Molecular Biology of the Cell
Vol. 16, 2275–2284, May 2005

Actin Depolymerization Transduces the Strength of B-Cell Receptor Stimulation[D]

Shengli Hao* and Avery August

Immunology Research Laboratories, Department of Veterinary Science, and Integrated Bioscience Graduate Program of the Huck Institute for Life Sciences, Cell and Developmental Biology Graduate Option, The Pennsylvania State University, University Park, PA 16802

Submitted October 8, 2004; Revised January 3, 2005; Accepted February 13, 2005
Monitoring Editor: Anne Ridley

Polymerization of the actin cytoskeleton has been found to be essential for B-cell activation. We show here, however, that stimulation of BCR induces a rapid global actin depolymerization in a BCR signal strength-dependent manner, followed by polarized actin repolymerization. Depolymerization of actin enhances and blocking actin depolymerization inhibits BCR signaling, leading to altered BCR and lipid raft clustering, ERK activation, and transcription factor activation. Furthermore actin depolymerization by itself induces altered lipid raft clustering and ERK activation, suggesting that F-actin may play a role in separating lipid rafts and in setting the threshold for cellular activation.

INTRODUCTION

The actin cytoskeleton regulates a number of cellular functions by a process that includes dynamic changes between monomer actin (G-actin) and filamentous or polymerized actin (F-actin) via polymerization and depolymerization. Several toxins can alter this G/F-actin balance. Jasplakinolide (JP), for example, is a toxin that specifically binds to F-actin and prevents it from depolymerizing, skewing the F/G actin balance to actin polymerization (Bubb *et al.*, 1994). By contrast, Latrunculin B (LatB), sequesters G-actin, leading to actin depolymerization (Spector *et al.*, 1983, 1989; Coue *et al.*, 1987). The actin cytoskeleton is important for lymphocyte antigen receptor signaling. Several lines of evidence suggest essential roles for the actin cytoskeleton in the transduction of antigen receptor signals. First, mutation or lack of proteins that regulate the actin cytoskeleton, such as the GTPase Rac, the guanine nucleotide exchange factor Vav, or WASP, lead to severe immune deficiencies (Derry *et al.*, 1994; Symons *et al.*, 1996; Penninger and Crabtree, 1999a; Zhang *et al.*, 1999; Gomez *et al.*, 2000; Tedford *et al.*, 2001; Gu *et al.*, 2003; Walmsley *et al.*, 2003). Second, disruption of the actin cytoskeleton with cytochalasin D terminates ongoing T-cell receptor (TCR) signals and abrogates cell proliferation and activation when T-cells are stimulated by antigen presenting cells (APC; Valitutti *et al.*, 1995; Delon *et al.*, 1998; Grakoui *et al.*, 1999). Third, actin polymerization or F-actin has been found to be involved in recruiting signaling molecules into lipid rafts, special lipid domains on the cell membrane that serve as signaling platforms (Cheng *et al.*, 1999; Janes *et al.*, 1999; Penninger and Crabtree, 1999b; Dustin and Cooper, 2000; Valensin *et al.*, 2002; Gupta and DeFranco, 2003). These data suggest that actin polymerization or F-actin plays a positive role in transducing lymphocyte antigen receptor signals. However, the exact role of F-actin in regulating lymphocyte antigen receptor signaling is still unclear.

Here we show that F-actin also plays a negative role in regulating B-cell receptor (BCR) signals. We show that the BCR induces an early rapid wave of actin depolymerization, which is dependent on the level of BCR cross-linking. Disrupting F-actin blocks BCR signals, whereas induction of partial depolymerization of actin leads to enhanced BCR signals. Furthermore, actin depolymerization alone can activate signaling pathways used by the BCR. These dynamic actin changes enhance BCR signals by enhancing lipid raft clustering and duration, leading to enhanced BCR signaling.

MATERIALS AND METHODS

Cells and Reagents

WT DT40 cells were generously provided by Dr. T. Kurosaki (Kansai Medical University and RIKEN Research Center for Allergy and Immunology, Moriguchi, Japan). They were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% chicken serum (Sigma, St. Louis, MO). The mouse B-cell line WEHI-231 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 supplemented with 10% FBS, 50 μM 2-mercaptoethanol and penicillin-streptomycin. Primary murine B-cells were purified from spleens of BALB/c mice (6–8 wk old) using the MACS B-cell isolation kit (Miltenyi Biotec, Auburn, CA). Protein-A HRP and rabbit anti-mouse HRP, Fura2-AM was from Sigma. Antibodies against phosphotyrosine (PY99), c-Myc (9E10), Syk (N-19), and bovine anti-mouse IgM rhodamine were from Santa Cruz Biotechnology (PY99, Santa Cruz, CA), against phospho-ERK and ERK from Cell Signaling (Waltham, MA), against Syk (N-19), goat anti-mouse IgM μ chain specific $F(ab')_2$ and Fab fragment unconjugated or conjugated with Rhodamine red-X were from Jackson ImmunoResearch Laboratories (West Grove, PA). Cholera toxin subunit B (CTB)-Alexa 647, phalloidin-Alexa 568, goat anti-mouse Alexa 488 were from Molecular Probes (Eugene, OR), Optiprep from Axis-shield PoC AS (Oslo, Norway), and CTB-HRP was from Sigma. SRF, NFκB, and NFAT luciferase reporter plasmids were from Stratagene (La Jolla, CA). Luciferase activities were detected using a Promega Luciferase Reporter Assay kit (Madison, WI). Goat anti-chicken IgM was from Bethyl Laboratories (Montgomery, TX).

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04–10–0881) on February 23, 2005.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

* Present address: Division of Biology, California Institute of Technology, 1200 E. California Boulevard, MC 147-75, Pasadena, CA 91125.

Address correspondence to: Avery August (axa45@psu.edu).

Western Blotting

Unstimulated or stimulated cells (5×10^6 cells/sample) were lysed in 100 μl Triton X-100 lysis buffer, denatured, resolved by 10% SDS-PAGE, and transferred to PVDF membranes (Pall Life Sciences, Glen Cove, NY). The indicated proteins were detected with the appropriate primary and secondary antibodies conjugated to HRP, and HRP activities were detected using the ECL plus system (Amersham, Piscataway, NJ). Images in Figure 6B were quantitated using NIH ImageJ, with the value from the unstimulated cells set at 1.

Ca^{2+} Measurements

Calcium measurements were performed using Fura2-AM as previously described (Hao *et al.*, 2003). Cells were stimulated with goat anti-chicken IgM antibodies and continuous fluorescence was detected at 22°C with a Hitachi F-2000 fluorospectrophotometer (Hitachi, San Jose, CA) at an excitation wavelength of 340 and 380 nm and an emission wavelength of 510 nm. Relative intracellular Ca^{2+} is shown as the ratio of fluorescence intensity at 340- to 380-nm excitation.

Cell Transfection and Luciferase Activity Assay

SRF and NFAT luciferase activities were performed as previously described (Hao *et al.*, 2003). NFκB-luciferase activities were performed in a similar manner. Cells were transfected with the relevant luciferase reporter plasmids, followed by a recovery period in growth medium for 8 h. Cells were then starved in serum-free RPMI 1640 supplemented with 10 mM HEPES, pH 7.2, for 12 h and then equally split into different groups that were either stimulated by adding 4 μg/ml anti-chicken IgM, other stimulants as indicated, or no stimulation as control for 8 h. Cells treated with inhibitors were pretreated for 30 min before stimulation unless stated otherwise. Cells were then harvested, counted, and lysed for luciferase assay using a Promega luciferase assay kit. The results are expressed as fold over nonstimulated control and are the mean ± SD of 3–6 independent experiments done in triplicate unless otherwise indicated.

