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Review

The pivotal position of the actin cytoskeleton in the initiation and regulation of B cell receptor activation ☆

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ABSTRACT

The actin cytoskeleton is a dynamic cellular network known for its function in cell morphology and motility. Recent studies using high resolution and real time imaging techniques have revealed that actin plays a critical role in signal transduction, primarily by modulating the dynamics and organization of membrane-associated receptors and signaling molecules. This review summarizes what we have learned so far about a regulatory niche of the actin cytoskeleton in the signal transduction of the B cell receptor (BCR). The activation of the BCR is initiated and regulated by a close coordination between the dynamics of surface BCRs and the cortical actin network. The actin cytoskeleton is involved in regulating the signaling threshold of the BCR to antigenic stimulation, the kinetics and amplification of BCR signaling activities, and the timing and kinetics of signaling downregulation. Actin exerts its regulatory function by controlling the kinetics, magnitude, subcellular location, and nature of BCR clustering and BCR signaling complex formation at every stage of signaling. The cortical actin network is remodeled by initial detachment from the plasma membrane, disassembly and subsequent reassembly into new actin structures in response to antigenic stimulation. Signaling responsive actin regulators translate BCR stimulatory and inhibitory signals into a series of actin remodeling events, which enhance signaling activation and down-regulation by modulating the lateral mobility and spatial organization of surface BCR. The mechanistic understanding of actin-mediated signaling regulation in B cells will help us explore B cell-specific manipulations of the actin cytoskeleton as treatments for B cell-mediated autoimmunity and B cell cancer. This article is part of a Special Issue entitled: Reciprocal influences between cell cytoskeleton and membrane channels, receptors and transporters. Guest Editor: Jean Claude Hervé.

Contents

1. Introduction . . . 570
2. B cell activation is initiated by BCR clustering . . . 570
3. Actin reorganization during BCR activation . . . 570
4. Roles of the actin cytoskeleton in BCR signaling . . . 572
   4.1. Actin in the initiation of BCR signaling . . . 572
   4.2. Actin-mediated amplification of BCR signaling . . . 572
   4.3. Actin in the downregulation of BCR signaling . . . 572
5. Actin in BCR internalization . . . 573
6. Signaling regulation of actin dynamics in B cells . . . 574
   6.1. Antigen-induced actin remodeling depends on BCR signaling . . . 574
   6.2. Actin regulators translate signaling into actin remodeling . . . 574
7. Perspectives and future directions . . . 576
References . . . 576

Abbreviations: Abp1, actin binding protein 1; BCR, B cell receptor; Btk, Bruton's tyrosine kinase; ERM, ezrin/radixin/moesin; ITAMs, immunoreceptor tyrosine-based activation motifs; mIg, membrane immunoglobulin; PI3K, phosphatidylinositol-3 kinase; $PI(3,4,5)P_3$, phosphatidylinositol-3,4,5-triphosphate; PLCγ2, phospholipase Cγ2; SSH, slingshot phosphatase; TLR, Toll-like receptor; N-WASP, neuronal Wiskott–Aldrich syndrome protein; WASP, Wiskott–Aldrich syndrome protein

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1. Introduction

B lymphocytes represent one of the two major branches of adaptive immunity. The primary function of B cells is to mount antibody responses upon encountering foreign antigen. In addition, B cells are critical regulators of the immune system. Apart from the immune regulatory function of antibody and antibody–antigen complexes, B cells can shape the functions of other immune cells by presenting antigen, providing co-stimulations, and secreting cytokines [1–4]. Because of their essential roles in immune protection, complex mechanisms have been evolved to regulate the functions of B cells, in order to mount the optimal antibody responses and to efficiently cooperate with other immune cells and systems during infections. While general cellular mechanisms are applicable to the regulation of B cell activation, the unique properties and functions of B cells suggest additional layers and distinct mechanisms for their regulation.

B cells originate from hematopoietic stem cells in the bone marrow. Throughout their maturation in the bone marrow and development in the periphery, B cells constantly face life–death and differentiation decisions. The fate of B cells is determined by the ability of B cells to express the B cell receptor (BCR) [5–8]. The binding of antigen to the BCR triggers B cell activation. Comprised of membrane immunoglobulin as its ligand binding domain, the BCR is capable of binding antigen as ligands in any possible form, including soluble and those on the surface of other cells [9,10]. The receptor transduces antigen binding into a series of cytoplasmic activities based on the nature of the antigen and receptor–antigen interactions [11–13]. Additionally, the BCR is responsible for capture, internalization and transport of bound antigen to the endosomal system, where antigen is transformed from its native form into a T cell recognizable form. This enables B cells to regulate T cell activation and to gain T cell stimulatory signals that are essential for B cell activation [3,4,14].

Knowledge accumulated from biochemical and molecular biology studies has defined most of the molecular components and enzymatic reactions in BCR signaling pathways, which have been extensively reviewed previously [11–13]. Recent advances in high resolution and live cell imaging, which have enabled us to examine cellular processes in multiple dimensions, have revolutionized the techniques of studying the cell biology of signal transduction and facilitated complex interpretations about signal transduction pathways. One of the major realizations from recent studies is the importance of the timing, location, and dynamics of molecular interactions in regulating signaling and the critical role of the actin cytoskeleton in controlling the spatiotemporal dynamics of molecular organization at the cell membrane [15–18]. While activation-induced actin remodeling in B cells was observed four decades ago, it is not until recently that actin-driven membrane dynamics has been identified as a key regulatory mechanism for B cell activation. This review summarizes the recent progress in our understanding of the molecular mechanisms that govern how the actin cytoskeleton regulates BCR-triggered B cell activation. This review further discusses how the newly developed ideas of actin-controlled molecular dynamics and organization at the cell membrane impact our understanding of B cell regulation.

