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Cofilin-Mediated F-Actin Severing Is Regulated by the Rap GTPase and Controls the Cytoskeletal Dynamics That Drive Lymphocyte Spreading and BCR Microcluster Formation

Spencer A. Freeman,*† Victor Lei,* May Dang-Lawson,* Kensaku Mizuno,‡ Calvin D. Roskelley,‡ and Michael R. Gold*

When lymphocytes encounter APCs bearing cognate Ag, they spread across the surface of the APC to scan for additional Ags. This is followed by membrane contraction and the formation of Ag receptor microclusters that initiate the signaling reactions that lead to lymphocyte activation. Breakdown of the submembrane cytoskeleton is likely to be required for the cytoskeleton reorganization that drives cell spreading and for removing physical barriers that limit Ag receptor mobility. In this report, we show that Ag receptor signaling via the Rap GTPases promotes the dephosphorylation and activation of the actin-severing protein cofilin and that this results in increased severing of cellular actin filaments. Moreover, we show that this cofilin-mediated actin severing is critical for the changes in actin dynamics that drive B and T cell spreading, for the formation of BCR microclusters, and for the increased mobility of BCR microclusters within the plasma membrane after BCR engagement. Finally, using a model APC, we show that activation of this Rap–cofilin signaling module controls the amount of Ag that is gathered into BCR microclusters and that this is directly related to the magnitude of the resulting BCR signaling that is initiated during B cell–APC interactions. Thus, Rap-dependent activation of cofilin is critical for the early cytoskeletal changes and BCR reorganization that are involved in APC-dependent lymphocyte activation. *The Journal of Immunology, 2011, 187: 5887–5900.

Lymphocytes are highly motile within lymphoid organs and make frequent contacts with potential APCs. T cells are activated by peptide/MHC complexes on APCs, whereas B cells can be activated by Ags or immune complexes that are captured by APCs (1). Signaling via the BCR or TCR induces rapid spreading of the lymphocyte across the surface of the APC (2, 3), allowing additional Ag receptors (AgRs) to encounter Ags and form microclusters (4, 5). Within 5–10 min, cell spreading is replaced by membrane contraction, ultimately leading to formation of an immune synapse (IS) in which Ag-bound TCRs or BCRs are concentrated into a central supramolecular activation cluster (2, 3). In contrast to the central supramolecular activation cluster, which may be primarily a site for AgR internalization (6, 7), AgR microclusters nucleate signalosome assembly and are thought to be the main site of AgR signaling (4, 5, 8). The extent to which lymphocytes spread on the surface of APCs, encounter Ag, and form AgR microclusters determines the magnitude of AgR signaling and whether lymphocyte activation ensues. Thus, elucidating the mechanisms that control cytoskeleton and microcluster dynamics is critical for understanding APC-induced lymphocyte activation.

The changes in cell morphology and AgR organization that occur during APC-mediated lymphocyte activation are driven by dynamic rearrangement of the submembrane cytoskeleton (9). In particular, cell spreading requires the formation of branched F-actin networks that exert outward force on the plasma membrane. The Rap and Rac2 GTPases have been implicated in AgR-induced actin polymerization and are important for B cell spreading and IS formation (10, 11). The active GTP-bound form of Rap1 binds multiple proteins that promote actin polymerization, including Vav2 and TIAM, upstream activators of the Rac and Cdc42 GTPases, respectively (12). Activated Rac and Cdc42 act via WAVE and WASP to activate the Arp2/3 complex, which initiates the formation of branched actin filaments. Rap1–GTP also binds RIAM and AF-6 (12), scaffolding proteins that recruit profilin, a protein that primes actin monomers for addition to the barbed ends of actin filaments.

In addition to actin polymerization, the severing of existing cortical F-actin filaments is likely to be a prerequisite for the cytoskeletal reorganization that underlies changes in lymphocyte morphology. Indeed, F-actin severing or depolymerization occurs within 15 s of BCR engagement (13). F-actin severing releases actin monomers that can be assembled into new filaments but also generates new barbed ends, which are preferred sites for the formation of branched actin filaments by the Arp2/3 complex (14). Thus, breakdown of the cortical cytoskeleton is coupled to the subsequent assembly of branched actin networks that drive cell spreading.

Abbreviations used in this article: ABD, actin binding domain; ABD–RFP, actin binding domain–red fluorescent protein fusion protein; AgR, Ag receptor; DC, dendritic cell; DN, dominant negative; ERM, ezrin–radixin–moesin; FRAP, fluorescence recovery after photobleaching; hlgM, human IgM; IS, immune synapse; mHBS, modified HEPES-buffered saline; mlgG, murine IgG; RFP, red fluorescent protein; ROI, region of interest; S3, serine 3; SHS, Slingshot phosphatase; SHS/C5, mutant Slingshot phosphatase with C→S replacement in its active site; TIRFM, total internal reflection fluorescence microscopy; WT, wild-type.

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F-actin severing is also likely to be important for the formation of AgR microclusters. In resting cells, the lateral diffusion of AgRs is limited to small membrane domains created by the submembrane actin cytoskeleton (“actin coralls,” “picket fences”), which is tethered to the membrane by ezrin–radixin–moesin (ERM) family proteins (15). Constraining AgRs within these actin coralls allows Ag-independent formation of small microclusters that provide tonic survival signals but prevents large-scale microcluster formation that would initiate lymphocyte activation (16). AgR signaling causes the breakdown of submembrane F-actin structures, as well as dephosphorylation and inactivation of ezrin (16, 17). Ezrin inactivation, which causes localized release of the plasma membrane from the submembrane cytoskeleton (18), enhances AgR mobility and microcluster formation (19). However, localized breakdown of the submembrane cytoskeleton may also be critical for these initial events in APC-mediated lymphocyte activation (9). The AgR signaling pathways that initiate cytoskeletal breakdown are not well understood, but they are likely to target F-actin–severing proteins such as gelsolin and cofilin. Indeed, cell-permeable peptides that block the binding of cofilin to F-actin prevent IS formation in T cells (20). Based on our previous findings that activation of the Rap GTPases is required for lymphocyte spreading and IS formation (10), we tested the hypothesis that AgR-induced Rap activation leads to increased F-actin severing and that this is required for the initial changes in cytoskeleton, membrane, and AgR dynamics that occur after Ag binding. We now show that the F-actin–severing protein cofilin is a downstream target of this Rap–cofilin module is essential for the changes in cytoskeletal dynamics that underlie B and T cell spreading and for BCR microcluster formation, mobility, and signaling.

Materials and Methods
Cell preparation and culture
Primary cells from C57BL/6 mice were isolated according to protocols approved by the University of British Columbia Animal Care Committee. Splenic B cells, as well as T cells from the spleen and lymph nodes, were isolated by depletion of non-B cells or non-T cells using kits from Stemcell Technologies. B cells were activated for 24–48 h with 5 ng/ml LPS plus 5 ng/ml IL-4 (R&D Systems), and T cells were activated for 24–48 h with 2.5 ng/ml IL-2 (R&D Systems) prior to being used for experiments. Dendritic cells (DCs) were obtained by culturing bone marrow cells in medium with 20 ng/ml GM-CSF for 8 d. A20 and WEHI-231 B-lymphoma cells, as well as Jurkat clone E6-1 cells, were obtained from American Type Culture Collection. A20 and WEHI-231 cells stably transfected with the pMSCVpuro vector (BD Biosciences) or with the pMSCV-FLAG–RapGAPII vector have been described previously (10, 21). A20 cells, as well as Jurkat clone E6-1 cells, were obtained from American Type Culture Collection. A20 and WEHI-231 cells stably transfected with the pMSCVpuro vector (BD Biosciences) or with the pMSCV-FLAG–RapGAPII vector have been described previously (10, 21).