Visualization of Lipid Rafts and F-actin by Confocal Microscopy

Cells (5×10^6 in 200 μl RPMI/1640, 20×10^6/ml) were pretreated with or without LatB or JP at 37°C for 1 h or as otherwise indicated and then incubated with cholera toxin B-FITC (CT-B, final concentration 25 μg/ml) in phosphate-buffered saline (PBS; 100×10^6/ml) containing 1% bovine serum albumin (BSA) at 4°C for 20 min in dark. The unbound CT-B was removed by washing twice with cold PBS. The cells were then resuspend in 200 μl PBS (or RPMI 1640) and stimulated with anti-IgM ($F(ab')_2$ fragments) or other stimulants at 37°C for the indicated times. The cells were then fixed immediately by adding 400 μl 3% paraformaldehyde solution (final 2%) and kept at 4°C overnight. The fixed cells were then washed with PBS and incubated with phalloidin-Alexa568 in 100 μl PBS containing 1% BSA at room temperature for 30 min in dark. The unbound dye was removed by washing twice with PBS. The cells were resuspended in 50 μl PBS containing 10% glycerol and analyzed by confocal microscopy. In some experiments, cells were stimulated first and then fixed in paraformaldehyde overnight before staining with CT-B, with no difference observed in the staining. All compared images were scanned using the same parameters. Three-dimensional (3D) images were reconstructed using AutoQuant (AutoQuant, Watervliet, NY). Actin polymerization levels were quantitated using ImagePro (MediaCybernetics, Silver Spring, MD) using Integrated Optical Density, scoring at least 25 cells for each condition for the relative amounts of F-actin in arbitrary units. Scores were then averaged for each time point and plotted with the zero time point set at 100%.

To assay BCR internalization, 5×10^6 WEHI-231 B-cells either pretreated or without pretreatment (control) were first incubated on ice with 20 μg/ml goat anti-mouse μ-chain–specific Fab Rhodamine red-X and 2.5 μg/ml CTB-Alexa 647 to detect the BCR or lipid rafts, respectively, and then either incubated with or without 5 μg/ml $F(ab')_2$ fragment of anti-goat Alexa 488 to cross-link the BCRs, followed by chasing at 37°C for 40 min. All staining procedures were operated at 0–4°C and unbound reagents at each step were washed away with large volume of ice-cold PBS before going to the next step. After experiments, the cells were immediately fixed using 4% paraformaldehyde for 20–30 min at room temperature. Lipid rafts, and the BCR bound either by monovalent antibody (Fab fragment) alone or by divalent antibody ($F(ab')2$ fragment) were individually visualized by sequential scanning with 633-, 547-, and 488-nm lasers and detecting through the channels with LP650, BP565–615IR, and BP500–530HQ filter, respectively, using a Zeiss LSM510 confocal microscope (Thornwood, NY). The images were analyzed with software LSM examiner. For acute treatments, $5–10 \times 10^6$ cells were treated with anti-IgM or LatB as indicated in the legends to the figures, and immediately were fixed using 4% paraformaldehyde (or 4% paraformaldehyde and 0.4% glutaraldehyde, which gave the same results) for 20–30 min at room temperature. After washing twice with PBS containing 100 mM glycine, lipid rafts, BCR, and F-actin were detected as described above and imaged using the confocal microscope.

Lipid Raft Isolation

Forty million WEHI-231 cells stimulated with anti-IgM or treated with 1 μM LatB, or left untreated as controls, were lysed in 200 μl TNET buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 4 mM EDTA, 1 mM EGTA, 0.5% Triton X-100) supplemented with phosphatase inhibitors (10 mM NaF, 1 mM sodium pyrophosphate, 1 mM Na_3VO_4) and protease inhibitor cocktail (Calbiochem, La Jolla, CA) on ice for 20 min, followed by passage through a 25-gauge syringe needle 20 times. After removal of nuclei by centrifugation at $1000 \times g$ for 10 min, the cell lysates were adjusted to 40% Optiprep and overlaid with 1 ml of 30%, 2 ml of 20%, 1 ml of 10%, and 400 μl 0% Optiprep in TNET buffer containing phosphatase inhibitors and protease inhibitors in a centrifuge tube. Subcellular fractions were separated by ultracentrifugation using Beckman SW55Ti rotor (Fullerton, CA) at $200{,}000 \times g$ for 4 h at 4°C. Fractions of 400 μl (13 total) were collected from the top and a 15-μl sample from each fraction was resolved by 10% SDS-PAGE and immunoblotted with appropriate antibodies.

Flow Cytometric Assay for BCR Internalization

WEHI-231 B-cells (2×10^6) either pretreated with 1 μM LatB at 37°C for 30 min or nontreated (control) and then treated with 10 μg F(ab')2 or Fab fragments of goat anti-mouse μ-chain–specific antibodies on ice for 15 min. Unbound antibodies were washed away with ice-cold PBS by centrifugation at $300 \times g$ for 30 s. Cells were then either kept on ice (as time 0) or warmed up to 37°C for 40 min. The amount of BCR still remaining on the cell surface were determined by staining with goat anti-mouse Alexa 488 in PBS containing 1% BSA and 0.1% NaN_3 on ice followed by analysis by FACS. Nonspecific staining determined by staining with secondary antibody alone was found to be very low and excluded by appropriate gate settings. For LatB pretreated cells, these reagents were always present as the same concentration during the staining steps.

RESULTS

The BCR Induces an Early Wave of Actin Depolymerization

Triggering the BCR leads to increases in actin polymerization within 3–5 min; however, it is not clear whether the actin cytoskeleton is also disassembled during BCR stimulation. To determine if the dynamics of the actin cytoskeleton is altered early after BCR stimulation, cells were stimulated with anti-IgM for short periods of time and analyzed for F-actin distribution. 3D reconstructed confocal images of the cells showed a significant decrease in F-actin levels within 15–30 s of BCR stimulation followed by repolymerization of F-actin in localized regions (Figure 1, A and C).

Lipid rafts are special domains in the plasma membrane rich in cholesterol and GM1 glycosphingolipids. Lipid rafts also contain lipid modified signaling molecules such as the Src family kinases Lck and Lyn, G proteins, Ras GTPase, and adaptor proteins such as LAT that are constitutively localized in the lipid rafts (Pizzo and Viola, 2003), whereas other molecules such as the TCR and the BCR are recruited into lipid rafts upon stimulation (Cheng *et al.*, 1999; Janes *et al.*, 1999; Sproul *et al.*, 2000; Tuosto *et al.*, 2001). Some negative signaling molecules such as SHP-1 and CD45 are excluded from lipid rafts (Janes *et al.*, 1999; Petrie *et al.*, 2000). Therefore, lipid rafts have been considered as signaling platforms for the assembly of signaling complexes and for signal transduction in various cell types including lymphocytes (Cheng *et al.*, 1999; Viola *et al.*, 1999; Petrie *et al.*, 2000; Tuosto *et al.*, 2001; Pizzo and Viola, 2003). We also analyzed lipid rafts during the same time periods. The data showed that BCR-induced depolymerization and repolymerization of actin accompanied lipid raft clustering (Figure 1A). Purified murine primary splenic B-cells also responded to BCR signals with a rapid early decrease in F-actin levels (Figure 1, D and E). Together, these data indicate that the BCR induces an early wave of actin depolymerization concomitant with lipid raft clustering.

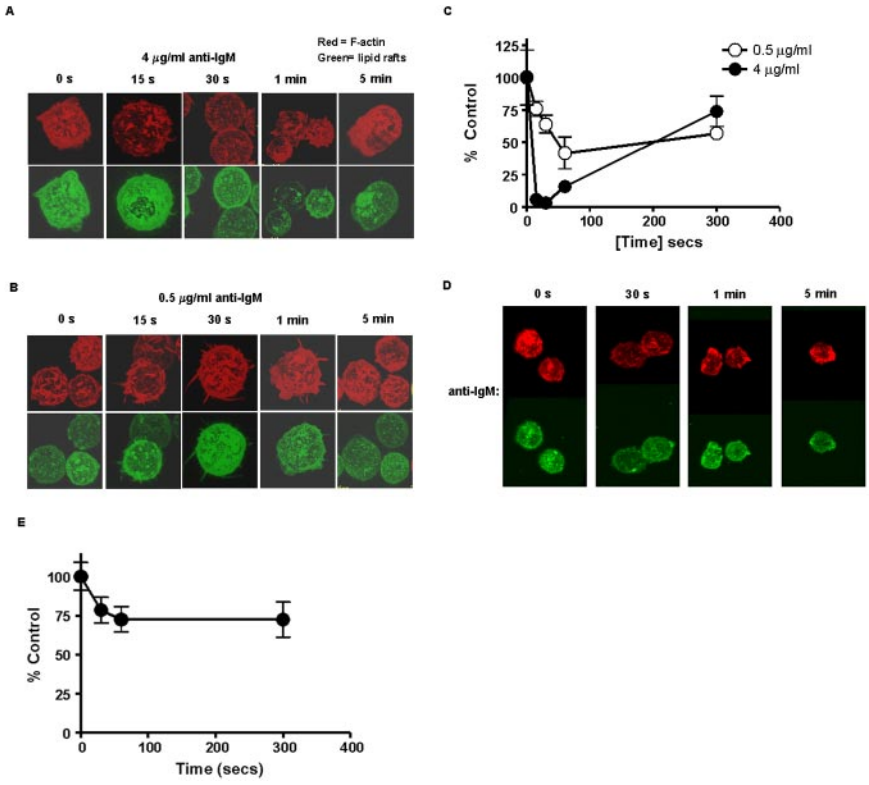

Figure 1. The BCR induces an early wave of actin depolymerization that is dependent on the stimulus strength. (A and B) The BCR induces an early wave of actin depolymerization in a dose-dependent manner. WT DT40 cells were left unstimulated (0 s) or stimulated with 4 μg/ml anti-IgM (A) or 0.5 μg/ml (B) for the indicated time periods and then analyzed for F-actin and lipid raft clustering. (C) Quantitation of actin depolymerization induced by the BCR. (D and E) Primary murine B-cells were left unstimulated or stimulated with 10 μg/ml anti-IgM $F(ab')_2$ fragments for the indicated time periods and then analyzed for F-actin. (E) Quantitation of actin depolymerization induced by the BCR in primary B-cells.