2. B cell activation is initiated by BCR clustering

B cells use clonally specific BCRs to survey the presence of foreign antigen. The BCR consists of membrane immunoglobulin (mIg) as the antigen binding unit and non-covalently associated Igα/β heterodimer as the signaling unit. The heavy chains of mIg and Igα/β chains are all single span transmembrane proteins, and their cytoplasmic tails can extend into the cortical actin network. The length of the mIg cytoplasmic domain is dependent on its isotypes, ranging from three amino acids in mIgM and mIgD to ~28 amino acids in IgG [19], thereby extending into the cortical actin network to different depths. The BCR does not have intrinsic kinase activity but contains immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of Igα/β, thereby requiring the recruitment of signaling molecules for activating signaling [13,20].

BCR activation is triggered by receptor clustering. Multivalent soluble antigen and membrane-associated antigen, but not monovalent soluble antigen, can effectively induce receptor phosphorylation and signaling cascades [21–23]. Based on data generated from single molecule and single receptor cluster tracking in live cells using total internal reflection fluorescence microscopy with high light sensitivity [24–27], BCR clustering during signaling activation can be temporally described as occurring in four stages. In the first stage, before BCR activation, surface receptors have been shown to exist as nanometer sized clusters and/or monomers and have a relatively limited lateral mobility (an average diffusion coefficient of IgM-based BCR in naïve primary B cells at ~0.03 $\mu m^2/s$) [23,25,28] (Fig. 1A). In the second stage in response to the binding of multivalent soluble antigen or membrane-associated antigen, the lateral mobility of BCRs transiently increases (~0.05 $\mu m^2/s$ for membrane-associated antigen), enabling interactions between BCRs and/or their nano-clusters to lead to the formation of microclusters [25,29,30]. When the antigen is membrane-associated, the formation of receptor microclusters takes place in the region of contact between the B cell membrane and the antigen-associated membrane (B cell contact zone) [23,25,31–33] (Fig. 1B). The diffusion of single BCR molecules inside clusters decreases dramatically [23]. In the third stage, the B cell spreads on antigen-associated membrane, thereby driving the formation of more BCR microclusters [31]. Newly formed BCR microclusters move centripetally to one pole of the cells or to the center of the cell surface contact zone, recruiting more BCRs and colliding with each other along the way [23,31,33] (Fig. 1C). Using live cell imaging by total internal reflection fluorescence microscopy, we have shown that BCR microclusters move at an average velocity of ~0.01 μm/s to the center of the B cell contact zone [33]. In the final stage, BCR microclusters coalesce with each other, resulting in the formation of a central cluster either at one pole of the cell (for soluble antigen) or in the center of the B cell contact zone [23,33,34] (Fig. 1D). The growth and coalescence of BCR microclusters reduces the lateral mobility of BCRs (an average diffusion coefficient of 0.02 $\mu m^2/s$) [29]. B cells stimulated with soluble antigen form a protrusion at the polarized BCR central cluster [35–37], while the cell membrane at the contact zone of membrane-associated antigen stimulated B cells undergoes contraction [31,33].

BCR clustering expands and stabilizes lipid rafts surrounding the receptor, bringing lipid raft-associated Src kinases physically close to the receptor [38–40]. Antigen binding and BCR clustering potentially cause receptor conformation changes, exposing ITAMs in the cytoplasmic tails [22], which enables receptor phosphorylation. The phosphorylated ITAMs provide docking sites for kinases, like Syk, and adaptor proteins, like BLNK, allowing the formation of signaling complexes and the propagation of signaling cascades in the cytoplasm [11,13].

3. Actin reorganization during BCR activation

Like other mammalian cells, the plasma membrane of B cells is supported by a cortical actin network. Because resting lymphocytes are small compared to other types of mammalian cells and most of their cytoplasm is occupied by their nuclei, cortical actin is the predominant actin structure in the cytoplasm of B cells. The cortical actin network consists of branched actin filaments that are held together by actin binding proteins and tethered to the plasma membrane through the interaction of ezrin/radixin/moesin (ERM) proteins with transmembrane proteins [41,42]. The linking of F-actin with plasma membrane-anchored proteins through ERM proteins creates spatially distinct compartments that can transiently confine the lateral movement of membrane proteins, such as receptors with their cytoplasmic domains extending into the cortical actin network. The size of these compartments has been estimated to be about 40–300 nm in diameter [43–46]. Therefore, microcluster formation requires either BCRs on the plasma membrane to overcome this spatial confinement or a

