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*J Immunol* 2011; 186:4088-4097; Prepublished online 21 February 2011;
doi: 10.4049/jimmunol.1001139

http://www.jimmunol.org/content/186/7/4088

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2011/02/22/jimmunol.1001139.DC1

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Conformational Switching in Ezrin Regulates Morphological and Cytoskeletal Changes Required for B Cell Chemotaxis

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B cell chemotaxis occurs in response to specific chemokine gradients and is critical for homeostasis and immune response. The molecular regulation of B cell membrane–actin interactions during migration is poorly understood. In this study, we report a role for ezrin, a member of the membrane-cytoskeleton cross-linking ezrin-radixin-moesin proteins, in the regulation of the earliest steps of B cell polarization and chemotaxis. We visualized chemokine-induced changes in murine B cell morphology using scanning electron microscopy and spatiotemporal dynamics of ezrin in B cells using epifluorescence and total internal reflection microscopy. Upon chemokine stimulation, ezrin is transiently dephosphorylated to assume an inactive conformation and localizes to the lamellipodia. B cells expressing a phosphomimetic conformationally active mutant of ezrin or those in which ezrin dephosphorylation was pharmacologically inhibited displayed impaired microvilli dynamics, morphological polarization, and chemotaxis. Our data suggest a 2-fold involvement of ezrin in B cell migration, whereby it first undergoes chemokine-induced dephosphorylation to facilitate membrane flexibility, followed by relocation to the actin-rich lamellipodia for dynamic forward protrusion of the cells. The Journal of Immunology, 2011, 186: 4088–4097.
initiating the inactivation of ERM proteins in chemokine-stimulated T cells (26).

We have previously reported that stimulation of the B cell Ag receptor results in dephosphorylation of ezrin at T567, transiently dissociating it from Csk-binding protein (or PAG), a transmembrane protein enriched in lipid rafts (27). Ezrin concomitantly dissociates from actin filaments, thus facilitating Ag-induced lipid raft coalescence (27). Thus, conformational changes in ezrin have the potential to regulate membrane–actin contacts when a B cell encounters chemokine gradients. Increased ezrin expression has been correlated with highly metastatic cancers, further supporting a role for this protein in migratory behavior of cells (28).

To investigate the participation of ezrin in the earliest morphological changes that precede cell migration, we performed high-resolution visual analysis of B cell morphology and ezrin in live resting B cells and those undergoing migration. We show that the regulatory threonine residue in ezrin and moesin is transiently dephosphorylated in response to chemokine stimulation. Preventing dephosphorylation or expressing a phosphomimetic mutant of ezrin inhibits B cell migration. Our data demonstrate that transient dephosphorylation of ezrin regulates membrane flexibility that the B cell must achieve during migration. Furthermore, switching between the tethering and untethering conformations of ezrin may facilitate rapid changes in membrane–actin interaction at the front of migrating B cells.

**Materials and Methods**

**Cells, Abs, and other reagents**

The murine 2PK3 B lymphoma cell line was cultured in B cell growth medium consisting of DMEM and 10% FBS. Primary B cells were purified from spleens of C57BL/6 mice by negative depletion using CD43 MicroBeads (Miltenyi Biotec). Rabbit polyclonal Abs to phospho-Thr-ERM, ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, Akt, and phospho-Akt were from Cell Signaling Technology, and Ab to ezrin was from Millipore. HRP-conjugated goat anti-rabbit secondary Abs and FITC-conjugated Ab to rabbit-IgG were from The Jackson Laboratory. Flow cytometry Abs, including biotin-conjugated CXCR5, FITC-conjugated B220, PE-conjugated CD19, PE-conjugated CD44, allophtocyanin-conjugated CD45.2, and PE-conjugated streptavidin were from BD Biosciences. Alexa Fluor 594-conjugated Ab to rabbit-IgG, rhodamine-conjugated phalloidin, and Alexa Fluor 488-conjugated phalloidin were from Invitrogen.

**Mice**

C57BL/6 and B6.SJL mice were purchased from The Jackson Laboratory and used at 8–12 wk of age. All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

**Plasmids and transfection**

Ezrin-pRRES2-EGFP was constructed by subcloning full-length ezrin (1–586) into the XhoI and EcoRI sites of the pRRES2-EGFP vector (CloneTech). The T567 residue in ezrin was mutated to aspartic acid (T567D) using the QuikChange II Site-Directed mutagenesis kit (Stratagene). The T567 residue in ezrin was mutated to aspartic acid (T567D) into the XhoI and EcoRI sites of the vector PEYFP-N1 (Clonetech). The T567 residue in the resulting fusion construct ( Ez-YPF ) was mutated to aspartic acid to generate a T567D-YPF fusion protein (TD-YPF). 2PK3 cells were transfected with 3–6 µg appropriate plasmids using Amaxa Nucleofector II (Lonza). Stable transfectants were generated by selecting for G418 resistance and sorting for high GFP expression using the FACSAria I cell sorter (BD Biosciences).

**Cell lysis and immunoblotting**

Purified splenic B cells or 2PK3 cells were stimulated with BLC or SDF-1α (R&D Systems) at 37 °C for 20 h. Lysates were subjected to SDS-PAGE followed by probing with appropriate primary and secondary Abs. In some experiments, prior to stimulation with chemokine, the cells were either pretreated for 30 min with the Ser/Thr phosphatase inhibitor calyculin A or pretreated for 10 min with the PLC inhibitor U-73122. The Journal of Immunology 4089

**Transwell migration assay**

Chemotaxis assays were performed with purified splenic B cells or transiently transfected 2PK3 cells using uncoated 5-µm transwell filters (Corning Costar) as described (29). Briefly, cells were added to transwell inserts seeded in wells coated with medium with or without the chemokine, harvested after 4 h, and counted by flow cytometry using timed acquisition for 1 min with time resolution set at 10 ms. Data were analyzed with FlowJo software (Tree Star).

