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Actin Reorganization Is Required for the Formation of Polarized B Cell Receptor Signalosomes in Response to Both Soluble and Membrane-Associated Antigens

Chaohong Liu,* Heather Miller,* Gregory Orlowski,† Haiyin Hang,‡ Arpita Upadhyaya,§ and Wenxia Song*

B cells encounter both soluble Ag (sAg) and membrane-associated Ag (mAg) in the secondary lymphoid tissue, yet how the physical form of Ag modulates B cell activation remains unclear. This study compares actin reorganization and its role in BCR signalosome formation in mAg- and sAg-stimulated B cells. Both mAg and sAg induce F-actin accumulation and actin polymerization at BCR microclusters and at the outer rim of BCR central clusters, but the kinetics and magnitude of F-actin accumulation in mAg-stimulated B cells are greater than those in sAg-stimulated B cells. Accordingly, the actin regulatory factors, cofilin and gelsolin, are recruited to BCR clusters in both mAg- and sAg-stimulated B cells but with different kinetics and patterns of cellular redistribution. Inhibition of actin reorganization by stabilizing F-actin inhibits BCR clustering and tyrosine phosphorylation induced by both forms of Ag. Depolymerization of F-actin leads to unpolarized microclustering of BCRs and tyrosine phosphorylation in BCR microclusters without mAg and sAg, but with much slower kinetics than those induced by Ag. Therefore, actin reorganization, mediated via both polymerization and depolymerization, is required for the formation of BCR signalosomes in response to both mAg and sAg. The Journal of Immunology, 2012, 188: 3237–3246.

Mature B cells encounter their cognate Ag when they circulate through the secondary lymphoid organs, where they are attracted into follicles through a CXCL13 gradient generated by follicular dendritic cells and fibroblastic reticular cells (1–3). The binding of Ag to the clonally specific BCR initiates B cell activation. In contrast to the TCR, the BCR can bind Ag in diverse forms. Two broad forms of Ag that B cells commonly encounter in the secondary lymphoid organs are soluble Ag (sAg) and membrane-associated Ag (mAg). Recent studies using multiphoton intravital microscopy have shown that sAgs with relatively small molecular mass (≤ 60 kDa), when injected s.c., rapidly reach B cell follicles in the draining lymph node, probably via gaps in the sinus floor (4) or the collagen-rich conduit network (5, 6). The conduits, which are secreted by fibroblastic reticular cells, passively deliver small molecules, such as Ag and the B cell chemokine CXCL13 (5, 6). Macrophages lining the lymph node subcapsular sinus capture and transport particulate Ag and immune complexes to follicles (7–9). Dendritic cells in the medullary sinus capture Ag and transport Ag to the B cell compartment. Moreover, follicular dendritic cells can capture sAgs in complexes with complement factors or Ab and retain them for long-term presentation (5, 10, 11). Ag captured by macrophages and dendritic cells is presented to B cells in a membrane-associated form.

Although in vitro B cells readily bind both sAg and mAg, how B cells are activated by different forms of Ag in vivo is not completely clear. Ag binding to the BCR can induce signaling cascades as well as Ag uptake, processing, and presentation. The cellular activities triggered by BCR–Ag interaction and signals from the microenvironment of B cells collectively determine the fate of B cells. The activation of B cells by both sAg and mAg has been studied extensively in vitro (12–16). Early studies, starting from the 1970s, mainly focused on sAg. These studies show that multivalent but not monovalent sAg induces the aggregation of surface BCRs into a central cluster at one pole of a B cell, which was called a BCR cap (17–19). Later, Cheng et al. (20) found that aggregated BCRs associated with lipid rafts, where Src kinases, such as Lyn, are constitutively present. The phosphorylation of the ITAMs in the cytoplasmic tails of the BCR by Src kinases leads to the activation of signaling cascades (15, 21). The requirement of multivalent sAg for BCR activation indicates the importance of Ag-induced BCR aggregation in BCR activation.

Recent studies using total internal reflection fluorescence microscopy (TIRFM) provide high-resolution live cell images of BCR signaling initiation events at the surface of B cells interacting with Ag tethered to planar lipid bilayers. Ag tethered to lipid bilayers is a widely used model for mAg. The binding of mAg, even monovalent mAg, to the BCR induces conformational changes and self-aggregation of surface BCRs (22, 23). The newly formed BCR microclusters reside in lipid rafts (24) and recruit signaling molecules, including Lyn, Syk (23), PLCγ2, Vav (25), and...
and costimulatory receptor CD19 (26). BCR microclusters increase in size over time by trapping more BCRs and eventually merge together to form a central cluster at the surface zone contacting Ag-tethered membrane, similar to the BCR cap. When the adhesion molecule ICAM is present on Ag-tethered membranes, the BCR central cluster is surrounded by ICAM, forming a surface macromolecular structure similar to the immunological synapse between T cells and APCs (27). Unlike T cells, ICAM facilitates but is not required for the formation of BCR signalosomes in response to mAg (27, 28).