B cells transfection kit was used for LPS/IL-4–activated primary B cells, as done previously (10), and the Amaza mouse T cell transfection kit was used for IL-2–activated primary T cells. Amaza transfection kit V was used for A20 cells, WEHI-231 cells, Jurkat cells, and DCs. Cells were used for experiments 24 h after transfection. Transfection efficiency ranged from 10 to 40%, depending on the plasmid used. For LPS/IL-4–activated primary B cells, transfection efficiencies for the vectors used in this study were generally 30–40%, as shown previously (see Fig. 2F in Ref. 10).

Abs
Goat anti-mouse IgM, goat anti-mouse IgG, goat anti-hamster IgG, goat anti-mouse IgG Fab fragment, and Cy3-labeled goat anti-mouse IgG (F(ab′)2) fragment were obtained from Jackson ImmunoResearch Laboratories. The OKT3 anti-human CD3 mAb, the 145-2C11 anti-mouse CD3 mAb, and the 37.5 anti-mouse CD28 mAb were obtained from eBioscience. The 9.3 anti-human CD28 mAb is described in Ref. 26. Rabbit Abs against cofilin and thulin that is phosphorylated on serine 3 (S3) were purchased from Cell Signaling Technologies. Mouse β-actin Ab was from Sigma-Aldrich. The biotinylated anti-Myc mAb (9E10) was from BD Biosciences. The rabbit anti–VSV-G Ab was from Abcam. Alexa 488- and Alexa 647-labeled goat anti-rabbit IgG or goat anti-rat IgG, as well as streptavidin–FITC, were obtained from Molecular Probes-Invitrogen. Rabbit anti–p–Tyr was from BD Biosciences.

Cell spreading
Tissue culture chambers (ibidi) were coated as in Ref. 10 with goat anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories) or with 10 μg/ml human fibronectin (Sigma-Aldrich) plus anti-mouse CD3/CD28 (2C11 and 37.51 mAbs) or anti-human CD3/CD28 (OKT3 and 9.3 mAbs). Cells (5 × 10^5) in 0.3% modified HEPES-buffered saline (mHBS): 25 mM sodium HEPES, pH 7.2, 125 mM NaCl, 5 mM KCl, 1 mM CaCl_2, 1 mM Na_2HPO_4, 0.5 mM MgSO_4, 1 mg/ml glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol) were added to the chambers and imaged in real time at 37°C using a UPlan Achromat 60×/1.4 numerical aperture objective on an Olympus FV1000 confocal microscope. In some experiments, the plasma membranes of the cells were labeled with CellMask Orange (Molecular Probes-Invitrogen) prior to imaging the cells. Videos were generated from time-lapse video recordings using Fluoview 1.6 software (Olympus). ImagePro software (Media Cybernetics) was used to quantify cell area and generate kymographs.

Cell stimulation and immunoblotting
B cells (5 × 10^6 in 0.4 ml mHBS) were stimulated in suspension with 10 μg/ml anti-IgM (splenic B cells), 10 μg/ml anti-IgG (A20 cells), or 100 μg/ml goat–Ig-coated beads prepared as in Ref. 10. T cells were stimulated in suspension with a mixture of 5 μg/ml anti-CD3, 10 μg/ml anti-CD28, and 10 μg/ml goat anti-hamster IgG. To stimulate cells with plate-bound Abs, tissue culture plates were coated with 10 μg/ml anti-Ig or with 10 μg/ml anti-CD3 plus 20 μg/ml anti-CD28, as in Ref. 10. For immunoblot analysis, cells were lysed in RIPA buffer [30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Igepal (Sigma-Aldrich), 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM PMSF, 1 mM leupeptin, 1 μg/ml aprotinin, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 1 μg/ml microcystin-LR]. Cell extracts were then separated by SDS-PAGE and transferred to nitrocellulose filters. Filters were blocked with TBS (10 mM Tris-HCl, pH 8, 150 mM NaCl) containing 1% (w/v) BSA or 5% (w/v) milk powder, and washed with TBS plus 0.1% Tween 20. Primary Abs were added for 1–2 h at room temperature or overnight in the cold. After washing, the filters were incubated with HRP-conjugated secondary Abs for 1 h at room temperature. Bands were visualized using ECL (GE Life Sciences).

Assays for G-actin/F-actin and in vitro actin-polymerizing activity
Using a G-actin/F-actin in vivo assay kit (Cytokeleton), cells were lysed and F-actin filaments stabilized according the manufacturer’s instructions. Cell extracts were separated into soluble and insoluble fractions by ultracentrifugation, and these fractions were analyzed by immunoblotting with a mouse β-actin Ab. To assay actin-polymerizing activity in cell extracts, cells were lysed in RIPA buffer containing 1 mM ATP and then sonicated for 1 min to destroy preexisting F-actin. The sonicated cell extracts were incubated at 37°C for 10 min to allow in vitro actin polymerization. F-actin stabilization buffer (Cytokeleton) was then added for 10 min before separating the extracts into soluble and insoluble fractions, which were analyzed by immunoblotting with a β-actin Ab.
Fluorescence recovery after photobleaching

Cells were incubated at 37°C on 8-well ibiTreat μ-slides (ibidi) that had been coated with either 10 μg/ml fibronectin, 10 μg/ml anti-Ig, or 10 μg/ml anti-CD23 plus 20 μg/ml anti-CD28. Fluorescence recovery after photobleaching (FRAP) was performed as described previously (27) using an Olympus FV1000 confocal microscope to image regions of interest (ROIs). After measuring the prebleach fluorescence signal, the ROI was photo-bleached using a 405-nm laser (100% intensity, 0.1 s). Fluorescence recovery within the ROI was then imaged over 1 min. Fluoview v1.6 software was used to quantify the fluorescence signal within the ROI, which was normalized to the prebleach intensity. Curves representing the single exponential fit of the data were generated using Prism 4 software (GraphPad).

Labeling of actin filament barbed ends

The incorporation of Alexa 488-actin at barbed ends of actin filaments was performed as described (28). Cells were stimulated as for spreading assays and then rendered semipermeable by a brief incubation with warm permeabilization buffer (20 mM HEPES, pH 7.5, 138 mM KCl, 4 mM MgCl₂, 3 mM EGTA, 0.4 mg/ml saponin, 1% BSA). Barbed ends were labeled by adding permeabilization buffer containing 1 mM ATP and 0.4 μM Alexa 488-actin. After 30 s at 37°C, cells were fixed by adding an equal volume of 8% paraformaldehyde in PBS. After 20 min at 20°C, the cells were stained with rhodamine–phalloidin for 20 min and imaged by confocal microscopy.