**In vivo B cell homing assay**

Five million per milliliter purified CD45.2+ splenic B cells from C57BL/6 mice were treated with calyculin A or DMSO for 30 min and washed. Ten million cells were injected i.v. into CD45.1+ B6.SJL recipients. Recipients were sacrificed 1.5 or 26 h later to harvest blood, lymph nodes, and spleen. Splenocytes were stained with Abs to B220 and CD45.2 followed by flow cytometry and data analysis.

**Scanning electron microscopy**

Cells were adhered on to poly-l-lysine (PLL)-coated coverslips and left unstimulated or stimulated with 10 µg/ml BLC, followed by immediate fixing in PFA and glutaraldehyde. Samples were subsequently rinsed, dehydrated with series of graded ethanol, subjected to critical point drying, and sputter coated with a thin layer of gold. Processed samples were viewed with a JEOL JSM 5310 Scanning Microscope (JEOL) at original magnification ×7500, and images were captured using JEOL Orion Image acquisition and handling controls (JEOL).

**Epi-fluorescence and total internal reflection fluorescence microscopy**

For ezrin and actin polarization experiments, splenic B cells, 2PK3 B cells, Ez-YFP-, or TD-YPF-expressing 2PK3 cells were adhered to PLL or fibronectin-coated coverslips, followed by stimulation with BLC. Cells were fixed immediately with 4% PFA followed by permeabilization and staining with rhodamine-conjugated phallolidin for 1 h at 4°C. After washing, the cells were covered with 200 µl PBS, and examined by microscopy. For staining endogenous ezrin and moesin fixed cells were blocked with 10 µg goat IgG for 15 min followed by staining with ezrin or moesin Ab, and washing. The cells were covered with 200 µl PBS, and examined by microscopy. For time-lapse imaging of Ez-YPF and TD-YPF, transiently transfected 2PK3 cells were adhered in wells of an eight-chambered slide at 5 × 106/chamber, rinsed with DMEM, and stimulated with 10 µg/ml BLC or DMEM in 100 µl. Recording was started 1 min before stimulation and resumed for 7 min after addition of BLC or plain medium. Images were collected every 20 s for a period of 8 min. For total internal reflection fluorescence (TIRF) microscopy, the focus was adjusted to the interface between the sample and coverslip, and the laser was aligned to achieve the optimum angle of reflection. All TIRF images were acquired at a depth of 110 nm. All images were acquired using a Leica-AM TIRF microscope DMi6000 (Leica Microsystems) with an attached Hamamatsu EM-CCD camera, using HCX PL APO 100 × oil objective with numerical aperture of 1.47 and appropriate filter cubes. The imaging was performed on cells maintained in B cell medium at 37°C using the Leica acquisition software LAS AF Version 2.2.0 (Leica Microsystems).

**Image processing**

Metamorph image analysis software was used for digital no-neighbors two-dimensional deconvolution, pixel intensity, line-scan calculation, and processing of time-lapse movies. Images acquired in the TIRF mode were deconvolved using Metamorph and analyzed further with ImagePro 6.1 using an algorithm specifically written to quantify the number of individual filaments in the contact area and their presence through the duration of imaging. Briefly, a region of interest (ROI) encompassing the contact area

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was drawn and extruded into multiple concentric regions of 10-pixel width each. The filaments were Top Hat filtered to enhance/equalize their appearance, thresholded, separated from one another (skeletonization, branch-point identification and subtraction), and subsequently counted in each concentric ROI across the stack of images (acquired every 20 s for 8 min). The total filament area in each ROI ring was calculated summing segmented filament pixels (Supplemental Fig. 6A). The heat maps were generated to represent the presence of each filament in the different image frames. Briefly, each frame of the segmented filament sequence was divided by 255 such that each positively segmented filament pixel was set to 1. A maximum intensity projection was then generated and pseudo-colored such that a look-up table ranging from blue to red represents filament pixels present in a single image frame (blue) to those persistent in all of the frames (red).

Statistical analyses

Graphs show the mean and SD observed in independent experiments. Exact p values were calculated using the nonparametric two-tailed Mann–Whitney U test for the unpaired data sets. Mann–Whitney U test is appropriate as the comparison is only between two groups, and each group contains a sample size of ≥10. The tests were performed with 95% confidence interval (α level = 0.05).