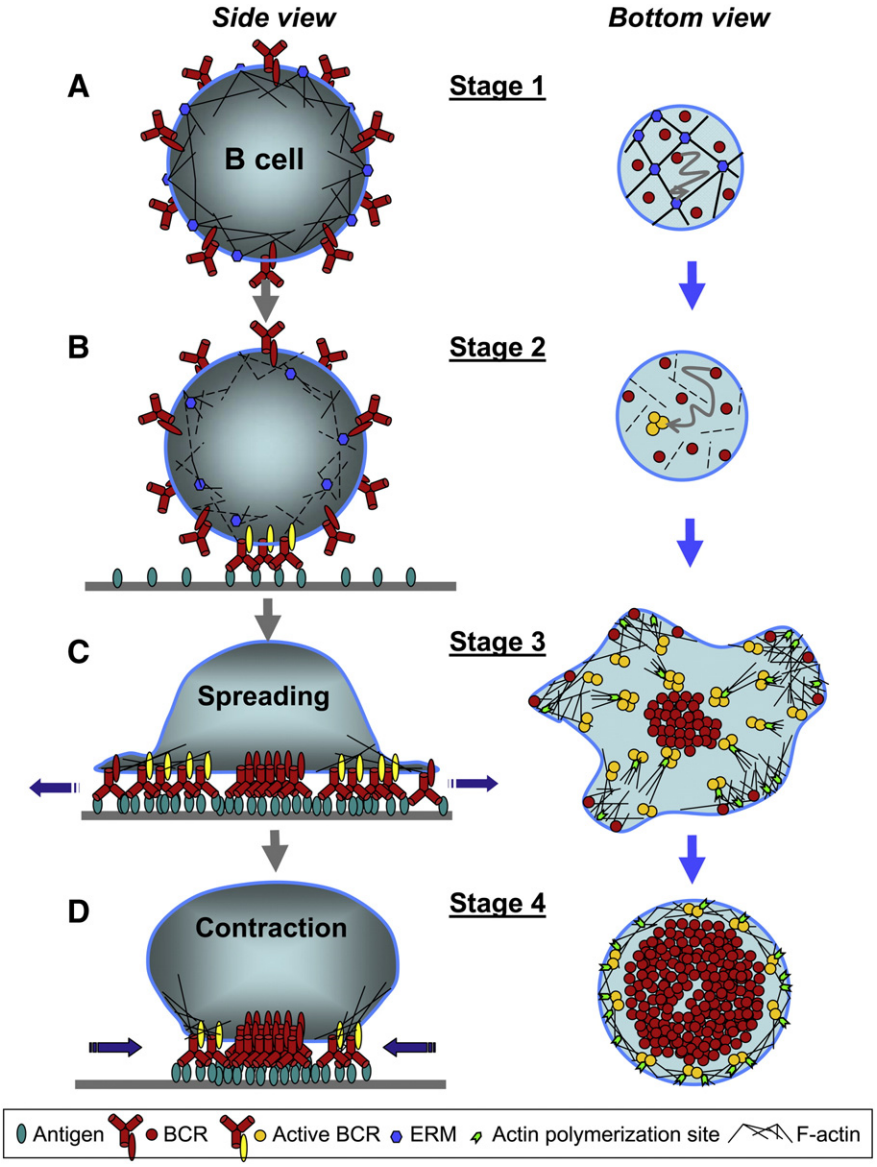

Fig. 1. The coordination of BCR and actin dynamics during signaling activation. Stage 1: In resting B cells, surface BCRs and BCR nano-clusters have a relatively limited lateral mobility, which is constrained by the cortical actin network tethered to the plasma membrane by the interaction of ERM proteins with membrane anchored proteins. (B) Stage 2: In response to the binding of membrane-associated antigen, the lateral mobility of BCRs is transiently increased, which is concurrent with the detachment of the cortical actin from the plasma membrane and a transient actin disassembly. This is followed by receptor immobilization and the formation of small clusters, triggering signaling activation. (C) Stage 3: B cell spreading on antigen-associated membrane increases the number and sizes of BCR clusters as the signaling level rises. Actin polymerizes and is accumulated at the leading edge of the spreading membrane and at BCR microclusters. (D) Stage 4: BCR microclusters merge into a central cluster at one pole of the cell while the B cell contracts. The actin cytoskeleton reorganizes away from BCR clusters to the outer rim of the BCR central cluster. The signaling activity of BCRs in the central cluster is reduced.

reduction in the degree of confinement by active remodeling of the actin cytoskeleton.

Actin remodeling following antigen engagement by the BCR was observed as early as 1970. The initial studies in 1970 reported a redistribution of the actin motor protein myosin from the cell periphery to the BCR central cluster in response to BCR cross-linking by antibody [47] and an accumulation of the actin cytoskeleton at the plasma membrane of B cells in response to mitogens [48,49]. BCR cross-linking was later found to cause the association of surface BCRs and proximal signaling components with detergent insoluble cytoskeletal fraction of cells [50–53]. These implicate a physical and functional relationship of actin with surface BCRs and BCR signaling complexes.

Recent studies using a combination of genetic tools and high resolution live cell imaging, particularly total internal reflection fluorescence microscopy, provide a much more detailed description of actin reorganization during B cell activation (Fig. 1). In resting B cells, the ERM protein ezrin couples the cortical actin network to the plasma membrane by binding to a transmembrane protein, Csk-binding protein, that resides in lipid rafts [54] (Fig. 1A). This interaction links lipid raft membrane compartments with actin-generated membrane compartments on the B cell surface. During the second stage of BCR clustering (Fig. 1B), the binding of antigen to the BCR induces a detachment of the cortical actin from the plasma membrane and a transient decrease in the overall levels of filamentous actin [30,55], indicating depolymerization of actin. The actin detachment is temporally associated with a transient dephosphorylation of ezrin [29,54], which results in the disassociation of ezrin from membrane anchor proteins [56,57]. Following the detachment of actin and transient actin depolymerization, actin polymerization becomes dominant in a polarized manner in the vicinity of BCR microclusters [30,33,35]. Upon stimulation with membrane-associated antigen, nascent BCR microclusters are colocalized with actin patches upon their emergence. The overall amount of F-actin in

the B cell contact zone increases over time during this stage of BCR clustering [33,35]. During the third stage of BCR clustering (Fig. 1C), as B cells spread, actin assembles preferentially at the leading edge of spreading cells concurrently with further BCR clustering at the cell periphery. Meanwhile, actin filaments display retrograde flow and undergo centripetal movement towards the center of the contact zone along with BCR microclusters [58]. During the last stage of BCR clustering (Fig. 1D), the levels of F-actin diminish in the center of the B cell contact zone as B cells contract and BCR microclusters coalesce into a central cluster. Upon the formation of the BCR central cluster, the actin cytoskeleton and de novo actin polymerization activity primarily localize to the outer edge of the BCR central cluster [33,35].