Immunofluorescence

Immunofluorescence was performed as described previously (27). After being fixed with 4% paraformaldehyde, cells were permeabilized with PBS containing 0.5% Triton X-100 for 3 min and then blocked with 2% BSA in PBS for 30 min. Primary Abs were added for 1 h and visualized with Alexa 488- or Alexa 647-labeled goat anti-rabbit IgG or goat anti-rat IgG, or with streptavidin–FITC. F-actin was visualized with rhodamine–phalloidin. Chambers or coverslips were treated with ProLong Gold anti-fade reagent containing DAPI (Molecular Probes). Images were captured using an Olympus FV1000 confocal microscope. Fluoview v1.6 and ImagePro software were used to analyze confocal images and quantify fluorescence.

In vitro F-actin severing

Cells were stimulated in suspension and lysed in 0.5 ml ice-cold lysis buffer (20 mMTris-HCl, pH8.5, 5 mM EDTA, 0.5 mM MgCl₂, 0.5% Triton X-100, 0.5 mM ATP, 5 mg/ml BSA, 6 mg/ml glucose, 100 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM Na₃VO₄, 25 mM β-glycerophosphate) for 20 min on ice. Insoluble material was removed by centrifugation. Seving assays were performed as described (29). Chamber slides (ibidi) were incubated with 20 μg/ml anti-biotin Abs in ISAP buffer (20 mM Tris-Cl, pH 7.5, 5 mM EDTA, 2 mM MgCl₂, 50 mM KC₁, 1 mM ATP, 1 mM DTT) for 1 h at room temperature, washed with ISAP buffer, and then blocked with ISAP buffer containing 0.5 mg/ml BSA for 5 min. Biotinylated fluorescent F-actin filaments were generated by incubating 0.2 μM Alexa 488–actin, 0.2 μM biotinylated actin, and 0.4 μM unlabeled actin (all from Cytoskeleton) in ISAP buffer for 1 h at 20°C. This mixture was then diluted 1:5 with ISAP buffer containing 5 mg/ml BSA and 100 mM DTT and added to the anti-biotin–coated chamber slide for 5 min. The chambers were washed with ISAP buffer containing 5 mg/ml BSA and imaged using an Olympus FV1000 confocal microscope to determine the preassay fluorescence intensity of adhered F-actin filaments. Cell extracts were then added for 5 min, after which the chambers were washed and imaged to determine the fluorescence intensity of remaining F-actin filaments. Fluorescence intensities were quantified using ImagePro 3D software.

In vivo F-actin severing

Cells were transiently cotransfected to express the F-actin binding domain (ABD) of utrophin fused to RFP (ABD–RFP) (23) as well as photo-convertible GFP (30) fused to β-actin. Peripheral ROIs containing F-actin structures were identified by ABD–RFP fluorescence, and the actin-GFP in the ROI was converted to a fluorescent conformation by illumination with a 405-nm laser with a SIM scanner (1–5% intensity, 0.1 s). GFP and RFP fluorescence within the ROI were then monitored in real time at 37°C. Single exponential fit curves of the GFP/RFP ratio were generated using Prism 4 software.

BCR microcluster formation on surrogate APCs

B16F1 melanoma cells (American Type Culture Collection) were grown in RPMI 1640 plus 8% FCS. Lipofectamine 2000 (Invitrogen) was used to transiently transfect these cells with a plasmid encoding a fusion protein consisting of a single-chain Fv generated from the 187.1 rat anti-IgG mAb, the hinge and membrane proximal domains of rat IgG1, and the transmembrane and cytoplasmic domains of H-2Kb (31). After 24 h, the B16F1 cells were detached in cell dissociation buffer (0.5 mM EDTA, 100 mM NaCl, 1 mM glucose, pH 7.4) and plated on chamber slides coated with 10 μg/ml fibronectin for 4 h to promote flattening and spreading. B cells (10⁴ cells in 100 μl tissue culture medium) were added and allowed to attach to the B16 cells for 5–10 min at 37°C. After fixation and permeabilization, the cells were stained with rabbit anti-p-Tyr and then with Alexa 568–goat anti-rabbit IgG to visualize p-Tyr. Alexa 488–goat anti-rat IgG to visualize the single-chain anti-IgG, and rhodamine–phalloidin to visualize F-actin before being imaged by confocal microscopy. Fluoview v1.6 and Image-Pro software were used to analyze confocal images and quantify fluorescence signals.

BCR microcluster mobility

Cells were incubated on ice with 100 ng/ml Cy3-labeled goat anti-mouse IgG Fab’/2 plus 1 μg/ml unlabeled goat anti-mouse IgG Fab for 10 min before being pelleted and resuspended in 30°C PBS. The cells were then plated on chamber slides coated with goat anti-human IgM for 2 min and imaged at 30°C to limit endocytosis. Live imaging of the cell–substrate interface by total internal reflection fluorescence microscopy (TIRFM) was performed using an inverted Zeiss Axiovert 200 microscope with a 100 × 1.45 numerical aperture TIRFM objective. For diffusion coefficient measurements, one-channel recordings from a 561-nm laser were acquired at 20 frames/s. Tracks that were followed for >1 s were analyzed with Slidebook 5.0 software (Intelligent Imaging Innovations).

Statistical analysis

Student two-tailed t test was used to compare sets of matched samples. GraphPad Prism software was used to generate nonlinear regression curves.

Results

AgR-induced cell spreading is associated with increased actin dynamics

Plating lymphocytes on AgR ligands that are immobilized on rigid substrates allows one to study lymphocyte spreading while limiting subsequent membrane contraction. Rapid and sustained cell spreading occurred when B cells contacted anti-IgM–coated surfaces and when T cells contacted anti-CD3/CD28-coated surfaces (Fig. 1A, Supplemental Videos 1, 2). This was associated with increased actin dynamics, as shown by transfecting primary B or T cells with actin-GFP and then monitoring fluorescence recovery after photobleaching (FRAP) in membrane-proximal ROIs containing F-actin structures (Fig. 1B, Supplemental Videos 3, 4). We found that plating cells on anti-IgM or anti-CD3/CD28 increased the maximal recovery of actin–GFP fluorescence in photobleached ROIs. This indicates that AgR signaling increased the incorporation of actin-GFP monomers into peripheral F-actin structures, relative to their release by depolymerization of actin filaments. Consistent with this, AgR signaling increased the amount of actin polymerizing activity present in B and T cell extracts (Fig. 1C). To show this, cell extracts were sonicated to destroy preexisting actin filaments and then incubated in vitro under conditions in which actin polymerization could occur, as indicated by the generation of insoluble F-actin that could be pelleted by centrifugation. Extracts from activated lymphocytes generated more F-actin than extracts from unstimulated cells. Similarly, when B cells were rendered semipermeable and then briefly incubated in the presence of Alexa 488-labeled actin monomers, cells plated on immobilized anti-IgM incorporated more Alexa 488–actin into F-actin than unstimulated cells (Fig. 1D). Much of this BCR-induced de novo actin polymerization occurred at the peripheral lamellipodia that are associated with cell spreading (Fig. 1D).