Results

Chemokine stimulation of B cells induces dephosphorylation of ezrin and moesin

We first examined the localization of ERM proteins in resting B cells by imaging endogenous ezrin and moesin in the epifluorescence and TIRF modes. Both proteins were localized in the cortical region beneath the membrane (Fig. 1A, left panels) and enriched in the cell-surface microvilli (Fig. 1A, right panels). To examine the effect of chemokine stimulation on the subcellular localization of ezrin, splenic B cells were stimulated with BLC and immunostained for endogenous ezrin and F-actin. In unstimulated resting B cells, ezrin was uniformly distributed at the cell cortex, but rapidly relocalized and copolarized with F-actin upon BLC stimulation (Fig. 1B). The line-scan measurements show an increase in pixel intensity of both ezrin and F-actin at the same position, indicating copolarization (Supplemental Fig. 1A). A similar copolarization of ezrin with F-actin was observed in the 2PK3 B lymphoma cell line upon BLC treatment (Supplemental Fig. 1B). Because relocalization of ezrin is expected to involve conformational change that depends on phosphorylation at the regulatory T567 site, we measured the phosphorylation of ezrin using a phospho-ThrERM–specific Ab. In naive, mature B cells, both ezrin and moesin were constitutively phosphorylated at T567 and T558, respectively (Fig. 1C), consistent with their predominantly cortical localization (Fig. 1A). Stimulation with BLC (Fig. 1C) or SDF-1α (Supplemental Fig. 1C) induced rapid but transient dephosphorylation of ezrin and moesin. Because phosphorylation at the regulatory threonine residue defines the active conformation of ERM proteins, our data indicate that exposure to chemokine induces a conformational switch from the open membrane-tethering form to the closed dormant form. Chemokine-induced relocalization of ezrin and copolarization with F-actin was also observed when purified splenic B cells (Supplemental Fig. 2A) or 2PK3 B cells (Supplemental Fig. 2B) were adhered on the physiological substrate fibronectin.

Preventing regulatory threonine dephosphorylation of ERM proteins inhibits B cell migration

To test whether dephosphorylation of the regulatory threonine residues in ezrin and moesin plays a role in B cell migration, we treated B cells with a cell-permeable Ser/Thr phosphatase inhibitor, calyculin A. Treatment of B cells with calyculin A resulted in a dose-dependent inhibition of BLC-induced dephosphorylation of ezrin and moesin (Fig. 2A). We performed dose titration to optimize the concentration of BLC for in vitro migration of B cells and threonine dephosphorylation of the ERM proteins and chose 1 μg/ml for our experiments (Supplemental Fig. 3). We performed transwell migration assay to examine the effect of preventing dephosphorylation of ezrin and moesin on the ability of B cells to undergo chemotaxis. Calyculin A treatment inhibited BLC-

![FIGURE 1. BLC stimulation induces copolarization of ezrin with actin and dephosphorylation of ezrin at T567. A, 2PK3 cells were stained for ezrin and moesin and imaged in the epifluorescence or TIRF mode at an additional original magnification ×1.6 with a 100× objective. Scale bar, 5 μm. B, Purified splenic B cells were stimulated with 10 μg/ml BLC for the indicated times and fixed prior to staining for ezrin and F-actin. Scale bar, 5 μm. C, Splenic B cells were stimulated with 3 μg/ml BLC for indicated lengths of time, and lysates were probed with p-ThrERM or ezrin Ab. The numbers shown below are ratio of p-ThrEzrin to total ezrin calculated from densitometric analysis of the immunoblots. Data are representative of two independent experiments.](http://www.jimmunol.org/Downloadedfrom/)
induced chemotaxis of B cells in a dose-dependent manner (Fig. 2B). Next, we adoptively transferred CD45.2+ C57BL/6 B cells treated with calyculin A into congenic CD45.1+ B6.SJL recipients and examined their ability to home to the lymph nodes and spleen using an in vivo homing assay. A larger number of calyculin A-treated B cells was found in the blood of the recipient mice (Fig. 2C) than those treated with DMSO at 1.5 h after adoptive transfer. Accordingly, only half of calyculin A-treated B cells were able to home to the recipient spleen at this time (Fig. 2D). A similar defect was also observed in the homing of calyculin A-treated B cells to the lymph nodes and spleen at 26 h posttransfer (Fig. 2E, 2F). These results indicate that ERM dephosphorylation is an important feature of B cell migration. However, incomplete inhibition of B cell homing suggests that additional factors play a role in regulating B cell migration in vivo.

As cell polarization and extension of lamellipodia are early events in cell migration, we examined the effect of Ser/Thr phosphatase inhibition on B cell morphology using high-resolution scanning electron microscopy (EM). The resting B cell surface was largely covered with microvilli and very few membrane ruffles (Fig. 2G, top panels). Calyculin A treatment impaired formation of surface microvilli and ruffles in resting B cells (Fig. 2G, bottom panels). Upon stimulation of B cells with BLC, most of the cells showed reabsorption of microvilli and an increase in the extension of membrane ruffles within 2 min (Fig. 2H, black bars). At 5 min poststimulation, a majority of the cells retained membrane ruffles but started showing a reappearance of microvilli. Most of the stimulated cells reverted back to their original resting morphology within 10 min. The presence of ruffles or microvilli on the B cells was not mutually exclusive, and cells that had both ruffles and microvilli were present in the population at all times. However, with increasing duration of stimulation, the percentage of cells that showed either ruffles or microvilli changed. Ten percent of the calyculin A-treated cells extended a few stubby projections upon BLC stimulation, but failed
to extend membrane ruffles (Fig. 2H, gray bars). These data show that threonine dephosphorylation of ERM proteins is necessary for initiation of morphological changes associated with cell migration.