Concurrent with BCR aggregation, mAg also induces B cell spreading and contraction on Ag-tethered membranes (29). Such morphological changes have been shown to increase Ag gathering and BCR aggregation at the B cell surface. B cell morphological changes and amplified BCR aggregation are dependent on BCR signaling mediated by CD19, Bruton’s tyrosine kinase (Btk), Vav, and Rac2 (25, 26, 30), suggesting that BCR proximal signaling induced by mAg provides a positive feedback for the BCR signalosome formation. Similar to the B cell response to mAg, morphological changes have also been observed in sAg-stimulated B cells, where these B cells form membrane protrusions in the vicinity of the BCR central cluster (19).

The actin cytoskeleton has been shown to be a critical factor in B cell activation (31). It was first observed more than 30 y ago that sAg induced F-actin accumulation at the BCR cap (32–35). A link between the actin cytoskeleton and BCR signaling was first suggested by Baeker et al. (36), who showed that disrupting the actin cytoskeleton by cytochalasin D induced Ca2+ flux. Later studies reported that perturbation of the actin cytoskeleton delayed the attenuation of protein tyrosine phosphorylation (37) and enhanced activation of the MAPK, ERK, and the transcription factors, SRF, NF-κB, and NFAT, induced by sAg (38). The underlying mechanism by which the actin cytoskeleton contributes to sAg-induced BCR activation remains to be elucidated. Recent studies using TIRFM show that the cortical actin network secured to the B cell membrane by ezrin controls the lateral movement of surface BCRs (39, 40). Disrupting the cortical actin network inhibits surface BCR aggregation and B cell spreading in response to mAg (29). However, in the absence of mAg, perturbation of cortical actin increases the lateral diffusion of surface BCRs and induces spontaneous signaling (39, 41). Our recent study demonstrates that the BCR proximal signaling molecule Btk and its negative regulator SHIP-1 positively and negatively regulate actin reorganization induced by mAg, respectively. Btk activates actin nucleation promoting factor, Wiskott–Aldrich symptom protein (WASP), promoting BCR aggregation and B cell spreading (42, 43). In contrast, SHIP-1 inhibits the activation of Btk and WASP, promoting the contraction of B cells and the merger of BCR microclusters into a central cluster (43). These data collectively reveal a critical role for the actin cytoskeleton in BCR activation induced by mAg. It remains unclear whether the actin cytoskeleton plays a similar role in sAg-induced BCR activation.

In this study, we compared the role of the actin cytoskeleton in BCR activation induced by sAg and mAg. Our results show that both mAg and sAg induce F-actin accumulation, actin polymerization, and the recruitment of the actin regulatory factors to BCR clusters, even though the kinetics and magnitude of these events are different in the two cases. Inhibition of actin reorganization by stabilizing F-actin blocks BCR clustering and tyrosine phosphorylation induced by both forms of Ag. Depolymerization of F-actin leads to unpolarized microclustering of BCRs and tyrosine phosphorylation in BCR microcluster without sAg and mAg. Our results demonstrate a similar role for the actin cytoskeleton in the initiation of BCR activation induced by mAg and sAg.

Materials and Methods

Mice and cells

Mice (CBA/CaJ) (The Jackson Laboratory, Bar Harbor, ME) of 6–12 wk old were used. To purify splenic B cells, mononuclear cells isolated using Ficoll density-gradient centrifugation (Sigma-Aldrich, St. Louis, MO) were 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 1 h to remove monocytes and dendritic cells.

Model Ags

Alexa Fluor 546 (AF546)-conjugated monobiotinylated Fab’ anti-mouse IgM and IgG Ab (AF546-nbFab’–anti-Ig) was used to label the BCR. It is generated from the F(ab)2 fragment (Jackson ImmunoResearch, West Grove, PA) using a published protocol (44). To activate B cells with sAg, splenic B cells were incubated with AF546–mB-Fab’–anti-Ig (2 μg/ml) mixed with mB-Fab’–anti-Ig (8 μg/ml) for 30 min and streptavidin (1 μg/ml) for 10 min at 4˚C. As a control, streptavidin was omitted. The cells were washed and warmed up to 37˚C for varying lengths of time. To activate B cells with mAg, cells were incubated with AF546–mB-Fab’–anti-Ig and mB-Fab’–anti-Ig tethered to planar lipid bilayers by streptavidin at 37˚C for varying lengths of time. As a control for mAg, surface BCRs were labeled with AF546–Fab’–anti-mouse IgM (2 μg/ml) at 4˚C, washed, and the B cells were incubated with transferin (TF)-tethered lipid bilayers where the molecular density of Tf on lipid bilayers was equivalent to that of AF546–mFab’–anti-Ig.