Despite the increased incorporation of actin monomers into actin filaments, AgR engagement did not cause a significant in-
crease in the total amount of F-actin within the cells (Fig. 1D, middle graph, and 1E). This suggests that AgR signaling causes reorganization of the actin cytoskeleton in which new actin polymerization is coupled to the severing of existing actin filaments. This is consistent with the idea that actin polymerization in cells occurs mainly by addition of actin monomers to the uncapped barbed ends of actin filaments (32), which are generated by actin severing (29). Therefore, we investigated the role of actin severing in lymphocyte spreading.

AgR signaling stimulates cofilin-mediated F-actin severing

Jasplakinolide, a drug that stabilizes polymerized actin filaments, inhibited B and T cell spreading (Fig. 2A), indicating that F-actin severing is essential for lymphocyte spreading. To test

FIGURE 1. B and T cell spreading is associated with increased actin dynamics and actin reorganization. A, Kymographs showing the spreading of representative LPS/IL-4–activated murine B cells or IL-2–activated murine T cells were generated from DIC images of the cell–substrate contact that were collected in real time (see Supplemental Videos 1, 2). Scale bars, 10 μm. The cell area (mean ± SD for >60 cells from three experiments) was determined at the indicated times after the cell first contacted the substrate. ***p < 0.001 (relative to time 0). B, FRAP analysis of membrane-proximal ROIs in LPS/IL-4–activated B cells or IL-2–activated T cells that had been transfected with actin–GFP and then plated on fibronectin (unstimulated) or fibronectin plus immobilized anti-IgM or anti-CD3/CD28 (stimulated) for 1 h. After recording the prebleach (PB) fluorescence intensity in a membrane-proximal ROI indicated by the red box, the ROI was photobleached, and fluorescence recovery was measured at 5-s intervals over 1 min (see Supplemental Videos 3, 4). Representative fluorescence images for single cells are shown in the left panels. Scale bars, 10 μm. The recovery of actin–GFP fluorescence after photobleaching (mean ± SD for 30 cells from three experiments) is graphed in the right panels. C, Primary B cells were stimulated in suspension with 10 μg/ml anti-IgM. Primary T cells were stimulated in suspension with anti-CD3 plus goat anti-hamster IgG to cluster the TCR, along with anti-CD28. After the indicated stimulation times, the cells were solubilized. The cell extracts were sonicated to destroy preexisting actin filaments and then incubated at 37 C for 10 min before being separated into soluble and insoluble fractions containing G- and F-actin, respectively. A representative blot is shown, and the amount of F-actin generated in vitro, expressed as a percent of the total F- plus G-actin (mean ± SEM from three experiments), is graphed. *p < 0.05, **p < 0.01 (relative to time 0; unstimulated cells). D, De novo actin polymerization was assessed in splenic B cells that were left in suspension (unstimulated) or plated on anti-IgM. Cells were permeabilized and incubated with Alexa 488–actin for 30 s. Total F-actin was visualized with rhodamine–phalloidin. Representative images of z-axis projections and single z-slices along the dotted lines are shown. The graphs show newly incorporated Alexa 488–actin (top), total F-actin (middle), and Alexa 488–actin incorporation relative to total F-actin (bottom), which were calculated from three-dimensional images of 20 cells from three experiments (mean ± SD). Scale bars, 10 μm. **p < 0.01, ***p < 0.001 (relative to time 0). E, Murine B or T cells were stimulated in suspension for the indicated times, as in C. Soluble and insoluble fractions containing G- and F-actin, respectively, were analyzed by immunoblotting with a β-actin Ab. A representative blot is shown, and data from three experiments are graphed as the percent of total actin present as F-actin (mean ± SEM).
whether AgR signaling increases cellular actin-severing activity, we used an assay (29) in which a mixture of biotinylated, Alexa 488-conjugated, and unlabeled actin monomers is assembled into filaments in vitro and then attached to slides coated with anti-biotin. The cells were then treated for 1 min with DMSO or with 2 μM jasplakinolide (Jasp) to stabilize F-actin filaments before being added to chamber slides coated with anti-IgM or anti-CD3/CD28. Fluorescence images of the cell–substrate interface were collected in real time using confocal microscopy. The left panels show representative still images taken when the cells first contacted the substrate (0 min) or after 20 min of contact with the substrate. Scale bars, 10 μm. Cell–substrate contact areas were calculated from these images and are graphed (mean ± SD for >100 cells from three experiments) in the right panels. ***p < 0.001 (compared with DMSO-treated cells at the same time point). B, Actin filaments containing Alexa 488-labeled and biotinylated actin monomers were immobilized on slides using anti-biotin Abs and imaged before and after a 5-min incubation with extracts from unstimulated or anti-IgM–stimulated (for 10 min) murine splenic B cells. Representative images of the fluorescent actin filaments remaining after incubation with the indicated cell lysate are shown. Each image represents a 64 μm x 64 μm field. Where indicated, a cofilin Ab or rabbit IgG was added to the stimulated cell extract. The percent of F-actin released was calculated according to the equation: [1 – (fluorescence after incubation with cell extract)/(fluorescence before adding cell extract)] × 100%. For each condition, the mean ± SEM for three random fields from each of three experiments is graphed. ***p < 0.001 (compared with unstimulated cells). Cell lysis buffer alone did not cause detectable F-actin severing (data not shown). C–E, Phosphorylation of cofilin on serine 3 (pS3 cofilin) in LPS/IL-4–activated murine splenic B cells, murine T cells, or Jurkat cells was assessed by immunoblotting. For each panel, similar results were obtained in two to four experiments.

FIGURE 2. AgR signaling induces cofilin-mediated F-actin severing and cofilin dephosphorylation. A. LPS/IL-4–activated murine splenic B cells or IL-2–activated murine T cells were incubated with CellMask Orange to label their plasma membranes. The cells were then treated for 1 min with DMSO or with 2 μM jasplakinolide (Jasp) to stabilize F-actin filaments before being added to chamber slides coated with anti-IgM or anti-CD3/CD28. Fluorescence images of the cell–substrate interface were collected in real time using confocal microscopy. The left panels show representative still images taken when the cells first contacted the substrate (0 min) or after 20 min of contact with the substrate. Scale bars, 10 μm. Cell–substrate contact areas were calculated from these images and are graphed (mean ± SD for >100 cells from three experiments) in the right panels. ***p < 0.001 (compared with DMSO-treated cells at the same time point). B, Actin filaments containing Alexa 488-labeled and biotinylated actin monomers were immobilized on slides using anti-biotin Abs and imaged before and after a 5-min incubation with extracts from unstimulated or anti-IgM–stimulated (for 10 min) murine splenic B cells. Representative images of the fluorescent actin filaments remaining after incubation with the indicated cell lysate are shown. Each image represents a 64 μm x 64 μm field. Where indicated, a cofilin Ab or rabbit IgG was added to the stimulated cell extract. The percent of F-actin released was calculated according to the equation: [1 – (fluorescence after incubation with cell extract)/(fluorescence before adding cell extract)] × 100%. For each condition, the mean ± SEM for three random fields from each of three experiments is graphed. ***p < 0.001 (compared with unstimulated cells). Cell lysis buffer alone did not cause detectable F-actin severing (data not shown). C–E, Phosphorylation of cofilin on serine 3 (pS3 cofilin) in LPS/IL-4–activated murine splenic B cells, murine T cells, or Jurkat cells was assessed by immunoblotting. For each panel, similar results were obtained in two to four experiments.
a phospho-site-specific Ab, stimulating murine splenic B cells with soluble, bead-bound, or plate-bound anti-IgM resulted in dephosphorylation of cofilin on S3 (Fig. 2C). Dephosphorylation of cofilin on S3 also occurred when murine splenic T cells or Jurkat T cells were stimulated with soluble or plate-bound anti-CD3/CD28 (Fig. 2D, 2E). Thus, AgR signaling induces cofilin dephosphorylation, which correlates with increased F-actin–severing activity within the cells.