Expression of a constitutively active form of ezrin inhibits B cell migration

Because calyculin A inhibits Ser/Thr phosphatase activity nonspecifically, it is likely to prevent other dephosphorylation events within the B cell in addition to dephosphorylation of ERM proteins. To specifically address the requirement for dephosphorylation of ERM proteins on B cell morphology and migration, we used a phosphomimetic mutant of ezrin that is trapped in an irreversible constitutively phosphorylated state. In the phosphomimetic mutant of ezrin, the regulatory threonine at position 567 was mutated to aspartic acid (T567D), affording constitutive binding to transmembrane proteins and F-actin (24). The plasmid pIRES2-EGFP expressing the active T567D (Supplemental Fig. 4A) mutant of ezrin was transiently transfected into 2PK3 B cells, and bicistronic expression of EGFP was used as a reporter of transfection efficiency (Supplemental Fig. 4B, left panel). Because the T567D mutant of ezrin is untagged, overexpression of the T567D mutant was assessed by increase in intensity of the protein band corresponding to ezrin in the immunoblot (Supplemental Fig. 4C). Expression of moesin remained unaffected by expression of the T567D mutant of ezrin (Supplemental Fig. 4C). We examined the effect of transiently overexpressing the T567D mutant on B cell migration using an in vitro transwell migration assay. Whereas 93% of the mock-transfected 2PK3 cells migrated in response to BLC, only 61% of GFP+ cells expressing the constitutively active T567D mutant of ezrin were able to migrate (Fig. 3A). CXCR5, the specific receptor for BLC, was similarly expressed in mock-transfected and T567D mutant-expressing 2PK3 cells (Supplemental Fig. 4B, right panel).

Constitutively active ezrin inhibits microvilli formation in resting B cells and prevents BLC-induced membrane ruffling

Next, we tested if 2PK3 cells expressing the T567D mutant are able to change their morphology upon BLC stimulation. Given the heterogeneity of the transiently transfected population and the inability to distinguish between untransfected and transfected cells by scanning EM, we generated 2PK3 B cell transfectants that stably expressed the T567D mutant of ezrin. We selected stable transfectants of 2PK3 cells exhibiting 100% GFP expression (Supplemental Fig. 4D, left panel) and CXCR5 expression that was comparable to that of untransfected cells (Supplemental Fig. 4D, right panel). The T567D-expressing stable transfectants showed 3-fold overexpression of ezrin as compared with the untransfected cells (Supplemental Fig. 4E). Expression of moesin was similar between untransfected 2PK3 cells and those stably expressing the T567D mutant (Supplemental Fig. 4E). Untransfected 2PK3 cells or those stably expressing the T567D mutant were stimulated with BLC and subjected to scanning EM. Similar to splenic primary B cells, the 2PK3 B cells were rich in surface microvilli (Fig. 3B, top left panel). Within 5 min of BLC stimulation, a majority of the untransfected 2PK3 cells resorbed their microvilli (Fig. 3B, 3C, open black bars) and extended membrane ruffles (Fig. 3B, 3C, filled black bars). In contrast, expression of the constitutively active T567D mutant not only reduced microvilli formation on most of the resting 2PK3 cells, making them appear bald (Fig. 3B, bottom left panel), but also impaired the induction of membrane ruffles in response to BLC (Fig. 3B, bottom panels). Stimulation of the T567D-expressing cells with BLC induced the appearance of stubby projections in a small fraction of these cells. The quantification of untransfected and T567D-expressing 2PK3 cells bearing microvilli or ruffles or lack thereof is presented in Fig. 3C. Together, these data demonstrate that restricting membrane-cytoskeletal dynamics by forced expression of the conformationally active mutant of ezrin alters resting B cell morphology and inhibits chemokine-induced polarization, explaining the reduced B cell migration observed in the presence of the T567D mutant of ezrin.

We further examined whether the BLC-dependent signal transduction was affected in the presence of the T567D mutant. Untransfected 2PK3 cells or those stably expressing the T567D mutant of ezrin were stimulated with BLC and cell lysates probed for phosphorylation-dependent activation of signaling proteins in the MAPK and PI3K pathways. Activation of ERK1/2, JNK1/2, and Akt pathways was similarly induced in control and T567D mutant-expressing 2PK3 cells (Supplemental Fig. 5A). These data indicate that the defects observed in migration of B cells expressing the active mutant of ezrin are not a consequence of impaired proximal signaling but result from defect in morphological changes induced by the chemokine. The importance of chemokine-induced PLC activation in initiating the inactivation of ERM proteins is well established in T cells (26). We tested if PLC activation would affect ERM inactivation in B cells and found that inhibition of PLC prevented dephosphorylation of ERM proteins (Supplemental Fig. 5B, 5C). These data indicate that regulation of

![FIGURE 3. Expression of constitutively active ezrin in B cells decreases in vitro B cell migration blocks surface microvilli formation and BLC-induced membrane ruffling. A, Transwell migration of 2PK3 cells that were mock transfected (empty bars) or transfected with the T567D mutant of ezrin (filled bars) in response to 1 μg/ml BLC. The bar graph shows percent migration for GFP+ cells. B, Scanning EM images of untransfected 2PK3 cells or those stably expressing the T567D mutant that were left unstimulated or stimulated with 10 μg/ml BLC for the indicated times. Scale bar, 1 μm. C, Quantification of untransfected 2PK3 cells (black bars) or those expressing the T567D mutant (gray bars) that showed presence of microvilli (empty bars) or membrane ruffles (filled bars) on their surface or were devoid of both microvilli and ruffles and hence identified as bald (patterned bars). Data are shown as a percent of total cells (n = 20) analyzed and are representative of two independent experiments.](http://www.jimmunol.org/)
membrane PIP<sub>2</sub> levels by PLC activity may influence the activation and localization of ERM proteins in B cells as well.