Actin Depolymerization May Serve as a Transducer of the Strength of BCR Stimulation

The induction of actin depolymerization in response to BCR stimulation prompted us to reconsider the role of actin depolymerization in transduction of BCR signals. We hypothesized that actin depolymerization transduces the strength of BCR stimulation and that the degree of actin depolymerization induced by anti-IgM stimulation would behave in a dose-dependent manner. We tested this idea by stimulating cells with low-dose (0.5 μg/ml) or high-dose (4 μg/ml) anti-IgM. We confirmed that a high dose of anti-IgM induced higher activation of the transcription factors SRF and NFAT, which we have previously shown is activated by the BCR via a PLCγ-Ca^{2+}–dependent pathway (Hao *et al.*, 2003). We also observed more sustained ERK phosphorylation and higher and more sustained tyrosine phosphorylation (Supplementary Figure 1). As expected, the degree of actin depolymerization and lipid raft clustering was also higher in cells treated with high-dose anti-IgM (Figure 1, cf. B with A, and C), correlating with transient induction of tyrosine phosphorylation by low dose stimulation (Supplementary Figure 1).

Actin Depolymerization Enhances BCR-induced Transcription Factor Activation

In fibroblasts, the actin dissembler LatB (Spector *et al.*, 1983, 1989; Coue *et al.*, 1987) leads to inhibition of SRF activation by serum (Sotiropoulos *et al.*, 1999). Conversely, actin stabilizer JP (Bubb *et al.*, 1994) by itself can activate SRF and further enhance serum-mediated SRF activation (Sotiropoulos *et al.*, 1999). We therefore determined whether altering the actin cytoskeleton would affect BCR-mediated activation of the transcription factor SRF. We found that actin depolymerization induced by low levels of LatB (0.25–2 μM) did not inhibit but rather enhanced BCR signals leading to SRF activation (Figure 2A). This difference was not due to the SRF reporter we used, because we confirmed that these same concentrations of LatB inhibited serum-induced SRF activation in Chinese hamster ovary cells using the same SRF-Luc reporter (unpublished data). High concentrations of LatB (>5 μM) similar to that used by other investigators to inhibit T-cell activation inhibited BCR activation leading to SRF activation (Figure 2A). Pretreatment of cells with another actin depolymerization agent, cytochalasin D (CD; Cooper, 1987) leads to similar results (Figure 2B), although less effective than LatB as previously reported (Sotiropoulos *et al.*, 1999). Furthermore, stabilization of F-actin by JP inhibited BCR-induced SRF activation (Figure 2C and unpublished data).

Changes in actin dynamics exerted a general effect on BCR signaling because actin depolymerization enhanced, whereas actin polymerization inhibited BCR-induced NFAT and NFκB activation, as measured by luciferase reporter assays (Figure 2, D and E). These data suggest that actin cytoskeleton depolymerization enhances common BCR signaling pathways leading to transcription factor activation.

Actin Depolymerization Prolongs BCR-induced ERK Activation

We and others have previously shown that ERK activation is essential for SRF, NFAT, and AP-1 activation by the BCR (Richards *et al.*, 2001; Brummer *et al.*, 2002; Antony *et al.*, 2003; Hao *et al.*, 2003). To test whether enhancement of BCR activation of these transcription factors by actin depolymerization was via modulating ERK signals, we pretreated DT40 cells with LatB or JP and then stimulated the cells with anti-BCR antibody followed by Western blotting for phospho-ERK. As expected, actin depolymerization by LatB prolonged BCR-induced ERK activation particularly at the 60-

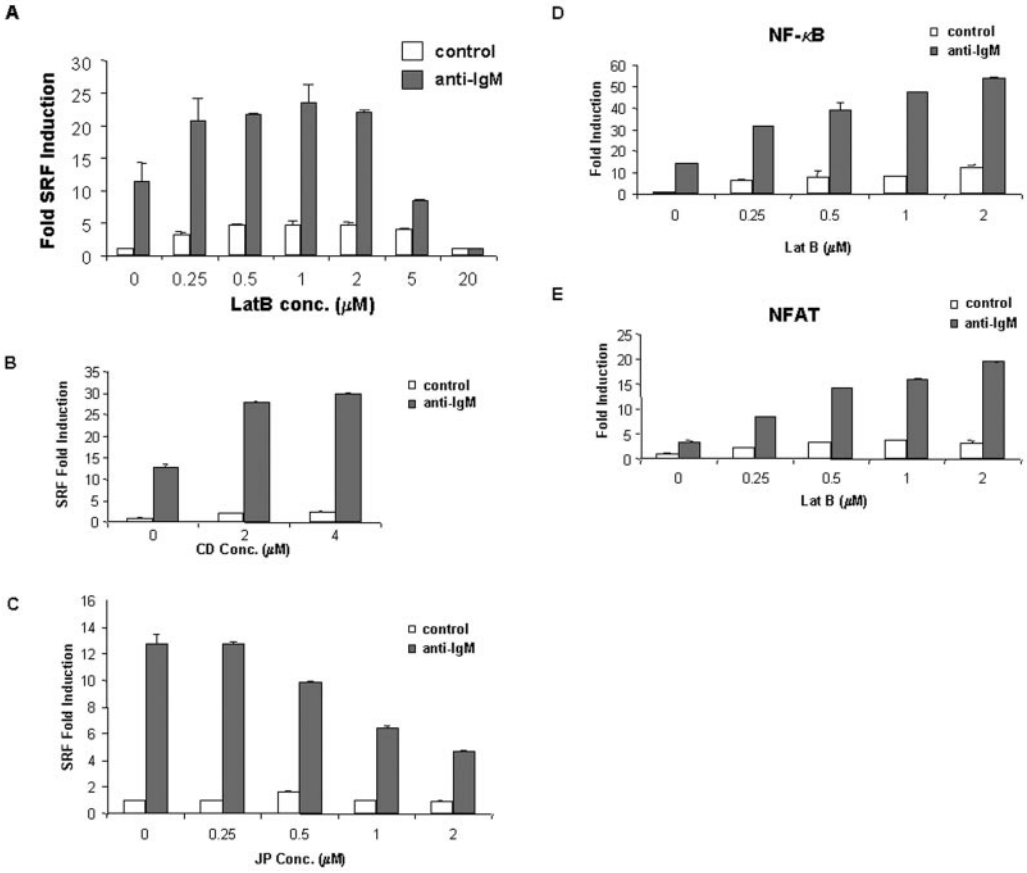

Figure 2. Actin depolymerization enhances BCR-induced SRF, NFAT, and NFκB activation. (A) LatB enhances BCR-induced SRF activation. SRF luciferase activities were determined in DT40 B-cells pretreated with vehicle or the indicated concentrations of LatB. Cells were then left unstimulated or stimulated with 4 μg/ml anti-IgM, and the results were expressed as fold increase over nonstimulated cells, which was set at 1. (B) CD enhances BCR-induced SRF activation. SRF luciferase activities were determined in DT40 B-cells pretreated with vehicle or the indicated concentrations of CD, followed by stimulation and luciferase assay as in A. (C) Stabilization of F-actin inhibits BCR-induced SRF activation. SRF luciferase reporter activities were determined in DT40 B-cells treated with or without the indicated concentrations of JP to stabilize F-actin and then treated as in A. (D and E) LatB enhances BCR-induced NFκB (D) and NFAT (E) activation. NFκB- or NFAT-luciferase activities were determined in DT40 B-cells pretreated with vehicle or the indicated concentrations of LatB. Cells were then treated as in A.

min time point (Figure 3A), whereas stabilization of F-actin by JP dramatically inhibited BCR-induced ERK activation (Figure 3B). Similar results were observed in purified primary splenic B-cells (Figure 3C).