**FIGURE 3.** Rap activation is required for peripheral actin dynamics, F-actin severing, and actin incorporation at barbed ends. A, Cells were transiently transfected with actin–GFP, plated on anti-IgG–coated slides, and imaged in real time by confocal microscopy for 10 min. Videos recordings of the GFP and DIC channels (see Supplemental Video 5) were combined to generate kymographs from representative cells. Scale bar, 10 μm. B, FRAP analysis of actin dynamics in membrane-proximal ROIs of cells that were transiently transfected with actin–GFP and plated on anti-IgG for 1 h. Representative images show FRAP in single cells (left panels). Scale bars, 10 μm. Fluorescence recovery over time (mean ± SEM for 30 cells from three experiments) is graphed (right panel). C, Cells expressing utrophin ABD–RFP and photo-convertible (PC) actin–GFP were plated on anti-IgG. After initiating actin–GFP fluorescence in a membrane-proximal ROI (white box) by brief ultraviolet illumination, GFP and RFP signals were monitored in real time by confocal microscopy. Representative images show the loss of GFP fluorescence from the ROI. Scale bars, 10 μm. The graph shows the ratios of the actin–GFP signal to the ABD–RFP signal (a measure of total F-actin) in the ROI, relative to the time 0 value. Each point is the mean ± SEM for 30 cells from three experiments. D, Cells were plated on anti-IgG for 1 h and then permeabilized for 30 s in the presence of Alexa 488–actin. F-actin was visualized with rhodamine–phalloidin. Scale bars, 10 μm. The graphs show Alexa 488–actin incorporation and the ratio of this value to the total F-actin in the cell (mean ± SEM for 20 cells from three experiments). *p < 0.05, **p < 0.01, ***p < 0.001 (compared with A20/vector cells at the same time point). PA, preactivation (i.e., prior to UV activation of actin–GFP); PB, prebleach.
Because BCR-induced activation of the Rap GTPases is required for B cell spreading and IS formation (10), we assessed whether Rap activation was required for the increased actin dynamics and cofilin-mediated F-actin severing that accompanied lymphocyte spreading. To assess the effects of blocking Rap activation, we used A20 cells (an IgG⁺ B cell line) that had been stably transfectected with RapGAP11 (10), a Rap-specific GTase-activating protein that enzymatically converts active GTP-bound Rap to the inactive GDP-bound state. We also interfered with Rap activation and function by transiently expressing Rap1N17, a DN form of Rap1.

Actin–GFP–expressing A20 cells underwent rapid spreading when plated on immobilized anti-IgG, and this was accompanied by the incorporation of actin–GFP into peripheral F-actin structures (Fig. 3A, Supplemental Video 5). In contrast, when Rap activation was blocked by expression of RapGAP11, the cells did not spread and incorporated less actin–GFP into peripheral F-actin structures (Fig. 3A). Similarly, expressing Rap1N17 in Jurkat cells blocked anti-CD3/CD28-induced spreading (see Fig. 5C). Real-time confocal imaging of the cell–substrate contact region revealed dynamic incorporation of actin–GFP into peripheral actin structures when murine splenic B cells, A20 cells, and Jurkat cells contacted surfaces coated with anti-Ig or anti-CD3/CD28 Abs (Supplemental Fig. 1). In contrast, GFP-labeled F-actin structures in RapGAP11-expressing A20 cells and Rap1N17-expressing Jurkat cells were more static (Supplemental Fig. 1B, 1C). FRAP analysis also showed that peripheral F-actin structures were more dynamic in A20/vector cells than in A20/RapGAP11 cells (Fig. 3B). In A20/vector cells, 70–80% of the actin–GFP fluorescence in peripheral ROIs was recovered by 60 s after photobleaching, compared with ~30% in A20/RapGAP11 cells. Thus, Rap activation is required for lymphocyte spreading and for the underlying peripheral actin dynamics.

Rap activation was also required for BCR engagement to stimulate both actin severing and actin polymerization at barbed ends. To assess actin severing in live cells, we developed an assay for visualizing the release of fluorescent actin monomers from F-actin filaments in real time. Cells were cotransfected with a photo-convertible form of actin–GFP, which assumes a fluorescent conformation when subjected to UV illumination, and with the ABD of utrophin fused to RFP, which can be used to visualize actin filaments in live cells (23). Real-time imaging of a membrane-proximal ROI allowed us to visualize dissociation of the actin–GFP from F-actin structures, as indicated by loss of the GFP signal from the ROI. This showed that BCR-induced dissociation of actin–GFP from actin filaments occurred to a greater extent in A20/vector cells than in A20/RapGAP11 cells (Fig. 3C). Rap activation was required not only for BCR-induced F-actin severing but also for actin polymerization at the barbed ends of actin filaments, as judged by the de novo incorporation of Alexa 488–actin into F-actin structures in semipermeabilized cells (Fig. 3D).

Consistent with the finding that Rap activation was important for BCR-induced F-actin severing, AgR-induced dephosphorylation and activation of cofilin also required Rap activation. Blocking Rap activation by expressing RapGAP11 in A20 cells prevented the dephosphorylation of cofilin induced by soluble, bead-bound, and plate-bound anti-Ig (Fig. 4A–C). Similarly, expressing the DN Rap1N17 protein in Jurkat cells blocked anti-CD3/CD28-induced cofilin dephosphorylation (Fig. 4D). Importantly, the increase in cofilin-mediated F-actin–severing activity caused by BCR engagement was completely abrogated when Rap activation was blocked (Fig. 4E). Thus, Rap activation is required for AgR-induced cofilin activation, F-actin severing, and subsequent actin polymerization at barbed ends.