**Irreversible tethering of the membrane and actin cytoskeleton inhibits BLC-induced resorption of microvilli**

The inability of B cells treated with calyculin A or those expressing the constitutively active mutant of ezrin to form surface microvilli prompted us to closely examine the microvilli dynamics in live B cells stimulated with chemokine. We employed TIRF microscopy to visualize the association of ezrin with microvilli and chemokine-induced changes in ezrin localization on the B cell surface. We transiently expressed YFP fusion proteins of wild-type ezrin (Ez-YFP) and the T567D mutant of ezrin (TD-YFP) in 2PK3 cells and performed time-lapse TIRF imaging to visualize surface dynamics of these proteins in the presence of BLC. To control for perturbations introduced during addition of BLC, the cells were also imaged upon addition of medium without BLC. To quantify individual microvilli dynamics, the contact area in the images was segmented into ROI (Supplemental Fig. 6A). The fluorescence intensity of individual microvilli filaments was used to estimate the total filament area in each concentric ROI. The lifespan of individual filaments following stimulation was determined by plotting filament area in each ROI throughout the time-lapse imaging period as described in Materials and Methods. Most 2PK3 cells expressing Ez-YFP revealed a profusion of distinct and spontaneously dynamic microvilli at the site of contact (Fig. 4A, top left, Supplemental Video 1). Medium-treated 2PK3 cells expressing Ez-YFP (Fig. 4A, left panels, Supplemental Video 1) or TD-YFP (Fig. 4A, right panels, Supplemental Video 2) showed no variation in total filament area (Fig. 4C, 4D). Heat mapping of medium-treated 2PK3 cells expressing Ez-YFP (Fig. 4C) showed mostly yellow or red colored filaments, indicating that most filaments persisted throughout the duration of imaging. BLC stimulation of cells expressing Ez-YFP induced resorption of microvilli with time (Fig. 4B, left panels, Supplemental Video 3), as reflected in the mostly blue- and cyan-colored filaments in the heat map (Fig. 4E), and reduced total filament area following stimulation (Fig. 4F). 2PK3 cells expressing TD-YFP showed a greatly reduced number of microvilli at the site of contact (Fig. 4A, 4B, top right panels) and an almost flat bald surface, consistent with our previous scanning EM data (Fig. 3B). Stimulation of TD-YFP–expressing 2PK3 cells with BLC (Fig. 4B, right panels, Supplemental Video 4) resulted in no change in the lifespan of the minimal surface projections that were mostly green in color (Fig. 4F). TD-YFP–expressing 2PK3 cells showed a slight increase in the total filament area (Fig. 4F′) in response to BLC treatment that may be due to the appearance of stubby protrusions, similar to those observed with scanning EM imaging (Fig. 3B, bottom panels). Our live TIRF imaging data together with the high-magnification scanning EM images reveal that dynamic local changes in the conformation of ezrin are necessary for the formation and maintenance of microvilli on the B cell surface. Furthermore, resorption of microvilli is an early event in BLC-induced cell polarization and migration that involves conformational inactivation of ezrin.

**BLC-induced relocation of constitutively active ezrin is impaired**

Polarization of endogenous ezrin and F-actin in response to BLC suggested a potential role for ezrin at the front pole of migrating B cells. To compare chemokine-induced partitioning of wild-type and T567D mutant of ezrin in live cells, we performed time-lapse epifluorescence imaging of Ez-YFP and TD-YFP in 2PK3 cells. Similar to endogenous ezrin, the Ez-YFP fusion protein was localized predominantly in the cortical region of the cell (Fig. 5A), but underwent relocation to the lamellipodia following BLC stimulation (Fig. 5A 5D, Supplemental Video 5). 2PK3 B cells expressing TD-YFP showed two distinct patterns of localization, with 40% of cells showing uniform cortical distribution (Fig. 5B).
and 60% of cells showing prepolarized localization (Fig. 5C). Constitutively polarized TD-YFP was enriched at what appeared to be the contact point between the cells and the substratum. Therefore, we examined the localization of adhesion molecules such as CD44 that accumulate at sites of contact with the substratum. TD-YFP colocalized with CD44 (Supplemental Fig. 6B, 6C) regardless of the presence or absence of BLC. 2PK3 cells with uniform distribution of CD44 showed a similar uniform distribution of TD-YFP, and cells with polarized CD44 showed copolarization of TD-YFP. These results indicate that contact-induced polarization of adhesion proteins such as CD44 forces the constitutively active T567D mutant of ezrin to assume a polarized distribution at the site of strongest adhesion. Regardless of the initial localization, TD-YFP failed to redistribute to the leading edge in response to BLC stimulation (Fig. 5B–D, Supplemental Videos 6, 7). Our results indicate that the mobility and partitioning of the constitutively active mutant of ezrin is compromised.

Constitutively active ezrin is unable to copolarize with F-actin in response to BLC

Next, we examined whether BLC-induced polarization of Ez-YFP coincides with that of F-actin. In resting 2PK3 cells, Ez-YFP was uniformly codistributed with F-actin (Fig. 6A), as observed earlier with endogenous ezrin (Fig. 1B). Upon stimulation with BLC, Ez-YFP copolarized with F-actin (Fig. 6A, bottom three rows, 6C). The line-scan measurements show an increase in pixel intensity of both ezrin and F-actin at the same position, indicating copolarization (Supplemental Fig. 6D). In contrast, TD-YFP failed to copolarize with F-actin (Fig. 6B, bottom three rows, 6C), indicating a marked defect in the partitioning of ezrin. The line-scan measurements show an increase in pixel intensity only of F-actin and not ezrin, indicating absence of colocalization (Supplemental Fig. 6E). These data show that expression of TD-YFP did not affect the ability of cells to polymerize F-actin in response to BLC stimulation. Instead, the mobility of the T567D mutant in response to chemokine was grossly inhibited.