Actin Depolymerization Elevates Sustained Ca^{2+} Signal

In addition to ERK signals, Ca^{2+} signals and particularly the sustained phase of Ca^{2+} signal are also essential for the activation of these transcription factors (Healy *et al.*, 1997; Antony *et al.*, 2003; Hao *et al.*, 2003). We therefore asked whether modulation of the actin cytoskeleton could regulate transcription factor activation by modulating Ca^{2+} signals as well. Depolymerization of actin by LatB significantly elevated the sustained Ca^{2+} signal induced by BCR signals in DT40 B-cells, whereas stabilization of F-actin by JP specifically inhibited the sustained phase of Ca^{2+} signal without affecting the initial Ca^{2+} peak (Figure 3D). Note that the peak of calcium is reduced in cells treated with LatB to induce actin depolymerization. This suggests that delaying actin polymerization by LatB affects some of the early BCR signals as well as late responses.

Actin Depolymerization by Itself Can Activate Cellular Pathways

During the course of our studies, we noticed that LatB-induced actin depolymerization not only enhanced BCR-induced transcription factor activation, but also was sufficient to activate these pathways by itself. We ruled out that this is due to some other actin-independent effects of LatB, because stabilization of F-actin by JP fully abrogated LatB-induced SRF and ERK activation (Figure 4). JP itself did not induce SRF or ERK activation (Figure 4C, see also Figure 2C), suggesting that SRF and ERK activation resulted from actin depolymerization rather than the stress from actin perturbation. Of note, ERK phosphorylation induced by actin depolymerization is weaker and less sustained than BCR stimulation, consistent with its weaker activation of transcription factors compared with BCR (see e.g., Figure 2A). In addition, CD, another actin depolymerizing reagent, has similar effects as LatB, further confirming that depolymerization of F-actin activates these pathways (see Figure 2B and unpublished data).

Activation of the above transcription factors and ERK by depolymerization of F-actin prompted us to explore whether

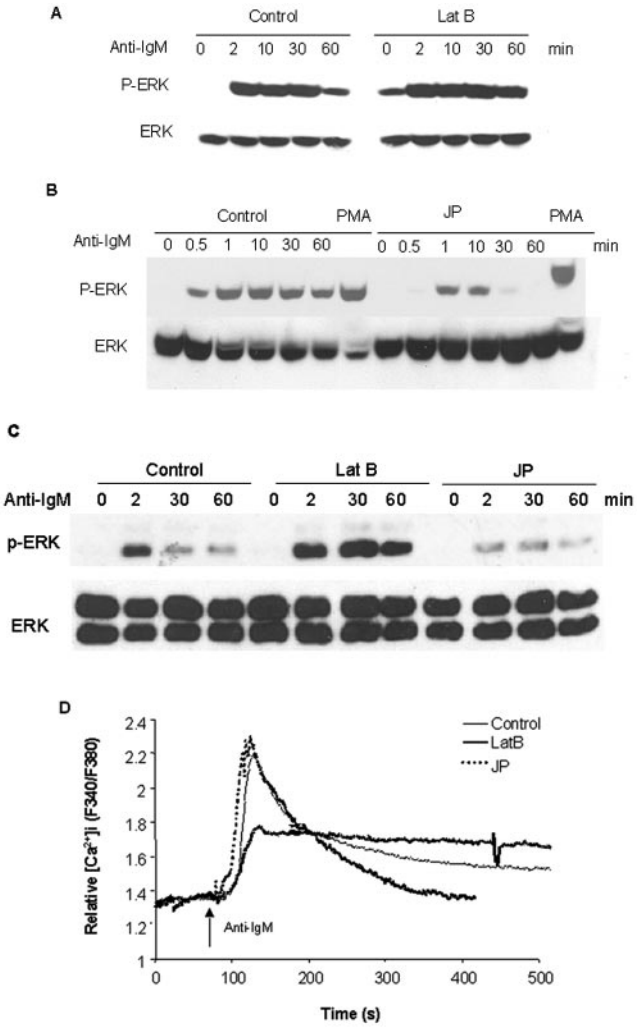

Figure 3. Actin depolymerization prolongs or enhances BCR-induced ERK activation and intracellular Ca^{2+} increase. (A and B) Actin depolymerization prolongs BCR-induced ERK activation. (A) WT DT40 B-cells were left unstimulated (time 0) or stimulated with anti-IgM in the presence or absence of pretreatment (with 0.5 μM LatB for 30 min) for the indicated time periods. ERK activation was then determined by probing blots with antibodies against activated ERK (top panels) or total ERK (bottom panels). (B) WT DT40 B-cells were left unstimulated (time 0) or stimulated with anti-IgM or PMA (as a positive control) in the presence or absence of pretreatment (with 2 μM JP for 1 h.) for the indicated time periods. Cells were then lysed and assayed for ERK activation. (C) Purified primary murine splenic B-cells were left unstimulated (time 0) or stimulated with anti-IgM $F(ab')_2$ in the presence or absence of pretreatment with LatB (0.5 μM for 2 h.) or JP (1 μM JP for 2 h.) for the indicated time periods. Cells were then lysed and assayed for ERK activation. (D) Actin depolymerization prolongs BCR-induced intracellular Ca^{2+} increase. WT DT40 cells were loaded with Fura-2AM, pretreated with 0.5 μM LatB or 1 μM JP for 1 h., and then stimulated with 4 μg/ml anti-IgM. Intracellular Ca^{2+} was analyzed as described in *Materials and Methods.*

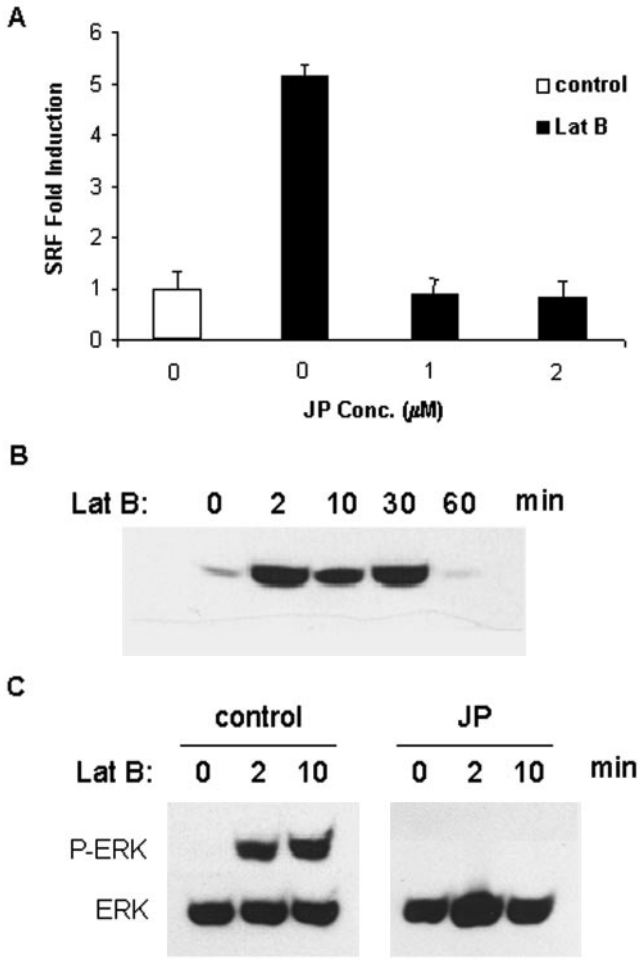

Figure 4. Actin depolymerization by itself activates SRF and ERK. (A) SRF luciferase activities were determined in DT40 B-cells pretreated with the indicated concentrations of JP for 1 h and then stimulated or not with 1 μM LatB, followed by luciferase analysis. (B and C) DT40 B-cells were left unstimulated (time 0) or stimulated with 0.5 μM LatB (B) or 0.5 μM LatB after pretreatment (with 1 μM JP for 1 h; C) for the indicated time periods. Cells were then lysed and equal amounts of protein assayed for ERK activation.