4. Roles of the actin cytoskeleton in BCR signaling

4.1. Actin in the initiation of BCR signaling

BCR-induced signaling is a rapid event, during which Ca^{2+} flux is triggered within seconds, thereby requiring a rapid clustering of surface BCRs. BCR clustering at the plasma membrane is dependent on the probability of receptor–receptor interactions in the two dimensional fluid plane of the plasma membrane, and is in part dictated by the lateral mobility of the receptors. BCRs in regions of the cell surface with relatively low levels of F-actin have greater lateral mobility than those in regions of the cell surface with relatively high levels of F-actin [25]. This indicates that the cortical actin network can control BCR lateral mobility and potentially create boundaries that constrain the lateral movement of surface BCRs and BCR signaling complexes. While the binding of multivalent antigen can provide BCRs with the initial force to overcome the lateral confinement, decreasing the degree of actin-generated confinement is probably critical to the fast kinetics of BCR clustering and the rapid amplification of signaling activation. Depolymerization of the actin cytoskeleton by treatment with latrunculin increases the lateral mobility of BCRs, which leads to slow but spontaneous BCR clustering and signaling activation in the absence of antigen [25,28,35]. In contrast, stabilization of filamentous actin by treatment with a saturated concentration of jasplakinolide prohibits BCR clustering and signaling activation even in the presence of multivalent antigen and membrane-associated antigen [35]. These results support the model that in resting and deactivated B cells the cortical actin network can inhibit BCR clustering by limiting receptor lateral mobility, and that dynamic reorganization of cortical actin can control the kinetics and magnitude of BCR clustering, consequently regulating the levels of both basal and activated signaling. The transient dephosphorylation of ezrin and actin depolymerization induced by BCR-antigen interaction are concurrent with a transient increase in the lateral movement of surface BCRs [25,29,30]. Manipulations of ezrin and moesin by siRNA knockdown, dominant negative and constitutive active mutants significantly affect the magnitude of BCR clustering [29]. These studies indicate that the detachment and disassembly of the cortical actin, which provides more lateral freedom for surface BCRs, is a critical step for the initiation of BCR clustering and signaling activation (Fig. 1A–B).

4.2. Actin-mediated amplification of BCR signaling

The kinetics and magnitude of BCR clustering show a close correlation with signaling. BCRs with relatively high affinity to an antigen induce faster and greater BCR clustering and signaling than those with relatively low affinity to the antigen [26]. The colligation of the BCR with the inhibitory coreceptor of B cells FcγRIIB by immune complexes inhibits BCR clustering and signaling [59–61]. The actin cytoskeleton has been shown to enhance BCR signaling by increasing the kinetics and magnitude of BCR clustering through two potential mechanisms: 1) modulating the mobility and directionality of the lateral movement of BCRs and BCR nano-clusters on the plasma membrane and 2) controlling B cell morphology. While the removal of lateral confinement generated by cortical actin can induce initial BCR clustering, the rapid formation and growth of BCR clusters requires actin reassembly and reorganization. This is shown by our finding that the spontaneous formation of BCR microclusters induced by latrunculin-mediated actin depolymerization is much slower than antigen-induced BCR aggregation. The slow BCR aggregation is associated with a slow increase in BCR signaling [35].

As discussed above, actin remodeling follows a distinct sequence in response to antigen binding to the BCR. The reassembly of actin at the outer edge of the B cell membrane contacting an antigen-associated surface is likely responsible for B cell spreading. Cell spreading expands the contact area of B cells with antigen-associated membrane, consequently increasing the number of antigen-engaged BCRs [31]. Inhibition of B cell spreading by BCR–FcγRIIB colligation and by gene deletion of the actin nucleation promoting factor Wiskott–Aldrich syndrome protein (WASP) significantly decreases the magnitude of BCR clustering and signaling [33,59]. Actin polymerization occurring at BCR microclusters [33,35] can push the clusters to one pole of the cell. Along the way, BCR microclusters potentially encounter and coalesce with other clusters, resulting in the growth of BCR clusters and the formation of BCR central clusters. Therefore, actin can amplify BCR signaling by increasing the antigen–BCR interaction and enhancing BCR cluster formation.

An early observation that antigenic activation influences the physical relationship of both BCR and signaling molecules with the cytoskeleton [50–53] suggests another mechanism for actin to facilitate signaling: the actin cytoskeleton with actin adaptor proteins potentially serves as a scaffold to recruit and position signaling molecules physically close to the BCR. The reassembly of the actin cytoskeleton and the re-association of active ezrin with the lipid raft-associated-Csk binding protein after the initial detachment and disassembly can compartmentalize membrane proteins for receptor clustering and receptor signaling complex formation.

4.3. Actin in the downregulation of BCR signaling

The actin cytoskeleton plays an important role in the negative regulation of BCR activation by inducing the coalescence of BCR microclusters into the central cluster and by mediating B cell contraction. Our recent studies [33] have established that there is a two phase relationship of BCR clustering and B cell morphological changes with BCR signaling in B cells interacting with membrane-associated antigen. In the first phase (0–5 min), the signaling activity of BCR microclusters, measured by tyrosine phosphorylation, is positively correlated with the number of BCRs in individual microclusters as well as with the extent of B cell spreading. In the second phase (5–10 min), the signaling activity of BCR clusters is inversely related with the number of BCRs in clusters. Upon reaching a certain size, the signaling activity of growing BCR clusters decreases, even as the number of BCRs in individual clusters continues to increase, resulting in signaling attenuation at the cell surface. This is further supported by the finding that surface BCRs transiently interact with lipid rafts and lipid raft-associated Src kinase Lyn, measured by fluorescence resonance energy transfer, within a time window equivalent to the first phase [38]. The transition from the first phase to the second phase is concurrent with the B cell morphological change from spreading to contraction [33]. Upon formation of the BCR central cluster, the surface (measured by TIRFM) and cellular (evaluated by flow cytometry) levels of tyrosine phosphorylation return to the basal levels [33]. B cell-specific gene deletion of SHIP-1 not only enhances BCR signaling and delays signaling attenuation, but also blocks the transition of BCR clustering from the first phase to the second phase as well as B cell morphological change from spreading to contraction. BCR clusters in SHIP-1 knockout B cells remain smaller in size and have higher signaling levels than those in wt B cells [33]. BCR clusters induced by latrunculin treatment, which are also unable to merge into a central cluster due to a lack of actin reassembly, show no sign of signaling

attenuation up to 30 min [35]. These data support the notion that the actin-driven formation of the BCR central cluster is a mechanism for signaling attenuation.