Discussion

We have used a combination of scanning EM, epifluorescence, and TIRF microscopy to perform a systematic visual analysis of the earliest changes occurring in chemokine-stimulated B cells. Our data provide evidence that dynamic changes in the conformation of ezrin regulate B cell morphology and migration. We show that chemokine-induced dephosphorylation of the regulatory threonine in ezrin regulates its polarization to the F-actin–rich front pole of migrating B cells. TIRF imaging of both live and fixed B cells showed an abundance of surface microvilli rich in ezrin and moesin. A proteomic analysis of microvilli was reported to show a similar enrichment of the ERM proteins ezrin and moesin in these membrane structures isolated from human T cells (30). As reported by others, we also observed chemokine-induced threonine dephosphorylation of both ezrin and moesin in B cells,
supporting the notion that these proteins function in a redundant manner to facilitate chemotaxis (31, 32). We have employed TIRF microscopy to image microvilli resorption in live B cells stimulated with chemokines. Inhibition of Ser/Thr phosphatase activity in B cells with calyculin A resulted in an impairment in the generation of surface microvilli altogether, suggesting that local and rapid changes in ERM conformation are necessary for the maintenance and dynamic behavior of microvilli. Calyculin A treatment also caused a loss of chemokine-dependent membrane ruffling and a marked reduction in B cell chemotaxis. Dephosphorylation of ERM proteins appears to be required for their detachment from the microvillar membrane and the supporting F-actin filaments to initiate resorption of the microvilli and extension of membrane ruffles. Inhibition of phosphatase activity in neutrophils was similarly reported to result in impaired neutrophil migration. Dephosphorylation of the regulatory T558 residue in moesin was suggested as a necessary step for release and retraction of the uropod during neutrophil migration (33). Inactivation of ERM proteins in chemokine-stimulated T cells is initiated by the reduction of membrane PIP2 levels upon activation of PLC (26). Our results show that inhibition of PLC prevents chemokine-induced ERM dephosphorylation in B cells, indicating that PLC activity may similarly regulate PIP2 levels in B cells to initiate chemotaxis.

Expression of the constitutively active mutant of ezrin also resulted in bald B cells with no surface microvilli, consistent with the effect of Ser/Thr phosphatase inhibition on B cell morphology. In contrast to untransfected 2PK3 B cells, even at 10 min after chemokine-stimulation, 2PK3 cells expressing the T567D mutant of ezrin failed to induce membrane ruffles. A similar dependence of chemokine-induced microvilli resorption on ERM dephosphorylation was reported in human peripheral blood T cells (31). Our results show that the constitutively active mutant of ezrin acts in a dominant-negative manner to restrict spontaneous microvilli formation and prevents chemokine-induced membrane ruffling. Restricted dynamics of these actin-rich membranous structures are likely to contribute toward reduced B cell migration observed in the presence of the constitutively active mutant of ezrin. The expression of the T567D mutant of ezrin only caused a partial inhibition of B cell migration observed in the presence of the constitutively active mutant of ezrin. The expression of the T567D mutant of ezrin only caused a partial inhibition of B cell migration, suggesting that ERM dephosphorylation is one of many factors contributing to the regulation of B cell migration. In our study, the constitutively active mutant of ezrin was found to spontaneously polarize in a majority of the 2PK3 B cells, whereas it was uniformly distributed in the remaining cells. Live imaging of BLC-stimulated 2PK3 B cells revealed that irrespective of its localization, the constitutively active mutant of ezrin was unable to relocate to the leading edge with F-actin. Our results show that polarization of adhesion molecules such as CD44, induced upon strong attachment to the substratum, can force polarization of the constitutively active mutant of ezrin. CD44 was reported to regulate adhesion and polarize to the uropod in chemokine-stimulated T cells (20, 34, 35). Our results suggest that the constitutively active nature of the phosphomimetic mutant of ezrin forces its irreversible association with CD44 and may block uropod detachment and retraction.

Signaling initiated by chemokine receptor engagement regulates cell adhesion and F-actin polymerization. In B cells, chemokine receptor-mediated activation of PI3K was reported to regulate B cell adhesion and migration (36). The expression of constitutively active ezrin in 2PK3 cells did not affect BLC-dependent activation of MAPK and PI3K activation. Actin polymerization also remained unaffected in B cells expressing the active mutant of ezrin, indicating that dephosphorylation of ezrin is not required for the pathway leading to the nucleation of new actin filaments.
Instead, ezrin dephosphorylation primarily acts by facilitating membrane–cytoskeletal tethering and untethering. Stimulation of mouse splenic B cells and 2PK3 B cells with BLC resulted in redistribution of ezrin at the F-actin–rich front edge. Our data indicate that transient dephosphorylation of ezrin mediates its relocation; however, the exact mechanism of relocation is unclear. LOK was recently shown to phosphorylate ERM proteins in human PBLs as well as those in murine peripheral lymphoid organs (25). Interestingly, lymphocytes from mice lacking LOK show enhanced F-actin polarization in response to chemokine stimulation, suggesting that this kinase plays an important role in regulating the equilibrium between phosphorylated and unphosphorylated ERM proteins, contributing to their localization during initiation of lymphocyte migration. In breast carcinoma cells, conformationally active ezrin recruited Cdc42/Rho-specific guanine nucleotide exchange factor Db1 to the GM1-containing lipid raft microdomains (37). This recruitment was proposed as a mechanism of activation of Cdc42 at the lamellipodia, facilitating morphological changes and directed cell migration (37). Inga parietal cells, the rapid turnover of T567-phosphorylated ezrin in the steady state is known to keep membrane activity in a dynamic state (38). We suggest that the redistribution of ezrin to the front of migrating B cells may serve to orchestrate rapidly evolving membrane–actin contacts at the lamellipodia. Because constitutively active T567D ezrin is already tightly associated with the membrane and cortical actin, it fails to dissociate and reassociate with the freshly polymerized and polarized F-actin at the front end and participate in lamellipodial protrusion.