The planar lipid bilayer was prepared as described previously (24, 45). Briefly, liposomes were made by sonication of 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-cap-biotin (Avanti Polar Lipids, Alabaster, AL) in a 1:100 molar ratio in PBS at a lipid concentration of 5 mM. Aggregated liposomes were removed by ultracentrifugation and filtration. Coverslip chambers (Lab-Tek Nalge, Nunc, Rochester, NY) were incubated with the liposomes (0.05 mM) for 10 min. After extensive washes, the coated coverslip chambers were incubated with 1 μg/ml streptavidin (Jackson ImmunolResearch), followed by 2 μg/ml AF546–mFab’–anti-Ig mixed with 8 μg/ml mB-Fab’–anti-Ig Ab or 16 μg/ml biotin-Tf.

Confocal fluorescence microscopy and image analysis

Splenic B cells were incubated with mAg or sAg at 37˚C for varying lengths of time. The cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.05% saponin, and stained with anti-coflin, phosphorylated coflin, gelsolin (Santa Cruz Biotechnology, Santa Cruz, CA), or phosphophorysin (4G10; Millipore, Billerica, MA) Abs. F-actin was stained by Alexa Fluor 488–phalloidin (Invitrogen) or total actin by anti–actin Ab (Sigma). Cells were analyzed using a confocal microscope (Zeiss LSM 710; Carl Zeiss Microscopy, Thornwood, NY). Series of z-sections were acquired at 0.4-μm intervals and reconstructed in three dimensions using Zen software from Zeiss. The fluorescence intensity profiles of F-actin, coflin, gelsolin, or phosphophorysin in relation to the BCR staining were generated using Zen software. Pearson correlation coefficients between different stainings were determined using LSM 510 software (46).

The ratio of fluorescence intensity at the cell pole of BCR aggregation to that at the opposite pole of the cells was determined using z-series of images and Andor iQ software (Andor Technology, Belfast, U.K.). For mAg-stimulated B cells, the fluorescence intensity sum of three z-sections close to lipid bilayers and that at the top of the cells were determined. For sAg-stimulated B cells, only B cells with the BCR central cluster located at the top of the cells were selected for the analysis. The fluorescence intensity sum of three z-sections close to the BCR central cluster and that close to the coverslip were determined. The level of tyrosine phosphorylation in individual mAg-stimulated B cells was determined by adding up the fluorescence intensity of phosphotyrosine staining in all z-sections of a cell using Andor IQ software. For each time point, the average fluorescence intensity was generated from ∼50 individual cells from two or three independent experiments.

Total internal reflection microscopy and image analysis

Images were acquired using a Nikon laser TIRF system on an inverted microscope (Nikon T2E000-PFS), equipped with a ×60, 1.49 NA Apo-achromat TIRF objective (Nikon Instruments, Melville, NY), a Coolscan HQ2 CCD camera (Roper Scientific, Sarasota, FL), and two solid-state lasers of wavelength 491 nm and 561 nm. For live cell imaging, time-lapse images were acquired at the rate of one frame every 3 s. Image acquisition started upon the addition of B cells onto Ag-tethered lipid bilayer and...
continued for 5 to 10 min at 37˚C. Interference reflection microscopy (IRM) images and fluorescence images at 491-nm excitation (for AF488) and 561-nm excitation (for AF546) were acquired sequentially.

To image intracellular molecules, B cells were incubated with mAg at 37˚C for varying lengths of time and then fixed with 4% PFA, permeabilized with 0.05% saponin, and stained for cofilin, gelsolin, phospho-tyrosine, and F-actin as described earlier. The B cell contact area was determined using IRM images and MATLAB software (The MathWorks, Natick, MA). The total fluorescence intensity (TFI) of each staining in the B cell contact zone was determined using Andor iQ software. Background fluorescence generated by Ag tethered to lipid bilayers in the absence of B cells or secondary Ab controls was subtracted. For each set of data, more than 20 individual cells from two or three independent experiments were analyzed.

Analysis of actin nucleation sites
Actin nucleation sites were labeled as previously described (47). For B cells that were incubated with sAg, cells were treated with 0.45 μM AF488–G-actin (Invitrogen) in the presence of 0.025% saponin during the last minute of incubation. For B cells that were activated by mAg, B cells were incubated with mAg in the presence of AF488–G-actin and 0.025% saponin for 5 min at 37˚C. The cells were fixed and analyzed using a Zeiss 710 confocal microscope to generate three-dimensional images and/or TIRFM.