Cofilin activation is required for lymphocyte spreading and actin dynamics

To test whether the activation of cofilin by its dephosphorylation on S3 is required for lymphocyte spreading, we expressed in A20 cells a catalytically inactive form of Slingshot phosphatase (SSH), the major phosphatase that acts on cofilin (24). Cells expressing this mutant SSH with a C→S replacement in its active site (SSH-C/S) had increased levels of phosphorylated cofilin, and when plated on immobilized anti-Ig spread to a lesser degree than neighboring untransfected cells or cells transfected with the empty vector (Fig. 5A). We also implicated cofilin activation in AgR-induced spreading by expressing a mutant form of cofilin that assumes an inactive conformation because of an S→D phospho-mimetic substitution at position 3. A20 cells expressing this cofilin S3D mutant as an mCherry fusion protein (Supplemental Fig. 2) did not spread on immobilized anti-Ig (Fig. 5B) and exhibited impaired actin dynamics (i.e., reduced recovery of actin–GFP fluorescence after photobleaching) (Fig. 5D, left panel). Thus, cofilin dephos-
phorylation and activation are required for BCR-induced cell spreading and the underlying actin dynamics. To test if Rap–GTP promotes cell spreading and actin dynamics primarily by controlling cofilin activation, we asked whether expressing a nonphosphorylatable “active” mutant form of cofilin (S→A mutation at position 3) could overcome the effects of blocking Rap activation. Indeed, expressing a cofilin S3A–mCherry fusion protein (Supplemental Fig. 2) restored the ability of A20/RapGAPII cells to spread on immobilized anti-Ig (Fig. 5B) and the ability of Rap1N17-expressing Jurkat cells to spread on anti-CD3/CD28 (Fig. 5C). Cofilin S3A expression also restored peripheral actin dynamics in both A20/RapGAPII cells and Rap1N17-expressing Jurkat cells, as shown by the increased recovery of actin–GFP fluorescence after photobleaching compared with A20/RapGAPII cells (Fig 5D, right panel) or Jurkat cells expressing only Rap1N17 (Fig. 5E). The inability of wild-type (WT) cofilin to bypass the block in Rap activation in A20/RapGAPII cells (Fig. 5B and 5D, right panel) indicates that cell spreading and actin

FIGURE 5. Rap-dependent cofilin dephosphorylation is required for cell spreading and actin dynamics. A, A20 cells were transiently transfected with Myc-tagged SSH-C/S, plated on immobilized anti-IgG for 1 h, and then stained for Myc (green), phospho-cofilin (blue), and F-actin (left panel). Representative images of z-axis projections and a single z-slice along the dotted line are shown. Scale bar, 10 μm. In the right panel, A20 cells were transiently transfected with either Myc-tagged SSH-C/S or the empty vector, plated on immobilized anti-IgG for 1 h, and then stained for Myc (green) and F-actin. The cell area for cells expressing Myc-tagged SSH-C/S or transfected with the empty vector (mean ± SEM for 30 cells from three experiments) is graphed. ***p < 0.001. B and C, A20/vector and A20/RapGAPII cells (B) were transiently transfected with actin–GFP as well as WT or mutant forms of cofilin fused to mCherry. Jurkat cells (C) were transiently transfected with actin–GFP and the indicated constructs. Cells were plated on anti-Ig or anti-CD3/CD28, and mCherry-expressing cells (inset, red) were imaged in real time as in Fig. 3A. Kymographs (upper panels) show superimposed DIC and actin–GFP images for representative cells. Images of cell–substrate contacts for representative mCherry-expressing cells (lower panels) are shown, along with cell area (mean ± SEM) for 30 cells from three experiments (right panels). Scale bars, 10 μm. **p < 0.01, ***p < 0.001. D and E, FRAP was performed as in Fig. 1B. Recovery of actin–GFP fluorescence within photobleached ROIs (mean ± SD for 30 cells from three experiments) is graphed.
dynamics require increased cofilin activity and not just increased levels of cofilin.

The Rap–cofilin pathway is required for B cells to spread across APCs and gather Ag into microclusters

The spreading of lymphocytes on APCs bearing cognate Ag allows additional AgRs to encounter Ag and promotes the formation of AgR microclusters that initiate signaling. To model these events, we generated surrogate APCs by transfecting B16F1 cells with a dimeric transmembrane form of a single-chain rat anti-mouse Igκ Ab (31), which can cluster κ-chain–containing BCRs (Fig. 6A). After allowing B cells to bind these APCs, we used Alexa 488–anti-rat IgG to detect the single-chain anti-Igκ Ab and anti–p-Tyr staining to visualize BCR signaling. LPS/IL-4–activated splenic B cells that bound to these APCs gathered the single-chain anti-Igκ surrogate Ag into microclusters that corresponded to sites of p-Tyr signaling (Fig. 6B, 6C). Very similar responses were observed when we imaged the interaction of IgM+ WEHI-231 B-lymphoma cells (see Supplemental Fig. 3B) or IgG+ A20 B-lymphoma cells (Fig. 7A) with B16F1 cells expressing the single-chain anti-Igκ Ab. Analysis of splenic B cells that had adhered to B16F1 expressing different levels of the single-chain anti-Igκ surrogate Ag revealed a roughly linear relationship between the amount of Ag gathered by a B cell and the amount of p-Tyr signaling (Fig. 6D). We were also able to express the single-chain anti-Igκ at a low frequency in bone marrow-derived DCs and showed that splenic B cells formed an IS when they adhered to these transfected DCs (Supplemental Fig. 3A).

We then used the B16F1 surrogate APC system to assess how the Rap–cofilin pathway affects Ag gathering, BCR microcluster formation, and BCR signaling in the context of B cell–APC interactions. Imaging the B cell–APC interface showed that A20/ vector cells that were allowed to attach to the surrogate APCs for 5 min extended F-actin–rich membrane processes along the surface of the APC and formed small p-Tyr–rich microclusters that colocalized with the surrogate Ag (Fig. 7A). By 10 min, the size and number of these microclusters increased as the A20 cells began to contract (Fig. 7A). In contrast, A20/RapGAPII did not spread to the same extent on the surface of the APC, as indicated by lower amounts of F-actin at the contact site. Importantly, this correlated with a significant reduction in the amount of Ag gathered, the number and size of BCR microclusters, and the amount of p-Tyr signaling (Fig. 7A). Similar results were obtained when we compared vector control and RapGAPII-expressing WEHI-231 B cells (Supplemental Fig. 3B). As well, both BCR microcluster formation and p-Tyr signaling were greatly decreased when the DN Rap1N17 protein was expressed in primary B cells (Fig. 7B). Thus BCR-induced Rap activation is required for B cells to spread on the surfaces of APCs, gather Ag, and form BCR microclusters that initiate p-Tyr signaling.

To assess whether Rap–GTP promotes Ag gathering and BCR microcluster formation via its ability to initiate cofilin-dependent

FIGURE 6. Surrogate APCs expressing a single-chain anti-Igκ Ab induce B cell spreading, BCR microcluster formation, and BCR signaling. A, Schematic of BCR engagement by B16F1 cells expressing a transmembrane form of a single-chain anti-Igκ Ab. B–D, LPS/IL-4–activated splenic B cells were plated on these APCs for 10 min, and conjugates were stained for the anti-Igκ surrogate Ag (green), p-Tyr (red), and F-actin (blue). Representative three-dimensional images show multiple B cells (arrowheads) bound to a single APC (B). Enlarged images of a single B cell show colocalization of the surrogate Ag and p-Tyr signaling (C). The relationship between the fluorescence signals representing the amount of Ag gathered and the amount of p-Tyr signaling, expressed as arbitrary units (a.u.), is plotted (D). Each dot is an individual B cell. Scale bars, 10 μm.
F-actin severing, we modulated cofilin activity in A20 cells. Preventing cofilin activation by expressing SSH-C/S, as well as by expressing the cofilin S3D phospho-mimetic mutant, significantly reduced the gathering of Ag into microclusters and the p-Tyr signaling at B cell–APC contact sites (Fig. 8). Conversely, expressing the activated cofilin S3A mutant protein in A20/RapGAPII cells overcame the defect in Ag gathering and microcluster-based p-Tyr signaling caused by blocking Rap activation (Fig. 8). For all cell types analyzed, there was a roughly linear relationship between the amount of Ag gathered by the B cell at the contact site and the amount of microcluster-based p-Tyr signaling (Supplemental Fig. 4). Thus, the Rap–cofilin module controls the ability of B cells to gather Ag, and this is directly related to the amount of BCR signaling that ensues.