LatB was acting through a new signaling pathway or uses the BCR signaling pathway. We and others have shown that DAG is essential for the BCR-mediated activation of SRF, NFAT, AP-1, and NFκB (Petro and Khan, 2001; Antony *et al.*, 2003; Hao *et al.*, 2003). We found that LatB also activates SRF via Lyn, Syk, Btk, BLNK, PLCγ, Ca^{2+}, and DAG, indicating that LatB-induced actin depolymerization may either directly activate the BCR or act through the same BCR signal transduction machinery (Supplementary Figure 3; Petro and Khan, 2001; Antony *et al.*, 2003; Hao *et al.*, 2003).

Actin Depolymerization Prolongs BCR Signals by Delaying Lipid Raft Internalization

To explore the mechanism by which actin cytoskeleton depolymerization prolongs BCR signals, we analyzed its effects on lipid raft clustering. At the resting state, lipid raft clusters were found to be small and randomly distributed on the plasma membrane, consistent with previous reports (Janes *et al.*, 1999; Gupta and DeFranco, 2003; Pizzo and Viola, 2003). The small clusters may be caused by the binding of the CT-B used to detect lipid rafts as previously reported (Mayor *et al.*, 1994). In addition, the actin cytoskeleton was found exclusively in the cortical region of the cells, and some clusters of lipid rafts were apparently colocalized with F-actin (Figure 5A, panels 1–3). Stimulation of the cells through the BCR for 5 min resulted in more extensive clustering of lipid rafts at

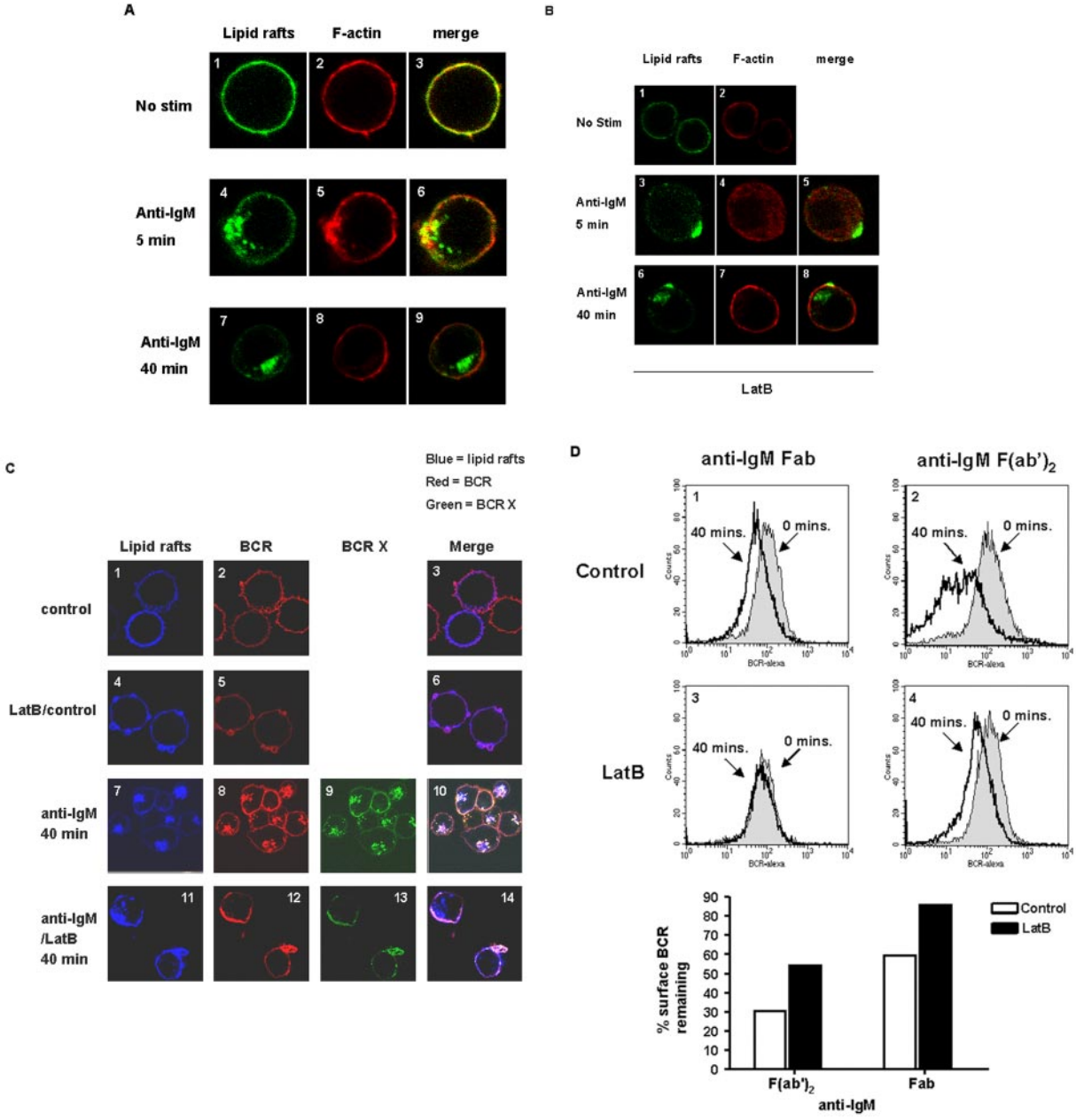

Figure 5. Actin depolymerization inhibits lipid raft internalization after BCR stimulation. (A) DT40 cells were left unstimulated (panels 1–3) or stimulated with 4 μg/ml anti-IgM for 5 min (panels 4–6) or 40 min (panels 7–9). Cells were fixed and stained to detect lipid rafts (panels 1, 4, and 7), or F-actin (panels 2, 5, and 8). Panels 3, 6, and 9 represent the merging of the lipid raft and F-actin images, with overlapping localization depicted in yellow. (B) WT DT40 cells were pretreated with 0.5 μM LatB and then left unstimulated (panels 1–2) or stimulated with 4 μg/ml anti-IgM for 5 min (panels 3–5) or 40 min (panels 6–8). Cells were fixed and stained to detect lipid rafts (panels 1, 3, and 6) or F-actin (panels 2, 4, and 7). Panels 5 and 8 represent the merging of the lipid rafts and F-actin images, with overlapping localization depicted in yellow. (C) WEHI 231 B-cells were pretreated with vehicle (panels 1–3) or pretreated with 0.5 μM LatB (4–6), left unstimulated (panels 1–6), and then stained with Rhodamine-conjugated goat anti-mouse IgM Fab fragments to detect the BCR and Alexa-647-conjugated CTB-B to detect lipid rafts on ice (panels 1–6). Some cells were stimulated with Alexa-488–conjugated anti-goat IgG F(ab')$_2$ fragments to cross-link and detect the BCR for 40 min at 37°C (panels 7–14). Cells were then fixed and analyzed by confocal microscopy. Panels 1, 4, 7, and 11: lipid rafts; panels 2, 5, 8, and 12: goat anti-mouse IgM Fab fragments to detect the BCR; panels 9 and 13: anti-goat IgG F(ab')$_2$ fragments to cross-link and detect the BCR. Panels 3, 6, 10, and 14 represent the merging of the lipid rafts and BCR images, with overlapping localization depicted in purple/white. (D) WEHI 231 B-cells were treated with vehicle control (panels 1 and 2) or pretreated with 0.5 μM LatB (panels 3 and 4) and then stained on ice with goat anti-mouse IgM Fab (panels 1 and 3) or (Fab')$_2$ fragments (panels 2 and 4). Cells were then either left on ice (unstimulated, shaded lines in all panels) or shifted to 37°C for 40 min and then chilled and remaining cell surface BCRs detected using Alexa-488–conjugated anti-goat IgG to detect BCRs remaining on the cell surface (open lines in all panels). Bottom: quantitation of BCR internalization. The MFI intensity of the cell surface BCRs were determined and the percent remaining determined. □, control stimulation; ■, stimulation in the presence of 1 μM LatB.

regions rich in F-actin (Figure 5A, panels 4–6). Notably, internalization of lipid rafts was also apparent at this time point. In contrast, the internalization of lipid rafts was not apparent in the cells treated with LatB (Figure 5B, panels 3–5). By 40 min post-BCR stimulation, almost all lipid raft clusters in control cells were apparently internalized (Figure 5A, panels 7–9). However, cells pretreated with LatB still had significant amounts of lipid raft patches on the cell surface (Figure 5B, panels 6–8). To determine if the BCR was affected by these treatments, we also analyzed the localization of the BCR in the presence or absence of LatB in the WEHI231 murine B-cell line. This analysis indicated that the BCR behaves in a similar manner to that of the lipid rafts, with significant internalization occurring upon cross-linking after 40 min (Figure 5C, panels 7–10), which was significantly prevented by LatB pretreatment (Figure 5C, panels 11–14). We confirmed that LatB prevented BCR internalization by analyzing surface expression of the BCR before and after triggering for 40 min by flow cytometry. Figure 5D demonstrates that triggering of the BCR either with a divalent antibody (anti-BCR $F(ab')_2$ fragments) or monovalent antibody (anti-BCR Fab fragments) resulted in internalization of the BCR after 40 min. However, in agreement with our microscopy studies, pretreatment of these cells with LatB resulted in a significant reduction of BCR internalization (Figure 5D, quantitated in the bottom panel). These results suggest that LatB-induced depolymerization of actin delays internalization of lipid rafts and the BCR and may explain why actin depolymerization prolongs BCR-induced signals such as ERK activation.