Treatment of latrunculin B and jasplakinolide
B-cells were pretreated with latrunculin B (Lat; 10 μM) or jasplakinolide (Jas; 2 μM (Calbiochem, Gibbstown, NJ) for 30 min at 37˚C before the incubation with Ag in the presence of Lat or Jas. When B cells were treated with Lat alone, the time course started at the addition of Lat.

Flow cytometry analysis
The tyrosine phosphorylation levels of sAg-stimulated B cells were determined using flow cytometry. B cells were incubated with mAg, B cells, or Jas plus sAg for varying lengths of time at 37˚C. Then cells were fixed by 4% PFA, permeabilized by 0.05% saponin, and stained with anti-phosphotyrosine mAb. After postfix with 2% PFA, the cells were analyzed using BD Canto II flow cytometer.

Statistical analysis
Statistical significance was assessed by the Mann–Whitney U test using Prism software (GraphPad Software, San Diego, CA).

Results
Both sAg and mAg induce the recruitment of F-actin to BCR aggregates
To compare actin reorganization induced by mAg and sAg, we chose a pseudo Ag system that can be applied as both soluble and membrane-associated forms. AF546-conjugated, monomobiotinylated Fab’ fragment of goat anti-mouse IgG+M (AF546–mB-Fab’-anti-Ig) was used to label surface BCRs. Labeled BCRs were aggregated either with soluble streptavidin (sAg) or streptavidin tethered onto lipid bilayers (mAg). The reorganization of the actin cytoskeleton and surface BCRs in B cells stimulated with sAg was examined using three-dimensional confocal fluorescence microscopy (CFM) and that in B cells stimulated with mAg was examined using both three-dimensional CFM and TIRFM. The surface area of the cell in contact with Ag-tethered lipid bilayer (B cell contact zone) was visualized by IRM. Upon incubation with mAg but not with Tf-tethered lipid bilayers, surface BCRs on splenic B cells aggregated into small clusters (3 min) and then formed a centralized cluster (7 min) at the B cell contact zone. This was concurrent with a rapid increase of the B cell contact zone in the early stages (1–6 min) and subsequent decrease (7 min) (Fig. 1A–C). During BCR aggregation, the actin cytoskeleton underwent a dramatic redistribution, from a uniform distribution at the periphery of unstimulated cells to accumulation at the contact zone (Fig. 1A–C). By 7 min, the actin cytoskeleton was primarily concentrated at the outer edge of centralized BCR clusters and did not

![FIGURE 1](http://www.jimmunol.org/)

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colocalize with BCR clusters extensively (Fig. 1A, 1B, 1E). In B cells stimulated with sAg, surface BCRs also formed micro-clusters at 3 min and then a polarized central cluster at 7 min (Fig. 1D), which was similar to what was seen in mAg-stimulated B cells. However, BCR clusters induced by sAg appeared less dense than those induced by mAg (Fig. 1A, 1D, bottom panels). sAg also induced the recruitment of F-actin to BCR clusters. F-actin accumulated not only at the periphery but also at the center of the BCR central cluster (Fig. 1D), colocalizing with the BCR extensively (Fig. 1E). To compare the kinetics and magnitude of actin redistribution in mAg- and sAg-stimulated cells, the ratio of F-actin fluorescence intensity in the CFM image slices at the BCR central cluster to that at the opposite pole of the BCR central cluster was determined. The two-pole ratio of F-actin was ∼1 in unstimulated B cells that interacted with polylysine-coated glass or Tf-tethered lipid bilayers, and it increased over time in both mAg- and sAg-stimulated B cells (Fig. 1F). This indicates a redistribution of F-actin from unpolared to polarized distribution toward BCR clusters. The two-pole ratio in mAg-stimulated B cells increased faster and was 2-fold higher than that in sAg-stimulated B cells at all the tested time points (Fig. 1F). Taken together, these results indicate that both mAg and sAg induce the recruitment of the actin cytoskeleton to BCR clusters along with BCR aggregation. mAg appears to induce a greater degree of polarized actin redistribution than that of sAg.

Both sAg and mAg induce actin polymerization at BCR aggregates

The actin cytoskeleton is reorganized by rapid polymerization and depolymerization. To compare actin remodeling induced by mAg with that induced by sAg, we analyzed the cellular distribution of de novo actin polymerization sites in relation to surface BCRs. Actin polymerization sites were detected by the incorporation of AF488–G-actin into polymerizing ends of actin filaments. AF488–G-actin was introduced into cells in the presence of a low concentration of non-ionic detergent during the last minute of incubation with sAg or during the entire incubation time with mAg. Intracellular incorporation of AF488–G-actin was significantly increased in splenic B cells stimulated by mAg compared with that in B cells incubated with Tf-tethered lipid bilayer (Fig. 2A). Both three-dimensional CFM (Fig. 2A) and TIRFM (Fig. 2B) analysis showed that actin polymerization sites were preferentially located at the outer edge of BCR clusters after a 5-min incubation with mAg. Similarly, actin polymerization was undetectable in the absence of sAg (Fig. 2C, top panels). After sAg stimulation, de novo actin polymerization sites were first colocalized with BCR microclusters and later were exclusively found at the outer rim of BCR central clusters as they formed (Fig. 2C). These results show that both sAg and mAg induce actin polymerization and that actin polymerization occurs at BCR microclusters and at the outer edge of the BCR central cluster.