ERM protein inactivation, which uncouples the plasma membrane from the cytoskeleton and decreases membrane rigidity (18, 34), is important for lymphocyte spreading and microcluster formation (19). However, we found that abrogating ERM-mediated coupling of the plasma membrane to the cytoskeleton did not bypass the requirement for cofilin-mediated actin severing. Overexpressing a truncated, DN form of ezrin that cannot bind actin, and which presumably prevents endogenous ERM proteins from linking the plasma membrane to the cytoskeleton, did not restore normal levels of Ag gathering in A20 cells in which cofilin-mediated actin severing was blocked by expressing either SSH-C/S or RapGAPII (Fig. 9, Supplemental Fig. 3C). Thus, F-actin severing is important for BCR microcluster formation, and its role in this process is not redundant with that of ERM protein inactivation.

**The Rap–cofilin pathway controls the mobility of BCR microclusters**

The submembrane cytoskeleton acts as a barrier to BCR diffusion, thereby preventing spontaneous BCR signaling (9). Treating B cells with actin-depolymerizing drugs results in increased BCR mobility, Ag-independent microcluster formation, and Ag-independent BCR signaling (13, 16). We found that disrupting the actin cytoskeleton with latrunculin A induced Ag-independent ERK phosphorylation to the same extent in A20/vector and A20/RapGAPII cells (Fig. 10A). This finding suggests that the main function of Rap activation in the initiation of BCR signaling is to promote cytoskeletal disassembly and allow the formation of signaling-active BCR microclusters.

To test whether the Rap–cofilin pathway regulates the mobility of nascent BCR microclusters, we used A20 cells that express two different BCRs, one containing the endogenous murine IgG (mIgG) and the other containing human IgM (hIgM). This allowed us to initiate signaling by the hIgM-containing BCRs using anti-IgM antibodies and then measure the mobility of the resulting BCR microclusters (Fig. 11). We found that in A20/vector cells, the hIgM-containing BCRs formed microclusters that were immobile, whereas in A20/RapGAPII cells, the hIgM-containing BCRs formed microclusters that were highly mobile, similar to those formed by the mIgG-containing BCRs (Fig. 11B). This suggests that the Rap–cofilin pathway controls the mobility of BCR microclusters, and that this pathway is important for the proper functioning of BCR signaling.

**FIGURE 7.** Rap activation is important for B cell spreading, Ag gathering, and BCR signaling during B cell–APC interactions. A, A20/vector cells (upper panels) and A20/RapGAPII cells (lower panels) were allowed to adhere to B16F1 cells expressing the single-chain anti-IgG Ab for 5 or 10 min and then stained as in Fig. 6. Images of a single z-slice along the dotted line (top rows), the z-projection (middle rows), and the xy-plane at the B cell–APC contact site (bottom rows) are shown for representative cells. Graphs show quantification in arbitrary units (a.u.) of F-actin in the A20 cells, the amount of Ag gathered, and the p-Tyr signal in the A20 cells at the plane of contact with the APC (mean ± SD for >60 APC-associated A20 cells from three experiments). Scale bars, 10 μm. ***p < 0.001. B, LPS/IL-4–activated splenic B cells were transiently transfected with a 1:3 ratio of a plasmid encoding GFP and either pcDNA3.1 or pcDNA3.1-Rap1N17. The cells were allowed to adhere to B16F1 cells expressing the single-chain anti-IgG Ab for 10 min and then stained with rabbit anti-p-Tyr and with Alexa 568–anti-rat IgG to image the single-chain anti-IgG. Representative images of GFP-positive cells (inset, white) are shown. Scale bar, 10 μm.
hIgM immobilized on a coverslip while inducing the formation of small, mobile mIgG-containing microclusters using a low concentration of Cy3-labeled anti-mIgG F(ab’2) plus a 10-fold excess of unlabeled anti-mIgG Fab fragments. By using cells transfected with actin–GFP, we could employ real-time TIRFM to image the mobility of nascent mIgG-containing BCR microclusters at the contact site, relative to the F-actin cytoskeleton. Although BCR microclusters exhibited a wide-range of diffusion coefficients, Rap1N17-expressing and SSH-C/S–expressing cells had fewer fast-diffusing microclusters than control cells (Fig. 10B,10C, Supplemental Videos 6–9). The mean diffusion coefficient of mIgG microclusters in cells expressing Rap1N17 or SSH-C/S (0.026 and 0.034 μm²/s, respectively) was about half that in A20 cells transfected with the empty vector (0.069 μm²/s) (Fig. 10D) and was similar to the mean diffusion coefficient of BCR microclusters in unstimulated A20 B cells (16). This suggests that BCR signaling increases BCR microcluster mobility by activating the Rap–cofilin pathway and promoting F-actin severing. Consistent with this idea, the density of submembrane F-actin, as assessed by TIRFM, was greater in Rap1N17-expressing and SSH-C/S–expressing cells than that in control cells (Fig. 10D, Supplemental Videos 7–9). Taken together, these data indicate that activation of the Rap–cofilin pathway by the BCR removes cytoskeletal barriers that limit the formation of BCR microclusters and their mobility within the plasma membrane (Fig. 11).

Discussion

Severing of the cortical membrane cytoskeleton is a rate-limiting step in cell shape change (35) and an initiating event in which Arp2/3-mediated actin polymerization at newly created barbed ends leads to a dynamic branched F-actin network that exerts outward force on the plasma membrane. We show for the first time to our knowledge that cofilin activation and cofilin-mediated F-actin severing are regulated by the Rap GTPases. Previous work had shown that activated Rap promotes actin polymerization via effector proteins such as Vav2, TIAM1, and RIAM (12). Thus, Rap acts as a master regulator of cell morphology and cytoskeletal organization via its ability to promote both cofilin-mediated F-actin severing and subsequent branched actin polymerization.