Actin Depolymerization Facilitates Altered Clustering of Lipid Rafts

Lipid rafts serve as signaling platforms and are considered to be where the antigen receptor signals are initiated. We thus hypothesized that activation of ERK and downstream transcription factors by actin depolymerization is through direct regulation of lipid raft clustering. To test this, DT40 cells were costained with CT-B-FITC to detect lipid rafts and Phalloidin-Alexa 568 to detect F-actin as described before. In these experiments, rather than imaging a single section of the cells, a series of sections from the top to the bottom of the cells, 0.2–0.3 μm apart was collected with a confocal microscope and a 3D image of the cell reconstructed. These resultant images show small lipid raft patches on nonstimulated cells randomly distributed all over the cell surface (Figure 6A; note that these patches may be caused by CT-B–mediated binding). However, within 1 min of stimulation by the BCR, the clustering of these lipid rafts is altered. Intriguingly and surprisingly, altered clusters of lipid rafts were also found in cells treated with LatB alone to induce actin depolymerization, and BCR stimulation of these cells lead to altered clustering of these lipid rafts (Figure 6A). It is unlikely that the observed clustering of lipid rafts is a result of cross-linking of lipid rafts by CTB itself because clusters were found in cells fixed with either 4% paraformaldehyde or 4% paraformaldehyde and 0.4% glutaraldehyde at room temperature for 30 min or longer, which has been shown to efficiently fix cell surface lipids (Mayor *et al.*, 1994). In contrast, JP-treated cells did not show this pattern of clustering of lipid rafts either with or without BCR stimulation (Figure 6A). Biochemical analysis of lipid rafts by fractionation confirmed that treatment of cells, in this case the murine B-cell line WEHI 231, with LatB resulted in movement of the BCR into lipid raft fractions (2.9-fold increase), similar to that seen after triggering of the BCR (2.4-fold increase), although the tyrosine kinase Syk did not seem to be affected as much

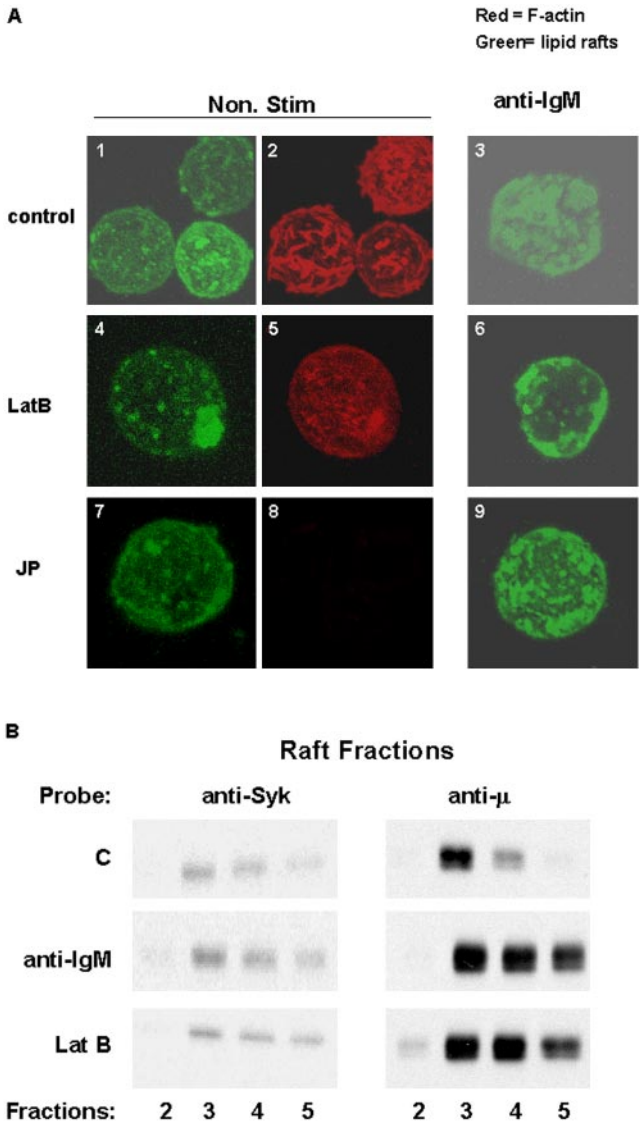

Figure 6. Actin depolymerization by itself facilitates lipid raft clustering. (A) WT DT40 cells were left untreated (panels 1–3) or treated with 0.5 μM LatB (panels 4–6) or 1 μM JP (panels 7–9) for 30 min and left unstimulated or stimulated with 4 μg/ml anti-IgM for 1 min (panels 3, 6, and 9). Cells were fixed and lipid rafts or actin detected, with 3D reconstructed images shown. (B) WEHI-231 B-cells were left unstimulated (top panels), stimulated with antimurine IgM (middle panels) or 1 μM LatB (bottom panels) for 5 min. Cells were lysed, and lipid rafts were isolated on a density gradient by centrifugation. Lipid raft fractions were separated by SDS-PAGE, transferred to PVDF membranes, and probed with anti-Syk (left panels) or anti-μheavy chain (right panels).

(2-fold increase after BCR triggering vs. 1.2-fold increase after LatB treatment, Figure 6B). Control experiments ruled out any contamination of these fractions with cytoplasmic fractions because c-myc was excluded from these fractions (and GM1 was found in the raft fractions by CTB reactivity; unpublished data). These results are consistent with the effects of these treatments on transcription factor and ERK activation. These results also suggest that at the resting state, the actin cytoskeleton may have a role in keeping the lipid rafts apart, which may be an important mechanism to prevent the generation of nonspecific signals due to spontane-

ous lipid raft clustering. Although these results suggest that the observed altered lipid raft clustering may be a passive process because actin depolymerization is sufficient to induce this pattern, our results cannot rule out that this may also be an active process (i.e., lipid rafts are pulled together by contractile forces from actin polymerization because polymerization of actin is not fully blocked at the concentrations we used) in response to antigen receptor signals. Altogether, our data suggest that actin depolymerization has a critical role in regulation of transcription factor activation and the duration of ERK and Ca^{2+} signals by controlling of lipid raft dynamics.

DISCUSSION

In this study, we report that the B-cell antigen receptor induces an early wave of actin depolymerization, which regulates lipid raft and B-cell antigen receptor clustering and enhances BCR signals. These experiments provide novel insight into the role of the actin cytoskeleton in regulation of signals from antigen receptor in lymphocytes by regulation of lipid raft dynamics.

Actin Polymerization May Play a Negative Role in the Regulation of BCR Signals

Although actin polymerization has been shown to be required for BCR signaling, our study suggests that the actin cytoskeleton (F-actin) may also play a negative role in regulation of lipid rafts. We propose that F-actin may keep lipid rafts apart in the resting state. It may also facilitate internalization of lipid rafts after BCR stimulation, which is a mechanism for terminating the BCR signal and allowing for antigen processing. Lipid rafts are randomly distributed on the cells (Valensin *et al.,* 2002; Pizzo and Viola, 2003) and whether these lipid rafts are free to move (diffuse) in the membrane and what causes lipid rafts to aggregate upon BCR stimulation is unclear. In our study, depolymerization of F-actin by LatB-induced altered clustering of lipid rafts, suggesting that movement of lipid rafts may be restricted in the membrane by F-actin fibers. Our work suggests that clustering of lipid rafts is probably spontaneous, because releasing F-actin inhibition seems to be sufficient to induce lipid raft clustering independently of BCR stimulation. Alternatively LatB may induce contractile forces that pull the lipid rafts together while shortening actin polymers. BCR stimulation also leads to depolymerization of F-actin and the degree of depolymerization is dependent on the strength of BCR stimulation, further confirming the idea that F-actin may have a negative role in keeping lipid rafts apart. It will be interesting to explore how F-actin restricts the movement of lipid rafts. One possible mechanism is that similar to anchoring of integrin molecules by F-actin through talin (Lub *et al.,* 1997; Stewart *et al.,* 1998; Sampath *et al.,* 1998), lipid rafts may be riveted in the membrane via F-actin through linker protein(s).