Ezrin is the only ERM family protein expressed in polarized epithelial cells of the small intestine (39, 40), whose surface is covered with dense brush border microvilli. Deficiency of ezrin in mice results in abnormal villus morphogenesis and neonatal death (41). Ezrin was shown to be critical for organizing the apical domain of the intestinal epithelial cells and its associated apical junctions (41). Further, ultra-thin section EM analysis of gastric parietal cells in mice shows a secretory canaliculus structure, which is composed of invaginations of the apical surface bearing numerous microvilli that interact with the tubulovesicular structures that underlie the apical canalicil. In ezrin knockdown mice, the parietal cells lack invaginated apical canalicil that do not expand even in the presence of histamine (42). Our data are in agreement with these studies in which ezrin localization was shown to be important for appropriate membrane contacts. Because the membrane localization of ezrin and its ability to link with actin filaments are regulated by phosphorylation of its regulatory T567 site, rapid and transient changes in phosphorylation of ezrin may orchestrate the dynamic membrane–actin contacts at the lamellipodial front.

An in-depth visual analysis of B cells using high-resolution and live cell imaging revealed a requirement of dephosphorylation of ERM proteins in B cell chemotaxis. Our data indicate that local changes in ezrin conformation are critical for the generation and maintenance of microvilli in resting B cells and their conversion to dynamic lamellipodia in the presence of a chemokine. The location of ezrin to the lamellipodia suggests a role for it in facilitating spatiotemporal molecular partitioning and/or in maintaining the dynamic nature of the front end of the migrating B cells.

Acknowledgments

We thank Twisharsi Dasgupta for help with construction of the YFP fusion proteins used in this study. Jennifer Powers for cell sorting, the Lerner Research Institute Imaging core personnel for help with imaging, and Dr. Amit Vasanji of the Biomedical Imaging and Analysis core for help with analysis of the TIRF data.

Disclosures

The authors have no financial conflicts of interest.

References


SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Chemokine-induced co-polarization of ezrin with F-actin in 2PK3 cells. (A) Line-scan histograms for images in Figure 1B. The line-scan histograms show the average pixel intensity across the dotted line (left) and the solid line (right) drawn on the merged images from Figure 1B. (B) 2PK3 cells were stimulated with 10 μg/ml BLC for the indicated times and fixed prior to staining for ezrin and F-actin. Scale bar, 5 μm. The line-scan histograms on the right show the average pixel intensity across the dotted line (left) and the solid line (right) drawn on the merged images. (C) SDF-1α induces dephosphorylation of ERM proteins in primary B cells. Splenic B cells were stimulated with 3 μg/ml of SDF-1α for indicated lengths of time and lysates were probed with pThrERM or ezrin antibody. The numbers shown below are ratio of pThr ezrin to total ezrin calculated from densitometric analysis of the immunoblots. Data are representative of two independent experiments.

Figure S2. BLC stimulation of B cells adhered to fibronectin induces co-polarization of ezrin with actin similar to that observed on poly-L-lysine. (A) Purified splenic B cells or (B) 2PK3 cells were stimulated with 10 μg/ml BLC for the indicated times and fixed prior to staining for ezrin and F-actin. Scale bar, 5 μm. Data are representative of two independent experiments. The line-scan histograms on the right show the average pixel intensity across the dotted line (left) and the solid line (right) drawn on the merged images.

Figure S3. Optimization of BLC concentration for in vitro migration and ERM dephosphorylation in B cells. (A) Purified splenic B cells were stimulated with 100 ng/ml, 300 ng/ml or 1 μg/ml of BLC for indicated lengths of time and lysates were probed with pThrERM
or ezrin antibody. **(B)** The bar graph represents quantification of the data in (A) showing the percent of phosphorylation remaining upon stimulation with the different doses of BLC. The ratio of pThrERM to total ezrin calculated from densitometric analysis of the immunoblots were normalized to the ratio in the unstimulated control. Data are representative of two independent experiments. **(C)** Transwell migration of purified splenic B cells in response to the indicated doses of BLC. **(D)** Transwell migration of 2PK3 cells in response to the indicated doses of BLC. The bar graphs in (C) and (D) show percent migration in response to BLC.