Both sAg and mAg induce the mobilization of actin regulators cofilin and gelsolin

Actin reorganization induced by Ag indicates that antigenic stimulation triggers the activation of actin regulators. Our previous studies have demonstrated that both mAg and sAg can induce Btk-dependent activation of WASP, an actin nucleation promoting factor (42, 43). In this study, we compared the effects of mAg and sAg on the cellular redistribution of actin regulators cofilin and gelsolin. Cofilin can either stabilize or destabilize F-actin depending on its concentration in the cytoplasm, and its F-actin binding activity is inhibited by phosphorylation (48). Gelsolin severs F-actin in a calcium-dependent manner (49). The cellular distribution of cofilin and gelsolin in splenic B cells was analyzed using specific Abs. In the control conditions where splenic B cells interact with Tf-tethered lipid bilayers (Fig. 3A, top panels) or with polylysine-coated glass slides (Fig. 3B, top panels), cofilin distributed evenly at the cell periphery. Reconstituted three-dimensional CFM images showed that in mAg-stimulated B cells, cofilin was recruited to BCR clusters as early as 3 min, surrounding the BCR central cluster (Fig. 3A). However, TIRFM did not detect a significant amount of cofilin at the B cell contact zone (Fig. 3C), suggesting that recruited cofilin does not associate with the plasma membrane and is at least 200 nm away from the contact zone. Reconstituted three-dimensional CFM images showed that cofilin was primarily located at the top of the BCR central cluster (Fig. 3A, bottom panels). In sAg-stimulated splenic B cells, cofilin was clearly recruited to BCR aggregates at early times and the central clusters at later times (Fig. 3B). Reconstituted three-dimensional images and fluorescence intensity profiles showed extensive colocalization of cofilin with the BCR central cluster (Fig. 3B, 3D), distinct from the distribution of cofilin in mAg-stimulated B cells (Fig. 3A, 3D). The colocalization of cofilin with the BCR in sAg-stimulated B cells, as quantified by Pearson’s correlation coefficients, increased over time for 10 min and decreased by 30 min as the BCR was internalized (Fig. 3E).

To determine whether cofilin recruitment is dependent on signaling, B cells were pretreated with the Src kinase inhibitor PP2. This treatment inhibited BCR central cluster formation and reduced the colocalization of cofilin with the BCR to basal levels in sAg-stimulated B cells (Fig. 3E, 3G). To determine whether the cofilin recruited to BCR clusters is activated, we stained for p-cofilin, which is incapable of binding to F-actin (50). In mAg-stimulated B cells, p-cofilin was recruited neither to the B cell contact zone (Fig. 3F) nor to the region above the contact zone (Fig. 3H) in comparison with B cells interacting with Tf-tethered lipid bilayers. In sAg-stimulated B cells, p-cofilin was largely excluded from BCR clusters (Fig. 3I) and did not colocalize with the BCR (Fig. 3J). The differential distribution of total cofilin and p-cofilin suggests that most of cofilin that colocalized with surface BCRs is active. Similar to F-actin recruitment, the fluorescence intensity...
The ratio of cofilin at the BCR central cluster to that at the opposite pole increased over time in both mAg- and sAg-stimulated B cells, but it increased much faster and reached higher levels in mAg-stimulated cells than in sAg-stimulated cells (Fig. 3K).

Incubation of splenic B cells with either mAg or sAg led to the redistribution of gelsolin to BCR clusters in splenic B cells, where gelsolin staining appeared punctate (Fig. 4A, 4C). Similar to cofilin, gelsolin surrounded the BCR central cluster in mAg-stimulated B cells (Fig. 4A) but colocalized with the BCR through the central cluster in sAg-stimulated B cells (Fig. 4C, 4D). There was no significant amount of gelsolin detected in the contact zone of mAg-stimulated B cells by TIRFM (Fig. 4B). The Pearson’s correlation coefficients showed a similar increase in the colocalization of gelsolin with the BCR over time as for cofilin (Fig. 4E).
The two-pole ratio of gelsolin fluorescence intensity increased over time and was much higher (~4-fold) in mAg-stimulated cells than that in sAg-stimulated cells (Fig. 4F).

Taken together, these results show that both mAg and sAg induce the recruitment of coflin and gelsolin to BCR clusters; however, their recruitment kinetics and magnitude and their distribution are different in mAg- and sAg-stimulated B cells.