Indeed, some recent studies have shown that F-actin does associate with lipid rafts and this association may play a negative role in TCR signaling. Cbp (Csk binding protein), also called PAG (phosphoprotein associated with GEMs), is a recently cloned protein that constitutively localizes to the lipid rafts (Brdicka *et al.,* 2000; Kawabuchi *et al.,* 2000). In addition to its association with Csk, a negative regulator of Src kinases, a recent study showed that Cbp also indirectly associates with F-actin via a linker protein EBP50, a member of actin-binding proteins ERM (Brdicka *et al.,* 2000; Kawabuchi *et al.,* 2000; Takeuchi *et al.,* 2000; Torgersen *et al.,* 2001; Itoh *et al.,* 2002). On TCR stimulation, Cbp is dephosphorylated and dissociates from EBP50-ERM. Over-expression of Cbp reduces the movement of lipid rafts and inhibits the formation of immunological synapse in T-cells (Itoh *et al.,* 2002). These studies support our finding that F-actin has a role in keeping lipid rafts apart at resting state. It is unclear how dephosphorylation of Cbp is regulated in response to TCR stimulation.

Our studies also provide evidence that polymerized actin (F-actin) may play a negative role in antigen receptor signaling. Previous reports have suggested a positive role for actin polymerization in antigen receptor signaling. We suggest that the actin cytoskeleton may play both positive and negative roles, dependent on the nature of stimuli and the stage of signal transduction in antigen receptor signaling. We believe that we have been able to observe this negative role for F-actin for the following reasons: in most studies in T- and NK-cells where F-actin has been shown to be critical for antigen receptor signals, the concentrations of LatB (5–20 μM) or CD (10–100 μM) are much higher than those used in our work (LatB 0.5–1 μM, CD 2–4 μM). Thus in our experimental system, actin polymerization is not fully blocked. Indeed, we found that F-actin increases after 1 min of BCR stimulation in the presence of LatB. Studies in mast cells support this view, where it has been shown that the high-affinity IgE receptor, (FcϵRI) signals and histamine release are enhanced by LatB or CD pretreatment, whereas JP has the opposite effects (Frigeri and Apgar, 1999; Oka *et al.,* 2002). However, higher concentrations LatB ($\sim$50 μM) fully blocks FcϵRI-mediated secretion (Pendleton and Koffer, 2001), confirming the idea that the actin cytoskeleton can play both positive and negative roles dependent on the concentrations used.

Rivas *et al.* (2004) have recently shown that treatment of T-cells with similar concentrations of LatB or CD also enhances T-cell proliferation, NFAT activation, and IL-2 production. They also observed that T-cell activation led to increased plasma membrane expression of the plasma membrane Ca^{2+} ATPase (PMCA), which was blocked by CD treatment, and suggested that this may be a mechanism by which actin depolymerization enhanced T-cell activation. Our data suggest that lipid raft dynamics may also be regulated by actin depolymerization, which can enhance early BCR signaling events. Indeed, we observe effects of LatB treatment on BCR stimulation of ERK activation, whereas Rivas *et al.* (2004) did not observe such changes in early T-cell activation.

The Actin Cytoskeleton Links Early BCR Signals to Sustained BCR Signals

Whether antigen receptor signals lead to cell activation and proliferation depends on the duration of the signal (Kundig *et al.,* 1996; Grakoui *et al.,* 1999); however, the mechanism that controls the duration of these signals is unknown. We have shown here that stabilization of F-actin by JP abrogates and induction of actin depolymerization by LatB enhances the sustained phase of ERK and Ca^{2+} increase, suggesting that the actin cytoskeleton may have a role in regulation of duration of antigen receptor signals as well. Indeed Song and colleagues have recently shown that blocking actin polymerization or depolymerization blocks BCR internalization; however, it was not clear in those studies whether this would result in enhanced BCR signals (Brown and Song, 2001). Our data suggest that this may be one consequence of reducing BCR internalization.

Early BCR Signals and Modulation of the Actin Cytoskeleton

Although it is not clear how early BCR signals could induce the depolymerization of the actin cytoskeleton, thus paving the way for enhanced lipid raft clustering, we suggest that early BCR signals trigger actin depolymerization via a rapid increase in localized intracellular Ca^{2+}. This would lead to activation of the F-actin–severing protein, gelsolin, which is activated by micromolar Ca^{2+} increase (Kinosian *et al.*, 1998; Gremm and Wegner, 2000). Thus early triggering of the BCR results in limited activation of the BCR pathway and rapid localized transient Ca^{2+} signals, which results in localized depolymerization of actin. This leads to lipid raft clustering and movement of the BCR into these lipid raft clusters, thus enriching the triggered BCRs with the signaling enzymes located in these lipid rafts, while excluding negative regulators such as SHP-1 and CD45. This then enhances the signals via the BCR, leading to full activation of the BCR signaling pathway. Thus weaker triggering of the BCR would lead to smaller changes in action depolymerization and lipid raft clustering, as we observe, and stronger triggering would lead to more extensive actin depolymerization and lipid raft clustering, and thus stronger signals. Actin depolymerization is also required to internalize the activated BCRs and attenuate the signal, and preventing this results in prolonged expression of the receptors at the cell surface and thus prolonged signals.

In conclusion, we have shown that the actin cytoskeleton plays a critical role in the transduction of BCR signals. In the resting state, F-actin probably has the role of keeping lipid rafts apart, thus preventing B-cell activation by nonspecific stimulation. On stimulation, BCR-induced signals reduce F-actin, thus perhaps releasing the inhibition of F-actin on lipid rafts. The degree of actin depolymerization determines the extent of lipid rafts clustering and BCRs recruited into clusters. In addition, the actin cytoskeleton may also regulate the internalization of lipid rafts and the BCR to terminate the signal. Taken together, these data suggest that the actin cytoskeleton plays an important role in regulating of the amplitude and duration of antigen receptor signals.

ACKNOWLEDGMENTS

We thank members of the Immunology Research Labs at Penn State for their critical feedback. We also thank the investigators mentioned in the materials section for the kind gifts of crucial reagents. This work was supported by a Johnson & Johnson Focused Giving Program Award and in part by grants from the American Heart Association (award 0330036N) and the National Institutes of Health (AI51626), all to A.A. S.H. was a graduate fellow of the Huck Institutes for Life Sciences at Penn State University.

REFERENCES

Antony, P., Petro, J., Carlesso, G., Shinners, N., Lowe, J., and Khan, W. (2003). B cell receptor directs the activation of NFAT and NF-kappaB via distinct molecular mechanisms. Exp. Cell Res. *291*, 11–24.

Brdicka, T. *et al.* (2000). Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. J. Exp. Med. *191*, 1591–1604.

Brown, B. K., and Song, W. (2001). The actin cytoskeleton is required for the trafficking of the B cell antigen receptor to the late endosomes. Traffic *2*, 414–427.

Brummer, T., Shaw, P., Reth, M., and Misawa, Y. (2002). Inducible gene deletion reveals different roles for B-Raf and Raf-1 in B-cell antigen receptor signalling. EMBO J. *21*, 5611–5622.

Bubb, M. R., Senderowicz, A. M., Sausville, E. A., Duncan, K. L., and Korn, E. D. (1994). Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. J. Biol. Chem. *269*, 14869–14871.

Cheng, P. C., Dykstra, M. L., Mitchell, R. N., and Pierce, S. K. (1999). A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. J. Exp. Med. *190*, 1549–1560.

Cooper, J. A. (1987). Effects of cytochalasin and phalloidin on actin. J. Cell Biol. *105*, 1473–1478.

Coue, M., Brenner, S. L., Spector, I., and Korn, E. D. (1987). Inhibition of actin polymerization by latrunculin A. FEBS Lett. *213*, 316–318.

Delon, J., Bercovici, N., Liblau, R., and Trautmann, A. (1998). Imaging antigen recognition by naive CD4+ T cells: compulsory cytoskeletal alterations for the triggering of an intracellular calcium response. Eur. J. Immunol. *28*, 716–729.

Derry, J. M., Ochs, H. D., and Francke, U. (1994). Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. Cell *78*, 635–644.