The inhibition of continuous growth and coalescence of BCR microclusters in SHIP-1 knockout B cells is accompanied by an increase in F-actin accumulation at the center of the BCR contact zone, an enhanced colocalization of F-actin with BCR microclusters, a decrease in the centripetal movement of BCR microclusters, and a delay in B cell contraction [33]. While these findings do not fully uncover the underlying mechanisms, they shed light on how the actin cytoskeleton potentially contributes to the signaling attenuation process. First, actin remodeling drives the centripetal movement of BCR clusters and facilitates the merger of BCR microclusters, which is potentially the beginning of signaling attenuation. Second, the reorganization of F-actin into regions spatially segregated from BCR clusters may be required for the formation of the BCR central cluster, by providing free space for BCR microclusters to merge. Third, actin-mediated cell contraction is probably important for bringing growing BCR clusters to a central location for merging. It is also reasonable to speculate that continuous local actin treadmilling found at the outer edge of the BCR central cluster [35] can build a lateral mobility boundary against BCRs moving away from the central cluster and thus inhibit the potential re-ignition of signaling.

The observation that BCRs in microclusters and in large central clusters exhibit different levels of signaling activity raises the question whether BCRs in emerging BCR microclusters and in BCR central clusters have different conformations and interact with each other in distinct manners. This idea is supported by recent studies which show that BCRs pre-exist as nanometer size clusters in resting B cells before antigen binding and actin reorganization. It has been suggested that the conformation and organization of BCRs in nano-clusters may inhibit BCR activation by physically blocking the accessibility of signaling molecules to the cytoplasmic tails of the BCR [62,63]. The studies by Tolar et al. suggest that antigen binding not only causes BCR clustering, but also conformational changes of the BCR, which expose ITAMs for the binding of signaling molecules [22]. The signaling inhibitory properties of the BCR central cluster implicate a possibility that BCRs in the central cluster interact with each other in a manner similar to those in resting B cells. Since both BCR nano-clusters in resting B cells and BCR central clusters in antigen-stimulated B cells are formed under the constraints of actin-based lateral boundaries, this suggests a role for the actin cytoskeleton in making switches between the inhibitory and activating states of BCRs.

5. Actin in BCR internalization

In addition to the induction of BCR clustering and signaling activation, antigen binding also triggers rapid BCR internalization [64–67]. BCR internalization leads to antigen uptake into the endosomal system, where protein antigens are fragmented and assembled with MHC class II molecules. Antigenic peptides are then presented by MHC class II at the B cell surface for T cell recognition [68,69]. The interaction of B cells with T helper cells through antigen presentation induces the formation of immunological synapses at the interface between B and T cells. Signals transduced through the synapse activate T cells, and activated T cells in turn provide B cells with stimulatory signals. The signals provided by T helper cells are essential for B cells to differentiate into memory subsets of B cells (see review, [3,70,71]). We have previously demonstrated that antigen-induced BCR internalization, but not the constitutive internalization of the receptor in the absence of antigen, requires actin remodeling [72]. BCR internalization has been shown as clathrin-mediated [65,73,74] and dynamin dependent [75,76]. Inhibition of actin remodeling results in the accumulation of antigen bound BCRs in long invaginated clathrin coated pits [72]. We have identified actin binding protein 1 (Abp1, HIP-55 or SH3P7) as a linker between the actin cytoskeleton and the endocytosis machinery, based on its ability to simultaneously bind F-actin and the proline-rich domain of dynamin 2 and the inhibitory effect of its gene knockout on BCR internalization [77]. Based on these data, the current model is that Abp1 recruits the actin cytoskeleton to the dynamin-restricted neck of clathrin coated pits, where actin generates a force, leading to the detachment of clathrin-coated vesicles from the plasma membrane (Fig. 2). Since the constitutive internalization of the BCR is not dependent on the actin cytoskeleton, a primary role of actin is to accelerate antigen uptake. The BCR can also be internalized via a clathrin-independent pathway, since clathrin deficiency decreases BCR internalization by 70% and not 100% [73]. Interestingly, the remaining 30% clathrin-independent BCR internalization was also found to depend on the actin cytoskeleton. How actin contributes to this BCR internalization pathway is not clear.

In addition to facilitating the membrane fission of clathrin-coated pits, actin and its motor non-muscle myosin II have recently been shown to be required for pulling membrane-associated antigen for internalization [78]. The actin cytoskeleton may also be involved in concentrating BCR–antigen complexes into clathrin-coated pits and in generating membrane curvature for the formation of clathrin-coated pits during BCR internalization. While the role of the actin cytoskeleton in generating BCR containing clathrin-coated pits has not been fully examined, the coordination of actin with Bar domain-containing proteins in membrane deformation has been extensively studied (see reviews, [79–81]). It is logical to speculate that BCR clustering facilitates BCR internalization. This hypothesis is supported by the finding that blockage of BCR clustering by the actin stabilizer jasplakinolide inhibits BCR internalization [72]. However, whether BCR internalization is directly regulated by the kinetics and magnitude of BCR clustering and whether it requires the formation of the BCR central cluster remain to be determined.

Apart from the initiation of antigen processing and presentation, BCR internalization can regulate receptor signaling function [82]. Chaturvedi et al. show that the BCR forms different signaling complexes at different cellular locations [76]. Phosphorylated Lyn, Syk and Erk are recruited to both the cell surface and endosomes, while the MAP kinases p38 and Jnk are recruited to endosomes but not the plasma membrane in response to antigenic stimulation. Inhibition of BCR internalization by the dynamin inhibitor dynasore [76] or by actin depolymerization [55] increases the phosphorylation of MAP kinases, MEK1 and Erk but decreases the phosphorylation of AKT, consequently deregulating the activation of downstream transcription factors. Our recent studies found that inhibition of BCR internalization by N-WASP conditional knockout increases the level of phosphorylated Btk but decreases the level of phosphorylated SHIP-1 at the B cell surface (Liu et al., unpublished data). While dynamin inhibition, actin depolymerization, and N-WASP gene deletion may alter BCR signaling independent of receptor internalization, these data collectively support the notion that actin-dependent

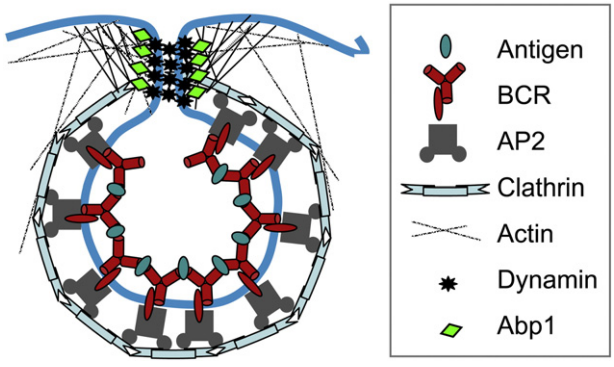

Fig. 2. Actin is required for BCR internalization. The actin cytoskeleton is essential for the fission step of clathrin-mediated endocytosis of the BCR. F-actin is linked to the dynamin-restricted neck of clathrin-coated pit by Apb1 that can simultaneously bind to F-actin and dynamin.