**Disruption of the actin cytoskeleton alters BCR activation in response to both sAg and mAg as well as Ag-independent BCR signaling**

To compare the role of the actin cytoskeleton in BCR activation induced by mAg and sAg, we disrupted the actin cytoskeleton using a pharmacological approach involving Lat and Jas. Lat promotes actin depolymerization by sequestering G-actin (51), whereas Jas stabilizes F-actin and binds F-actin at the same site as phalloidin (52). We used these two reagents at concentrations that abolished or bind most F-actin (Supplemental Fig. 1) on BCR aggregation and tyrosine phosphorylation in unstimulated B cells attached to lipid bilayers by Tf or attached to polylysine-coated glass slides. The reconstituted three-dimensional CFM analysis showed that when B cells were treated with Lat in the absence of Ag, surface BCRs formed microclusters in a nonpolarized manner (Fig. 6D, 6I). There were very few BCR microclusters detected by TIRFM in the B cell membrane zone contacting Tf-tethered lipid bilayers (Fig. 6E, bottom panels). BCR microclusters in Lat-treated cells formed at 10 min, which is later than those induced by mAg and sAg (data not shown), and remained randomly distributed at 30 min (Fig. 6D, 6I). These BCR microclusters were positive for p-tyrosine staining (Fig. 6C, 6H, 6K, 6L).

The actin cytoskeleton has been shown to regulate Ag-independent signaling in B cells by controlling BCR lateral mobility (39). To understand how actin is involved in signaling regulation in resting B cells, we determined the effect of Lat and Jas at the concentrations that eliminate or bind most F-actin (Supplemental Fig. 1) on BCR aggregation and tyrosine phosphorylation in unstimulated B cells attached to lipid bilayers by Tf or attached to polylysine-coated glass slides. The reconstituted three-dimensional CFM analysis showed that when B cells were treated with Lat in the absence of Ag, surface BCRs formed microclusters in a nonpolarized manner (Fig. 6D, 6I). There were very few BCR microclusters detected by TIRFM in the B cell membrane zone contacting Tf-tethered lipid bilayers (Fig. 6E, bottom panels). BCR microclusters in Lat-treated cells formed at 10 min, which is later than those induced by mAg and sAg (data not shown), and remained randomly distributed at 30 min (Fig. 6D, 6I). These BCR microclusters were positive for p-tyrosine staining (Fig. 6C, 6H, 6K, 6L), suggesting that tyrosine phosphorylation occurs at these microclusters. Quantitative analysis showed that Lat treatment alone increased the p-tyrosine level in unstimulated B cells. However, this Lat-induced p-tyrosine increase had much slower kinetics than that induced by Ag and did not show any sign of

**FIGURE 4.** The recruitment of gelsolin to BCR clusters in B cells stimulated by sAg or mAg. Splenic B cells were incubated with AF546–mB-Fab–anti-Ig tethered to lipid bilayers (mAg) at 37°C for indicated times. Cells were fixed, permeabilized, stained for gelsolin, and analyzed by CFM (A) and by TIRFM (B). Splenic B cells were incubated with AF546–mB-Fab–anti-Ig without (−) or with streptavidin (sAg) at 4°C, washed, and warmed to 37°C for varying lengths of time. After fixation and permeabilization, the cells were stained for gelsolin and analyzed using CFM (C). The fluorescence intensity profiles of BCRs and gelsolin were generated using Zen software (D). The Pearson’s correlation coefficients between BCR and gelsolin staining in sAg-stimulated cells were determined using Zen software (E). The two-pole ratio of gelsolin fluorescence intensity (FI) was determined using Andor IQ software (F). Shown are representative two- and three-dimensional images at indicated times and the average values (±SD) of ~50 cells from three independent experiments. Scale bars, 2.5 μm. *p < 0.01 [compared with no streptavidin (−) in (E) and compared with sAg in (F)].
attenuation for at least 30 min (Fig. 6K, 6L). In contrast, Jas treatment in the absence of Ag induced neither BCR aggregation nor tyrosine phosphorylation (data not shown).

These results collectively suggest that actin depolymerization itself is sufficient for BCR self-aggregation into microclusters and signal induction in BCR aggregates; however, actin polymerization may be essential for the fast kinetics of BCR aggregation and signal activation and the merger of BCR microclusters into the central cluster as well as signaling attenuation induced by both mAg and sAg.