Dustin, M. L., and Cooper, J. A. (2000). The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. Nat. Immunol. *1*, 23–29.

Frigeri, L., and Apgar, J. R. (1999). The role of actin microfilaments in the down-regulation of the degranulation response in RBL-2H3 mast cells. J. Immunol. *162*, 2243–2250.

Gomez, M., Tybulewicz, V., and Cantrell, D. (2000). Control of pre-T cell proliferation and differentiation by the GTPase Rac-I. Nat. Immunol. *1*, 348–352.

Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A.S., Allen, P. M., and Dustin, M. L. (1999). The immunological synapse: a molecular machine controlling T cell activation. Science *285*, 221–227.

Gremm, D., and Wegner, A. (2000). Gelsolin as a calcium-regulated actin filament-capping protein. Eur. J. Biochem. *267*, 4339–4345.

Gu, Y. *et al.* (2003). Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science *302*, 445–449.

Gupta, N., and DeFranco, A. L. (2003). Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B-lymphocyte activation. Mol. Biol. Cell *14*, 432–444.

Hao, S., Kurosaki, T., and August, A. (2003). Differential regulation of NFAT and SRF by the B cell receptor via a PLCgamma-Ca(2+)-dependent pathway. EMBO J. *22*, 4166–4177.

Healy, J., Dolmetsch, R., Timmerman, L., Cyster, J., Thomas, M., Crabtree, G., Lewis, R., and Goodnow, C. (1997). Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. Immunity *6*, 419–428.

Itoh, K., Sakakibara, M., Yamasaki, S., Takeuchi, A., Arase, H., Miyazaki, M., Nakajima, N., Okada, M., and Saito, T. (2002). Cutting edge: negative regulation of immune synapse formation by anchoring lipid raft to cytoskeleton through Cbp-EBP50-ERM assembly. J. Immunol. *168*, 541–544.

Janes, P. W., Ley, S. C., and Magee, A. I. (1999). Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. J. Cell Biol. *147*, 447–461.

Kawabuchi, M., Satomi, Y., Takao, T., Shimonishi, Y., Nada, S., Nagai, K., Tarakhovsky, A., and Okada, M. (2000). Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. Nature *404*, 999–1003.

Kinosian, H., Newman, J., Lincoln, B., Selden, L., Gershman, L., and Estes, J. (1998). Ca2+ regulation of gelsolin activity: binding and severing of F-actin. Biophys. J. *75*, 3101–3109.

Kundig, T. M., Shahinian, A., Kawai, K., Mittrucker, H. W., Sebzda, E., Bachmann, M. F., Mak, T. W., and Ohashi, P. S. (1996). Duration of TCR stimulation determines costimulatory requirement of T cells. Immunity *5*, 41–52.

Lub, M., van Kooyk, Y., van Vliet, S. J., and Figdor, C. G. (1997). Dual role of the actin cytoskeleton in regulating cell adhesion mediated by the integrin lymphocyte function-associated molecule-1. Mol. Biol. Cell *8*, 341–351.

Mayor, S., Rothberg, K., and Maxfield, F. (1994). Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. Science *264*, 1948–1951.

Oka, T., Sato, K., Hori, M., Ozaki, H., and Karaki, H. (2002). FcepsilonRI cross-linking-induced actin assembly mediates calcium signalling in RBL-2H3 mast cells. Br. J. Pharmacol. *136*, 837–846.

Pendleton, A., and Koffer, A. (2001). Effects of latrunculin reveal requirements for the actin cytoskeleton during secretion from mast cells. Cell Motil. Cytoskeleton *48*, 37–51.

Penninger, J., and Crabtree, G. (1999a). The actin cytoskeleton and lymphocyte activation. Cell *96*, 9–12.

Penninger, J.M., and Crabtree, G. R. (1999b). The actin cytoskeleton and lymphocyte activation. Cell *96*, 9–12.

Petrie, R. J., Schnetkamp, P. P., Patel, K. D., Awasthi-Kalia, M., and Deans, J. P. (2000). Transient translocation of the B cell receptor and Src homology 2 domain-containing inositol phosphatase to lipid rafts: evidence toward a role in calcium regulation. J. Immunol. *165*, 1220–1227.

Petro, J., and Khan, W. (2001). Phospholipase C-gamma 2 couples Bruton's tyrosine kinase to the NF-kappaB signaling pathway in B lymphocytes. J. Biol. Chem. *276*, 1715–1719.

Pizzo, P., and Viola, A. (2003). Lymphocyte lipid rafts: structure and function. Curr. Opin. Immunol. *15*, 255–260.

Richards, J., Dave, S., Chou, C., Mamchak, A., and DeFranco, A. (2001). Inhibition of the MEK/ERK signaling pathway blocks a subset of B cell responses to antigen. J. Immunol. *166*, 3855–3864.

Rivas, F., O'Keefe, J., Alegre, M., and Gajewski, T. (2004). Actin cytoskeleton regulates calcium dynamics and NFAT nuclear duration. Mol. Cell. Biol. *24*, 1628–1639.

Sampath, R., Gallagher, P. J., and Pavalko, F. M. (1998). Cytoskeletal interactions with the leukocyte integrin beta2 cytoplasmic tail. Activation-dependent regulation of associations with talin and alpha-actinin. J. Biol. Chem. *273*, 33588–33594.

Sotiropoulos, A., Gineitis, D., Copeland, J., and Treisman, R. (1999). Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. Cell *98*, 159–169.

Spector, I., Shochet, N. R., Blasberger, D., and Kashman, Y. (1989). Latrunculins—novel marine macrolides that disrupt microfilament organization and affect cell growth: I. Comparison with cytochalasin D. Cell Motil. Cytoskeleton *13*, 127–144.

Spector, I., Shochet, N. R., Kashman, Y., and Groweiss, A. (1983). Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. Science *219*, 493–495.

Sproul, T. W., Malapati, S., Kim, J., and Pierce, S. K. (2000). Cutting edge: B cell antigen receptor signaling occurs outside lipid rafts in immature B cells. J. Immunol. *165*, 6020–6023.

Stewart, M. P., McDowall, A., and Hogg, N. (1998). LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a Ca2+-dependent protease, calpain. J. Cell Biol. *140*, 699–707.

Symons, M., Derry, J. M., Karlak, B., Jiang, S., Lemahieu, V., McCormick, F., Francke, U., and Abo, A. (1996). Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. Cell *84*, 723–734.

Takeuchi, S., Takayama, Y., Ogawa, A., Tamura, K., and Okada, M. (2000). Transmembrane phosphoprotein Cbp positively regulates the activity of the carboxyl-terminal Src kinase, Csk. J. Biol. Chem. *275*, 29183–29186.

Tedford, K., Nitschke, L., Girkontaite, I., Charlesworth, A., Chan, G., Sakk, V., Barbacid, M., and Fischer, K. (2001). Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling. Nat. Immunol. *2*, 548–555.

Torgersen, K. M., Vang, T., Abrahamsen, H., Yaqub, S., Horejsi, V., Schraven, B., Rolstad, B., Mustelin, T., and Tasken, K. (2001). Release from tonic inhibition of T cell activation through transient displacement of C-terminal Src kinase (Csk) from lipid rafts. J. Biol. Chem. *276*, 29313–29318.

Tuosto, L., Parolini, I., Schroder, S., Sargiacomo, M., Lanzavecchia, A., and Viola, A. (2001). Organization of plasma membrane functional rafts upon T cell activation. Eur. J. Immunol. *31*, 345–349.

Valensin, S., Paccani, S. R., Ulivieri, C., Mercati, D., Pacini, S., Patrussi, L., Hirst, T., Lupetti, P., and Baldari, C. T. (2002). F-actin dynamics control segregation of the TCR signaling cascade to clustered lipid rafts. Eur. J. Immunol. *32*, 435–446.

Valitutti, S., Dessing, M., Aktories, K., Gallati, H., and Lanzavecchia, A. (1995). Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. J. Exp. Med. *181*, 577–584.

Viola, A., Schroeder, S., Sakakibara, Y., and Lanzavecchia, A. (1999). T lymphocyte costimulation mediated by reorganization of membrane microdomains. Science *283*, 680–682.

Walmsley, M., Ooi, S., Reynolds, L., Smith, S., Ruf, S., Mathiot, A., Vanes, L., Williams, D., Cancro, M., and Tybulewicz, V. (2003). Critical roles for Rac1 and Rac2 GTPases in B cell development and signaling. Science *302*, 459–462.

Zhang, J. *et al.* (1999). Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. J. Exp. Med. *190*, 1329–1342.