B cell activation in vivo may often involve the interaction of B cells with APCs bearing captured Ags. The ability of B cells to spread across the surface of an APC increases potential encounters with membrane-bound Ags, thereby enhancing the formation of BCR microclusters that initiate signaling. Thus, the cytoskeletal reorganization that promotes B cell spreading is a critical determinant of whether BCR signaling exceeds the threshold for B cell activation. Our data provide new insights into how these cytoskeletal changes are initiated (summarized in Fig. 11). We show that Rap-dependent activation of cofilin is required for BCR signaling to stimulate F-actin severing and to initiate the accelerated actin dynamics that drive cell spreading. Moreover, by using surrogate APCs, we show that activation of the Rap–cofilin signaling module...
FIGURE 10. The Rap–cofilin pathway controls BCR microcluster mobility. A, Cells were treated with latrunculin A for the indicated times. Cell extracts were probed for p-ERK and total ERK. B–D, A20 cells that express both hlgM and mlgG were transfected with actin–GFP plus Rap1N17, SSH-C/S, or the empty pcDNA3.1 vector. The cells were mixed with 100 ng/ml Cy3-labeled anti-mlgG F(ab')2 plus 1 μg/ml anti-mlgG Fab, plated on anti-hlgM for 2 min, and then imaged by TIRFM. B shows the diffusion coefficients for BCR microclusters (>500 tracks from >25 cells in five experiments). **p < 0.001 (compared with cells transfected with the empty vector). C shows the relative frequencies of mlgG microclusters with diffusion coefficients in the indicated ranges. D shows representative images of BCR microclusters (red) and their tracks (blue lines) alone (left panels) or merged with the actin–GFP signal (right panels). Scale bar, 10 μm. BCR microcluster movement is shown in Supplemental Videos 6–9.

promotes the formation of BCR microclusters and is a key determinant of the extent of Ag gathering by BCR microclusters and the magnitude of resulting BCR signaling. BCR-induced activation of the Rap–cofilin module also leads to increased mobility of nascent BCR microclusters, presumably by breaking down cytoskeletal barriers that limit BCR diffusion. This can facilitate the formation of larger, more mature microclusters with enhanced signaling capabilities. Thus, activation of the Rap–cofilin signaling module couples cell spreading with increased BCR mobility, thereby optimizing Ag encounter, microcluster formation, and BCR signaling.

Although ex vivo murine splenic B cells transiently extend membrane processes across Ag-bearing surfaces (3), LPS/IL-4–activated primary B cells exhibit much more dramatic spreading on Ag-bearing APCs than do resting B cells (S.A. Freeman and M.R. Gold, unpublished observations). This is consistent with previous work by Severinson and colleagues (36) showing that activated primary B cells exhibit a sustained spreading response when plated on immobilized Abs against membrane Ig, CD44, LFA-1, and other cell surface proteins, whereas resting B cells do not. By altering cytoskeletal regulation, danger signals such as TLR ligands may prime B cells for enhanced actin dynamics and BCR microcluster mobility upon APC encounter. This could increase the probability of the APC–B cell interaction leading to B cell proliferation and Ab production, especially when Ag densities are low. This may be a novel mechanism by which danger signals lower the threshold for B cell activation when Ags are presented in the context of microbial infections or adjuvants. It is intriguing to speculate that memory B cells are also primed for enhanced actin dynamics and undergo robust spreading in the absence of TLR stimulation, as is the case for the B cell lines we examined. Although the nature of this TLR-induced change in B cell cytoskeletal regulation is not known, preliminary results indicate that the basal level of phosphorylated cofilin is somewhat lower in activated B cells than in ex vivo B cells and that the extent of BCR-induced cofilin dephosphorylation is significantly greater in LPS- or CpG DNA-activated primary B cells than in ex vivo cells (S.A. Freeman and M.R. Gold, unpublished observations). It would be of interest to test whether B cell activation increases the expression or activity of SSH or other phosphatases that dephosphorylate cofilin and to determine whether this is the key regulatory switch that enhances actin dynamics and cell spreading in activated B cells.

To study the relationship between Ag gathering and BCR signaling, we developed a novel surrogate APC system that can be used with any B cell expressing mouse κ L-chain. Using this system, we validated observations made using lipid bilayers, demonstrating a relationship between the extent of lymphocyte spreading, the amount of Ag gathered into BCR microclusters, and the magnitude of resulting BCR signaling. Such surrogate APCs can facilitate detailed studies of the mechanisms that underlie the dynamic cytoskeletal and membrane reorganization that occur when B cells encounter Ag-bearing APCs. In this report, we show that both Rap activation and cofilin dephosphorylation are important for cell spreading, Ag gathering, microcluster formation, and BCR signaling induced by APC-associated Ags. Our observation that expressing the nonphosphorylatable S3A “active” form of cofilin overcame the impaired cell spreading and Ag gathering caused by blocking Rap activation indicates that promoting cofilin activation is one of the main functions of Rap during APC-induced B cell activation. Activated Rap also promotes integrin activation in B cells (21), which enhances B cell spreading in lipid bilayer models of B cell–APC interactions (37). However, expressing the cofilin S3A mutant protein did not restore the
ability of A20/RapGAPII cells to adhere to and spread on immobilized integrin ligands (data not shown). This indicated that the active cofilin S3A protein restored BCR-induced spreading in A20/RapGAPII cells via its effects on actin dynamics, as opposed to restoring integrin activation.

Dephosphorylation of cofilin on S3 exposes a globular region that can bind to actin filaments and induce a structural twist that leads to severing of the filament (38). The mechanism by which BCR-induced Rap activation promotes cofilin dephosphorylation remains to be determined. Cofilin is phosphorylated on S3 by LIM domain kinase, but BCR signaling did not reduce the amount of phosphorylated, active LIM domain kinase in B cells (data not shown). Instead, Rap–GTP could act through various effector proteins to increase the enzymatic activity of SSH or promote the release of SSH from 14-3-3 proteins that sequester it away from cofilin. Rap may also control cofilin dephosphorylation by regulating local cytoskeletal dynamics. Consistent with this idea, disrupting the actin cytoskeleton with latrunculin A increased cofilin phosphorylation in A20 cells, whereas stabilizing the actin cytoskeleton with jasplakinolide caused complete dephosphorylation of cofilin, even when Rap activation was blocked (data not shown).

Another question that remains to be addressed is which Rap proteins regulate cofilin dephosphorylation in B and T cells. There are five Rap proteins, Rap1A, Rap1B, Rap2A, Rap2B, and Rap2C, each of which is encoded by a separate gene. We have shown that the BCR activates both Rap1 and Rap2 proteins and that expressing RapGAPII, as we have done in this study, blocks the activation of both Rap1 and Rap2 by the BCR (21). Further studies using cells in which individual Rap genes are disrupted or silenced would be required to assess the relative roles of the different Rap proteins in regulating cofilin.

The submembrane skeleton of cortical actin filaments is linked to the plasma membrane by ERM family proteins. This creates cytoskeletal barriers that limit and spatially structure the diffusion pattern of membrane proteins and, in doing so, regulates receptor signaling (19, 39). In lymphocytes, these actin corrals and picket fences maintain cells in a resting state by limiting spontaneous AgR aggregation and signaling (16). The removal of these barriers in response to initial AgR signaling allows AgR microclusters to form and assemble signalosomes. These early events in AgR signaling appear to involve both the inactivation of ERM proteins (9) and F-actin severing. We found that BCR-induced activation of the Rap–cofilin signaling module is important for BCR microcluster formation and that it acts independently of ERM protein inactivation. Indeed, the activation of both Rap and cofilin was required for BCR-induced cell spreading and microcluster formation even when ERM protein-mediated coupling of the membrane to the cytoskeleton was abrogated by expressing a DN form of ezrin.

In summary, our demonstration that F-actin severing initiated by the Rap–cofilin pathway is important for AgR microcluster formation and microcluster-based signaling provides new insights into these critical early events in AgR signaling.

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