BCR internalization can regulate BCR signaling by modulating the cellular localization of the receptor. Because BCR internalization of membrane-associated antigen has not been well studied, it is unclear whether endocytosis occurs at BCR central clusters where receptors reduce their signaling activities or/and at signaling active BCR microclusters. In either case, BCR internalization is likely to cause a transition of the receptor into a different signaling stage.

6. Signaling regulation of actin dynamics in B cells

6.1. Antigen-induced actin remodeling depends on BCR signaling

Actin remodeling during BCR activation is triggered by BCR signaling. The biochemical cascades induced by BCR-antigen interaction have been extensively studied (see review [11,13,83–85]). Here, we briefly summarize the BCR signaling pathways that have been shown to be involved in actin reorganization. Antigen-triggered BCR clustering first activates Src and Syk kinases in lipid rafts, which induce the recruitment and phosphorylation of adaptor proteins, like BLNK and Grb2. Through these kinases and adaptor proteins, the BCR activates phospholipase Cγ2 (PLCγ2), phosphatidylinositol-3 kinase (PI3K), the guanine nucleotide exchange factor Vav and Ras-GTPase. PLCγ2 and PI3K activate phosphatidylinositide metabolism, $PI(3,4,5)P_3$-dependent Btk activation, and IP_3-dependent calcium flux. BCR activation also induces the phosphorylation of the costimulatory molecule CD19, and complement-opsonized antigen can further enhance CD19 phosphorylation. The phosphorylated CD19 enhances the activation of PI3K, Vav, and Btk [13,86–89]. Phosphorylated Vav activates Rho family GTPases, Cdc42 and Rac1/2 [11,90,91]. Activated Src kinases, such as Lyn, also activate inhibitory phosphatases, including the tyrosine phosphatase SHP and the phosphatidylinositol-5 phosphatase SHIP-1 that convert $PI(3,4,5)P_3$ into $PI(3,4)P_2$, which together downregulate BCR signaling [92–97]. Rho-family GTPases, phosphatidylinositol lipids and calcium are the major regulators of the actin cytoskeleton (see reviews [98–100]).

Actin-dependent early events of BCR activation, including BCR clustering and B cell morphological changes, have been shown to require PLCγ2, Btk, Vav, the Rho family GTPases Rac and Rap, as well as the BCR costimulatory molecule CD19. Deletion of any one of these genes results in impaired BCR clustering and B cell spreading in mouse primary B cells and/or B cell lines [32,101,102]. In contrast, the inhibitory phosphatase SHIP-1 was found to inhibit B cell spreading, but promote B cell contraction and coalescence of BCR microclusters into the central cluster. B cell-specific gene deletion of SHIP-1 results in an increase in B cell spreading, a delay in B cell contraction, and an inhibition in the growth and merger of BCR microclusters [33]. We have previously demonstrated that Btk is a major signaling molecule that is responsible for activating actin remodeling for B cell spreading and BCR clustering. These activities are dramatically decreased in Btk-deficient B cells. SHIP-1 inhibits B cell spreading and promotes the formation of the BCR central cluster by suppressing Btk activation. Treatment with a Btk inhibitor can correct the phenotype of SHIP-1 knockout B cells [33]. For actin-dependent BCR internalization, Btk, Vav1 and/or Vav3 but not Vav2, and Rac1/2 are required. Rac1/2 is downstream of Btk and Vav, and Btk activates Rac by inducing the phosphorylation of Vav [64,75,103,104]. All these findings show that the actin cytoskeleton is regulated by a large host of cell function specific factors, which enables it to finely tune BCR signaling.

6.2. Actin regulators translate signaling into actin remodeling

Signaling molecules regulate actin dynamics and organization by controlling the activity of a group of actin regulators that mediate the attachment of the actin cytoskeleton to the plasma membrane and govern the reassembly and disassembly of the actin cytoskeleton. The detachment of the cortical actin network from the plasma membrane, the earliest event of signaling-induced actin remodeling, is likely to be induced by a transient dephosphorylation of the conserved threonine 567 of ezrin [29,54], which dissociates ezrin from lipid raft-associated transmembrane proteins and returns ezrin to a closed inactive conformation [41,42,57] (Fig. 3A). The activation of ezrin and the maintenance of its open and active conformation require the binding of $PI(4,5)P_2$ to its N-terminal domain and the phosphorylation of T567 in its C-terminal domain [105]. The other members of ERM proteins, like moesin, work cooperatively with ezrin in mediating the attachment and detachment of the actin cytoskeleton to the B cell membrane, as demonstrated by the fact that double knockdown of ezrin and moesin has a stronger effect on B cell spreading and BCR clustering as compared to single knockdown of either protein [29]. ERM proteins have been shown to share both sequence homology and activation mechanisms with each other [41,42,57]. BCR-induced activation of PLCγ2, which converts $PI(4,5)P_2$ to IP_3, has been suggested to act as a trigger for ezrin inactivation [106]. However, Vav1 and Vav2 appear to have an even greater involvement since Vav1/2 double knockout has stronger inhibitory effects on BCR-induced disassociation of ezrin from the cell surface than PLCγ2 gene knockout [29] (Fig. 3A). This suggests a role for the Rho-family GTPases, Cdc42 and Rac1/2 that are downstream of Vav, in ezrin dephosphorylation. In contrast to this observation, constitutively active Cdc42 has been shown to induce ezrin phosphorylation by activating Cdc42 binding protein kinase during the filopodia formation when over-expressed in a fibroblast cell line [107]. While ezrin dephosphorylation can be induced by different upstream signals and mechanisms in different cellular processes and cell types, how Vav contributes to BCR-induced ezrin dephosphorylation and which phosphatases are responsible for ezrin dephosphorylation in B cells remain elusive.

