) and GAK (cyclin G associated kinase) (157). Interestingly GAK is the ubiquitously expressed form of the neuronal-specific protein auxilin, a protein involved in the decoating steps of CME (201). The interaction between Abp1 and GAK in mammalian systems has not yet been reported. However, GAK recruitment was shown to coincide with that of dynamin recruitment during CME (201). It will be interesting to test whether Abp1 is able to regulate GAK's function either directly or indirectly in mammalian systems and if this will have any impact on endocytosis.

Elucidating the link between the signals generated by antigen crosslinking of the BCR and the effective processing and presentation of the antigen will enable better understanding of the complexities of the B cell mediated immune response. This will provide important insight for a number of disease conditions that are mediated wholly or

in part by defects in the humoral immune response. These conditions include

autoimmunity, hypersensitivity, certain types of cancers and immunodeficiency.

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