Discussion

Two of the common physical forms of Ags that B cells encounter in the draining lymph node and spleen are sAg and Ag presented on the surfaces of dendritic cells and macrophages (3). How the physical presentation of Ag modulates BCR signaling has not been well studied. Previous studies have shown a role for the actin cytoskeleton in mAg-induced BCR activation (31). This study reveals that actin reorganization is equally important for BCR signaling triggered by sAg. Both mAg and sAg induce actin reorganization and recruitment of actin regulators to BCR aggregates. Inhibition of actin reorganization by stabilizing F-actin blocks BCR aggregation and tyrosine phosphorylation induced by both mAg and sAg. Elimination of most F-actin by depolymerization leads to the unpolarized formation of BCR microclusters and tyrosine phosphorylation in the BCR microcluster in the absence of Ag. These data indicate an essential role for the actin cytoskeleton in BCR activation, irrespective of the nature of Ag presentation.

Because of their different physical forms, sAg and mAg have distinct modes of interaction with surface BCRs. Whereas sAg can bind BCRs on the entire surface of B cells at the same time in a nonpolarized fashion, mAg can only bind BCRs at the surface zone contacting the Ag-tethered membrane, which provides a polarized stimulation. Although both sAg and mAg can induce BCR self-aggregation into microclusters and central clusters by cross-linking BCRs, they do so in distinct ways. Upon sAg stimulation, surface BCRs have to identify a pole of the cell laterally to migrate toward to form the BCR central cluster. Further, mAg induces more dramatic morphological changes in B cells than those by sAg. These observations suggest that the actin cytoskeleton plays distinct roles in mAg- and sAg-stimulated BCR activation. This study shows that whereas both mAg and sAg induce actin reorganization in splenic B cells, the magnitude of F-actin and actin regulator recruitment in mAg-stimulated cells is generally greater than that in sAg-stimulated cells, as revealed by comparing the fluorescence intensity ratio between the pole where the BCR central cluster forms and its opposite pole. In addition, mAg-induced BCR redistribution appears to lead to the formation of a more compact BCR central cluster than that by sAg. Because we formulated mAg and sAg using the same reagents with the same concentrations and examined both using the same approach, the observed differences suggest that the physical form of Ag has an impact on actin reorganization and surface BCR aggregation, and mAg appears to be more potent in triggering these events compared with sAg.

In addition to differences in the recruitment levels, the distribution patterns of F-actin, cofilin, and gelsolin are distinct in mAg- and sAg-stimulated B cells. F-actin, cofilin, and gelsolin are preferentially localized at the outer edge of the contact zone in mAg-stimulated B cells but are colocalized with the BCR throughout the central cluster in sAg-stimulated B cells. Their differential distribution is likely linked to the distinct mechanisms by which BCR aggregates grow and merge with each other in mAg- and sAg-stimulated cells. After initial contact with mAg, B cells undergo rapid spreading on Ag-tethered membrane, which is followed by contraction (29). B cell spreading has been shown to enhance BCR self-aggregation by allowing more surface BCRs to engage Ag, and B cell contraction after spreading facilitates the merger of small BCR aggregates into the central cluster (29, 43). The coaccumulation of WAVE, cofilin, and gelsolin with the BCR at the outer edge of the contact membrane suggests that actin polymerization (by WAVE), severing and/or depolymerization (by cofilin and gelsolin) occur at the leading edge of the spreading membrane, driving it outwards. This study also finds that in contrast to WAVE (43), cofilin and gelsolin are not concentrated in the B cell contact zone but are localized more than 200 nm above the BCR central cluster. This indicates that F-actin severing mediated by cofilin and gelsolin occurs above the BCR central cluster, which potentially promotes actin depolymerization at the cytoplasmic side of the actin cytoskeleton and provides G-actin monomers for WAVE-mediated actin polymerization at the B cell contact zone. Such a coordination of cofilin and gelsolin with WAVE and other actin regulators potentially leads to actin reorganization in a unique manner that generates both a lateral force for B cell spreading and contraction and a force that allows sustained adhesion of B cells to Ag-tethered membrane. A recent study by Freeman et al. (53) shows that Rap GTPase-dependent activation of cofilin leads to actin severing, which is required for
efficient B cell spreading and BCR aggregation, confirming a role for cofilin in BCR activation in response to mAg.

In contrast to mAg-stimulated B cells, sAg-stimulated B cells lack a target surface to adhere to and do not undergo dramatic morphological changes. The persistent colocalization of F-actin, cofilin, and gelsolin with surface BCR aggregates in sAg-stimulated B cells provides two hypotheses. First, the lateral movement of BCR aggregates and the dynamics of the actin cytoskeleton are functionally linked with each other. Second, both WASP-activated actin polymerization and cofilin- and gelsolin-mediated actin severing and/or depolymerization occur at the plasma membrane, which potentially induces lateral mobilization of actin filaments to drive the lateral movement of BCRs but not the formation of lamellipodia for B cell spreading. Further studies are required to test these hypotheses.