BCR-induced dephosphorylation of ezrin is concomitant with a transient decrease in the global level of F-actin indicating actin disassembly, and an equally transient increase in the lateral mobility of surface BCRs indicating a reduction in the spatial confinement of BCR at the cell membrane [29,54]. The disassembly of actin not only lowers the restrictions to BCR lateral movement, but also increases pools of free G-actin required for actin reassembly. Cofilin-mediated actin severing and depolymerization have been shown to be required for BCR induced actin disassembly as well as for the subsequent reassembly [30] (Fig. 3B). Cofilin, a member of the actin-depolymerizing factor protein family, can sever actin filaments by distorting the helical twist of filaments and depolymerize actin filaments by promoting the disassociation of ADP-bound G-actin from the minus end of filaments [99,108]. The F-actin binding ability of cofilin is inhibited by the phosphorylation at the serine 3 residue [99,108]. This phosphorylation is generally mediated by LIM domain kinase [109] and is dephosphorylated by Slingshot phosphatase (SSH). SSH is inhibited by 14-3-3 protein-mediated sequestration [110]. In B cells, BCR activation induced by both soluble and membrane-associated antigen triggers dephosphorylation of cofilin, activating its severing function [30,35]. Dephosphorylated cofilin is recruited to the vicinity of BCR microclusters and central clusters but not to the plasma membrane, indicated by the fact that cofilin was not detected by total internal reflection fluorescence microscopy [35]. This suggests that cofilin severs and depolymerizes F-actin from the cytoplasmic side of the cortical actin network. The dephosphorylation of cofilin and its actin severing activity are dependent on SSH and Rap activation, suggesting that BCR-induced activation of Rap can release SSH from 14-3-3 protein sequestering, consequently dephosphorylating and activating cofilin [30] (Fig. 3B). However, cofilin-mediated activity seems to be independent of the ERM protein ezrin [30], indicating that ezrin dephosphorylation-induced membrane detachment of the actin cytoskeleton from the plasma membrane does not trigger cofilin activation. BCR activation also induces the recruitment of another actin severing protein, gelsolin, to the B cell periphery [35], even though the contribution of gelsolin to BCR-induced actin severing has not been well defined. The F-actin binding activity of gelsolin requires its binding with calcium and phosphatidylinositol lipids [100,111]. This raises the

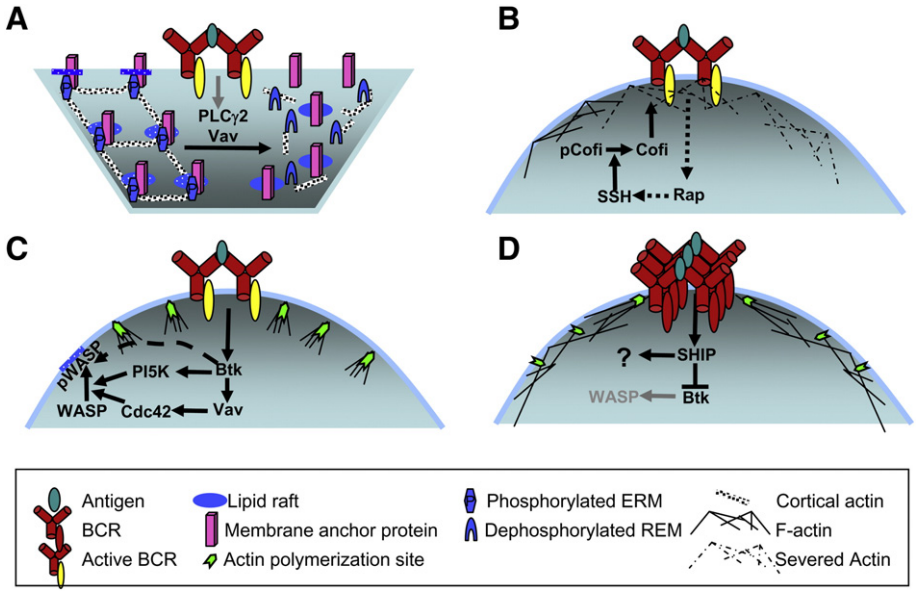

Fig. 3. Signaling regulation of actin dynamics through actin regulators. (A) BCR signaling triggers ERM dephosphorylation, which leads to the disassociation of ezrin from membrane anchor proteins and the detachment of the cortical actin from the plasma membrane. (B) BCR signaling induces the dephosphorylation of cofilin, which activates its actin severing activity. Cofilin dephosphorylation requires SSH and depends on Rap activation. (C) The BCR downstream signaling molecule Btk activates WASP through activating Vav and PI5K as well as WASP phosphorylation. WASP mediates actin reassembly that drives B cell spreading and the movement of BCR microclusters at the B cell surface. (D) The activation of the inhibitory phosphatase SHIP-1, induced by the BCR, inhibits WASP activation and induces B cell contraction and the formation of the BCR central cluster.

possibility of increased gelsolin activation and actin severing activity in response to BCR-induced calcium flux and phosphatidylinositide production.