**Figure S4. Generation of a phosphomimetic mutant of ezrin and stable transfectants of 2PK3 cells expressing the constitutively active mutant of ezrin.** (A) Schematic of the full length ezrin in pIRES2-EGFP vector in which threonine residue at position 567 was mutated to aspartic acid (T→D) to generate a constitutively active (T567D) mutant of ezrin. **(B)** GFP (left) and CXCR5 (right) expression on mock transfected (shaded histogram) 2PK3 cells or those expressing the T567D mutant of ezrin (black histograms). The gray unfilled histogram in the right panel represents unstained 2PK3 cells. **(C)** Expression of ezrin and moesin in lysates of 2PK3 cells 24 h after they were mock transfected or transfected with pI2E-T567D. **(D)** Untransfected 2PK3 cells (shaded histograms) or those stably expressing T567D (black histograms) were analyzed by flow cytometry for GFP (left) or CXCR5 expression (right). The gray unfilled histogram in the right panel represents unstained 2PK3 cells. **(E)** Expression of ezrin and moesin in lysates of untransfected 2PK3 cells or those stably expressing the T567D mutant of ezrin.
Figure S5. Expression of constitutively active ezrin in 2PK3 cells does not affect BLC-dependent MAPK and PI3K activation. (A) Mock-transfected 2PK3 cells (empty bars) or those stably expressing the T567D mutant of ezrin (filled bars) were left unstimulated or stimulated with 3 µg/ml of BLC for the indicated times. The cell lysates were probed with antibodies to ERK, pERK, JNK, pJNK, Akt and pAkt. The graphs show the ratio of pERK to total ERK (left), pJNK to total JNK (middle) and pAkt to total Akt (right) calculated from densitometric analysis of the immunoblots. Data are average of three independent experiments (mean±SD). (B) Inhibition of phospholipase C activity inhibits chemokine-induced threonine dephosphorylation of ezrin in B cells. Splenic B cells were pretreated with 2.5 µM of the PLC-inhibitor U-73122 and left unstimulated or stimulated with 1 µg/ml of BLC for 3 min and lysates were probed with pThrERM or ezrin antibody. (C) The bar graph shows the ratio of pThrERM to total ezrin calculated from densitometric analysis of the immunoblot shown in (B). Data are representative of two independent experiments.

Figure S6. (A) Concentric segmentation of TIRF images for quantification of microvilli resorption. The images shown in Fig. 4A and B were segmented into concentric regions of interest (ROIs). Each ROI encompassing the contact area was 10 pixels wide and the innermost region was numbered one with the count increasing towards the cell periphery. The individual filaments were counted in each ROI using a custom algorithm and total filament area (Fig. 4C’-F’) was calculated in each ROI at each time point of the time-lapse movies (Supplemental videos 1-4). (B-C) Constitutively active ezrin co-localizes with CD44 and fails to relocalize in response to BLC. 2PK3 cells were transiently transfected with TD-YFP, stimulated with 10 µg/ml BLC for the indicated times, stained for CD44 and imaged in the epifluorescence mode. B
cells displaying both uniform (B) and polarized (C) distribution of TD-YFP show co-localization of CD44 and ezrin. Scale bar, 5 μm. Data are representative of three independent experiments (n=20 per group). (D-E) Line-scan histograms for images in Figures 6A and 6B. The line-scan histograms (D) and (E) show the average pixel intensity across the dotted line (left) and the solid line (right) drawn on the merged images from Figure 6A and 6B, respectively.

DESCRIPTION OF VIDEOS

Supplemental Video 1: The B cell surface is covered with dynamic ezrin-rich microvilli.

2PK3 cells were transiently transfected with Ez-YFP (green) and imaged in the TIRF mode for 5 min in the absence of BLC stimulation. Images were acquired every 20 s with a 100x TIRF objective. The movie is set to play at 5 frames per second.

Supplemental Video 2: Constitutively active ezrin inhibits the formation of microvilli in B cells. 2PK3 cells were transiently transfected with TD-YFP (green) and imaged in the TIRF mode for 8 min in the absence of BLC stimulation. Images were acquired every 8 s with a 100x TIRF objective. The movie is set to play at 5 frames per second.

Supplemental Video 3: BLC stimulation induces resorption of ezrin-rich microvilli. 2PK3 cells were transiently transfected with Ez-YFP (green) and imaged in the TIRF mode for 8 min in the presence of 10 μg/ml BLC. Images were acquired every 8 s with a 100x TIRF objective. The movie is set to play at 5 frames per second.
Supplemental Video 4: Constitutively active ezrin inhibits the formation of microvilli in B cells. 2PK3 cells were transiently transfected with TD-YFP (green) and imaged in the TIRF mode for 8 min in the presence of 10 μg/ml BLC. Images were acquired every 8 s with a 100x TIRF objective. The movie is set to play at 5 frames per second.

Supplemental Video 5: BLC stimulation induces polarization of ezrin. 2PK3 cells were transiently transfected with Ez-YFP (green), stimulated with 10 μg/ml of BLC and imaged live in the epifluorescence mode for 8 min. Images were acquired every 20 s with a 100x objective. The movie is set to play at 5 frames per second.

Supplemental Video 6: Uniformly distributed constitutively active ezrin fails to polarize upon BLC stimulation. 2PK3 cells were transiently transfected with TD-YFP (green), stimulated with 10 μg/ml of BLC and imaged live in the epifluorescence mode for 8 min. Images were acquired every 20 s with a 100x objective. The movie is set to play at 5 frames per second.

Supplemental Video 7: Polarized constitutively active ezrin fails to relocalize upon BLC stimulation. 2PK3 cells were transiently transfected with Ez-YFP (green), stimulated with 10 μg/ml of BLC and imaged live in the epifluorescence mode for 8 min. Images were acquired every 8 s with a 100x objective. The movie is set to play at 5 frames per second.