The differences in distribution of F-actin and actin regulators in mAg- and sAg-stimulated B cells raise the question of how these two types of Ags induce distinct modes of actin reorganization. Ag-triggered actin reorganization has been shown to depend on BCR signaling. We have recently shown that the recruitment and activation of WASP are positively and negatively regulated by Btk and SHIP-1, respectively (42, 43). This study shows that cofilin and gelsolin, two actin regulators involved in actin severing and depolymerization, also coclustered with surface BCRs in response to both mAg and sAg. Furthermore, the coclustering of cofilin with BCRs depends on BCR signaling. Freeman et al. (53) have recently shown that in B cells, cofilin is activated in a Rap GTPase-dependent manner in response to Ag stimulation. Gelsolin is known to be activated by calcium flux (54). The activity of cofilin has been shown to be regulated via a growing number of cellular pathways, including its phosphorylation and dephosphorylation by LIM kinases and phosphatases chronophin and slingshot 1L, its binding to phosphatidylinositides, and its local concentration relative to F-actin levels (55). These collectively indicate that the activation and recruitment of cofilin and gelsolin provide an additional level of control for BCR signaling to regulate actin reorganization. The
physical presentation of Ags may regulate the spatiotemporal organization of BCR signalosomes, consequently leading to differential activation or distribution of these actin regulators.

How the physical presentation of Ag, soluble and membrane bound, affects the initiation event of BCR signaling remains to be addressed. Association with the membrane transforms Ag stimulation from a nonpolarized into a polarized one and potentially generates physical restraint on the Ag–BCR interaction. This increased physical restraint potentially enhances the ability of Ag to induce conformational changes in the BCR through putative mechanical feedback, thereby facilitating BCR self-aggregation. Tolar et al. (22) showed that a monovalent Ag, which cannot induce BCR activation as a soluble form, induced BCR aggregation and signaling when it was tethered to lipid bilayers. This monovalent mAg induces BCR self-aggregation probably by inducing conformational changes of the BCR, exposing the C4u4 domain of membrane IgM that has been shown to be involved in BCR self-aggregation. Therefore, the physical form of Ag potentially modulates BCR activation via Ag’s ability to induce conformational change. This study shows that the p-tyrosine stain has a different distribution pattern in mAg- compared with sAg-stimulated B cells. The p-tyrosine staining is concentrated at the outer edge of the central cluster in mAg-stimulated B cells but is present throughout the central cluster in sAg-stimulated B cells. This result suggests that mAg- and sAg-induced signalosomes differ in their spatial organization. Our finding that the distribution pattern of p-tyrosine staining in mAg- and sAg-stimulated cells is similar to those of F-actin and actin regulators coflin and gelsolin supports the notion that differential actin remodeling contributes to the distinct spatial organization of BCR signalosomes in mAg- and sAg-stimulated B cells.

Actin reorganization has been shown to be involved in the initiation of BCR signaling and the regulation of tonic signaling in resting B cells (31, 39–41). How actin dynamics regulate BCR signaling at different activation stages in response to different forms of Ag remains to be elucidated. Similar to previous findings (29, 39), this study found that elimination of most F-actin by Lat induced tyrosine phosphorylation in the absence of Ag. Our study further shows that actin depolymerization by Lat induces BCR aggregation and that the BCR aggregates are positive for tyrosine phosphorylation. This suggests that actin depolymerization alone is sufficient for the induction of BCR aggregation, and BCR aggregation is able to initiate BCR signaling. Additionally, our study found two distinct properties of Lat-induced BCR aggregates. First, Lat-induced BCR aggregation is slow, random, and unpolarized compared with BCR aggregation induced by mAg and sAg, which is concurrent with a slow increase in tyrosine phosphorylation. Second, Lat-induced BCR aggregates persist as microclusters and are unable to merge into a polarized central cluster, which is associated with a lack of attenuation of Lat-induced tyrosine phosphorylation. The second property of Lat-induced BCR aggregates is similar to what we previously found in SHIP-1–deficient B cells where BCR aggregates persist as microclusters and signaling attenuation is inhibited (43). This finding further supports the notion that the merger of BCR microclusters into the central cluster is associated with signal attenuation. These results collectively suggest that although actin depolymerization can induce BCR aggregation and BCR signaling, actin polymerization, in coordination with actin depolymerization, is important for the fast kinetics of BCR aggregation and activation as well as the merger of BCR aggregates into a central cluster and signaling attenuation.

Our studies demonstrate that actin remodeling is required for BCR activation in response to both mAg and sAg. In both cases, actin remodeling, including both polymerization and depolymerization, is essential for early and rapid BCR aggregation and the later growth and merger of these aggregates into a polarized central cluster. Actin remodeling induced by sAg and mAg exhibits different magnitude and spatial organization, which provides distinct feedback for the formation of BCR signalosomes. Future studies are required to define the molecular detail underlying the functional interaction between the actin cytoskeleton and BCR signaling during B cell activation.

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Disclosures
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References