WASP is well known for its function in actin reassembly following BCR-induced actin disassembly (Fig. 3C). The importance of this actin nucleation promoting factor in B cells is demonstrated by complex immune disorders, including immune deficiency, autoimmune diseases, and lymphatic cancers, caused by WASP deficiency [112–114] and B cell autoimmunity resulting from B cell-specific WASP gene deletion [115,116]. As the hematopoietic-specific member of the WASP-family proteins, WASP induces actin polymerization on existing filaments by binding to and activating the F-actin-associated actin nucleation factor Arp2/3 [117,118]. The molecular mechanism for WASP activation has been well defined. In the absence of stimuli, WASP maintains a closed autoinhibitory conformation. The conformational change from closed to open requires the binding of GTP-bound Cdc42 or Rac to the GTPase binding domain and $PI(4,5)P_2$ to the Pleckstrin homology domain of WASP [119]. The open conformation allows the phosphorylation of its conserved tyrosine and serines, which further stabilizes the open and active conformation of WASP [120,121]. We have demonstrated that Btk is the main upstream signaling molecule responsible for WASP activation. Btk activates WASP by inducing the phosphorylation of Vav that activates Cdc42 or Rac, an increase in $PI(4,5)P_2$ production by activating phosphatidylinositol-5 kinase (PI5K), and the phosphorylation of WASP [64] (Fig. 3C). Phosphorylated WASP localizes at BCR microclusters and is enriched at the leading edge of the spreading membrane similar to F-actin distribution. Even though the phosphorylation of WASP is significantly decreased in Btk-deficient mouse B cells [64], whether Btk phosphorylates WASP directly or indirectly by activating another kinase is not known. Our finding that WASP gene knockout has only modest effects on actin accumulation, B cell spreading, BCR clustering [33] and BCR internalization in mouse B cells [116] suggests that additional actin nucleation promoting factors are involved in the actin reassembly process. The stronger effects of WASP and N-WASP double knockout (with N-WASP as B cell-specific knockout) on B cell spreading on a surface coated with the cell adhesion protein CD44, peripheral B cell development, and B cell-mediated antibody responses [122] suggest an involvement of the ubiquitously expressed WASP homologue, N-WASP. Our recent studies show that antigen-induced actin reassembly, B cell spreading, BCR clustering and signaling are almost abolished in B cells from this double knockout mouse (Liu et al., unpublished data), further supporting this notion. These collectively suggest that WASP and N-WASP are the major players in actin reassembly following antigen-induced disassembly. Actin reassembly and reorganization mediated by WASP and N-WASP are responsible for B cell spreading and BCR clustering (Fig. 3C).

Following their formation, BCR microclusters move centripetally and coalesce into a central cluster in B cells stimulated with either soluble or membrane-associated antigen. In B cells interacting with membrane-associated antigen, this process is associated with a morphological transition of B cells from spreading to contraction. B cell contraction and BCR microcluster merger, but not B cell spreading and BCR clustering, are inhibited by B cell-specific SHIP-1 knockout [33], indicating SHIP-1 activation as an upstream signal for this process (Fig. 3D). We have demonstrated that SHIP-1 inhibits WASP activation by suppressing Btk activation [33]. SHIP-1 inhibits Btk as well as PLCγ2 and Akt by converting their plasma membrane docking site, $PI(3,4,5)P_2$ into $PI(3,4)P_2$ [96] (Fig. 3D). This may act as a potential mechanism to induce a transition of actin dynamics, from being located preferentially at BCR microclusters to being localized to the outer edge of the BCR central cluster. The decrease in WASP activation is concurrent with the formation of the BCR central cluster and the decrease of F-actin levels at the BCR central cluster [33]. Therefore, turning off WASP may help to create an actin-free space for gathering a large number of surface BCRs and BCR microclusters. Surprisingly, the overall level of cellular F-actin does not appear to decrease even as the level of F-actin decreases at the BCR central clusters [30]. This suggests that F-actin is spatially reorganized through rapid actin depolymerization and polymerization and/or through binding to different F-actin binding proteins at different cellular locations. It is possible that F-actin is re-localized from the cell surface to the neck of clathrin-coated pits by Abp1 and to BCR containing vesicles by N-WASP, for BCR internalization [77,123,124]. However, actin regulators, which are activated by SHIP-1 to mediate B cell contraction and to facilitate BCR microcluster coalescence into the central cluster, have not yet been identified.

7. Perspectives and future directions

The actin cytoskeleton in the cell is uniquely positioned to be a critical regulator of cell signaling. Various signaling pathways influence the physical reorganization of the actin cytoskeleton, while the dynamics of actin can in turn influence further signal transduction events. The actin cytoskeleton displays a high degree of plasticity with a wide range of changes in structure and organization in response to changes in chemical composition of its surroundings. Further, a host of actin regulators can fine tune the activity and organization of the actin cytoskeleton in response to signaling cues from inside as well as outside the cell. Therefore actin in the cell can act as a perfect means to couple chemical signaling with mechanical responses of the cell, such as movement of the cell periphery or dynamics of surface proteins, which are essential during B cell activation.

The actin cytoskeleton clearly plays critical roles in both the initiation and regulation of BCR signaling. The cortical actin network can modulate the signaling threshold by varying its organization beneath the plasma membrane, which constrains receptor lateral mobility in the plasma membrane and impacts the probability of BCR cluster formation. By virtue of its extreme plasticity, the actin cytoskeleton can enhance BCR activation by undergoing rapid disassembly and reassembly. Transient disassembly of cortical actin enhances the mobility of receptors in the plane of the plasma membrane and enables receptor–receptor interactions by dissolving confinement barriers. Reassembly of actin allows large scale morphological changes during cell spreading to maximize the area of contact between B cells and the antigen presenting surface. Most interestingly, actin has been found to be involved in downregulation of signaling at the B cell surface by facilitating the coalescence of BCR microclusters into a central cluster and by increasing the rate of BCR internalization. This actin-mediated signaling regulation is facilitated by close interactions between BCR-induced signaling pathways and actin regulators. The actin regulators that are activated by positive signaling, like Btk, and negative signaling, like SHIP-1, are able to generate feedback amplifications for both signaling activation and downregulation through different modes of actin remodeling. It is expected that actin regulators downstream of positive and negative signals work together to enable the switch from cell spreading mode to cell contraction mode and to facilitate the transition from signaling activation to attenuation. The downregulation of BCR signaling after initial activation is critical for maintaining B cell tolerance. However, actin regulators downstream of SHIP-1 have not yet been identified. The mechanisms underlying this transition remain elusive. Identification of actin regulators that are responsible for actin-facilitated signaling downregulation and delineation of the mechanisms for the transition from BCR signaling activation to attenuation should provide a means for manipulating B cell activation.

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