Biochemical and Biophysical Research Communications 427 (2012) 202–206

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Analyzing actin dynamics during the activation of the B cell receptor in live B cells

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ARTICLE INFO

Article history:
Received 5 September 2012
Available online 17 September 2012

Keywords:
Actin reorganization
BCR activation
Soluble and membrane antigens
Actin polymerization
Actin depolymerization

ABSTRACT

Actin reorganization has been shown to be important for lymphocyte activation in response to antigenic stimulation. However, methods for quantitative analysis of actin dynamics in live lymphocytes are still underdeveloped. In this study, we describe new methods to examine the actin dynamics in B cells induced by antigenic stimulation. Using the A20 B cell line expressing GFP–actin, we analyzed in real time the redistribution of F-actin and the lateral mobility of actin flow in the surface of B cells in response to soluble and/or membrane associated antigens. Using fluorescently labeled G-actin, we identified the subcellular location and quantified the level of *de novo* actin polymerization sites in primary B cells. Using A20 B cells expressing G-actin fused with the photoconvertible protein mEos, we examined the kinetics of actin polymerization and depolymerization at the same time. Our studies present a set of methods that are capable of quantitatively analyzing the role of actin dynamics in lymphocyte activation.

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1. Introduction

The actin cytoskeleton is essential for the formation of immunological synapses between T cells and antigen presenting cells [1–3]. Actin reorganization in T cells is triggered and regulated by both adhesion molecules and T cell receptor (TCR) [4]. T cells also undergo spreading and contraction during synapse formation, which is associated with a centripetal actin flow [5]. In addition to its well-known role in antigen presentation to T cells, data from previous studies suggest that actin reorganization also plays a crucial role in the activation of B cells. Antigen induced B cell receptor (BCR) activation is important for B cell function. BCR activation was shown to induce global actin depolymerization, followed by actin polymerization. Depolymerization enhanced BCR signaling, while blocking depolymerization reduced the BCR signaling [6]. Recent reports show that depolymerization of the actin cytoskeleton inhibits surface BCR clustering in response to membrane-tethered antigens [7], but increases the lateral diffusion rate of surface BCRs and induces signaling in the absence of antigen [8]. An ezrin- and actin-defined network was suggested to influence steady-state BCR diffusion by creating boundaries that restrict BCR diffusion; thus, the membrane skeleton could control signaling by influencing BCR dynamics [9]. Antigen binding to the BCR-induced kinetically and spatially controlled actin reorganization. This actin reorganization is required for B-cell spreading and contraction and controls BCR clustering in response to antigen. The stimulatory kinase Btk and the inhibitory phosphatase SHIP-1 have opposite regulatory roles in actin reorganization, B-cell spreading and contraction, surface BCR clustering, and B-cell surface signaling [10]. Previous research has also shown that actin is important for the presentation of antigens by B cells. Cytochalasin D treatment of F6 B lymphoma cells dramatically reduced the degradation of the invariant chain and delayed the appearance of stable forms of class II molecules, which reduced the efficiency of antigen presentation [11]. Also, Cytochalasin D treatment of CH27 cells decreases the internalization of BCR and blocks the movement of BCR from early endosomes to late endosomes [12].

Actin reorganization in lymphocytes has been traditionally studied by phalloidin staining and with labeled antibodies targeting to actin. FRAP analysis of membrane-proximal regions of interest (ROIs) in LPS/IL-4-activated B cells or IL-2-activated T cells that had been transfected with actin–GFP showed that Ag receptor-induced cell spreading is associated with increased actin dynamics [13]. Lifeact, a 17-amino-acid peptide that labels F-actin in eukaryotic cells, allows visualization of actin dynamics in nontransfectable cells without interfering with actin dynamics caused by GFP tags *in vitro* and *in vivo* [14].

Tracking of actin dynamics and reorganization in live cells is rarely performed after BCR activation in response to soluble antigens. However, the predominant form of antigen *in vivo* has been suggested to be attached to membrane surfaces, such that B cell membranes interact with other membranes to form BCR clusters and an immunological synapse [15]. Because of constraints due

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0006-291X/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2012.09.046

to mimicking of membrane-bound antigens and the required microscopy techniques, live cell tracking of actin dynamics and reorganization is rarely performed after BCR activation in response to membrane-bound antigens. It is important to quantify the actin dynamics in this physiological closed system. Experiments with G-actin incorporation indentify the *de novo* actin polymerization sites and dynamics, and can exclude the background caused by GFP–actin cell lines. Because of the tough environment caused by polymerization buffer, research on G-actin incorporation has been limited to splenic B cells. Actin depolymerization tracking is still a challenge because of the background levels of G-actin. Photoconvertible fluorescent proteins create a brighter signal and lower background, suitable for tracking the actin depolymerization events [16]. In this study, we tracked in real time the actin reorganization in GFP–actin mouse B lymphoma cells (A20) in response to soluble antigens by confocal microscopy and quantified actin flow speed in response to membrane antigens using total internal reflection fluorescence (TIRF) microscopy. *De novo* actin polymerization sites and dynamics were examined in primary B cells by TIRF microscopy. Finally actin polymerization and depolymerization was investigated using photo-convertible fluorescent actin (mEos-actin) expressed in A20 cells by confocal microscopy.

2. Materials and methods

2.1. Mice and cells

Wild type (wt) (CBA/CaJ), 6–10 weeks old mice (Jackson Laboratories, Bar Harbor, ME) were used. To isolate splenic B cells, mononuclear cells were subjected using Ficoll (Sigma–Aldrich, St Louis, MO) density-gradient centrifugation, treated with anti-Thy1.2 monoclonal antibodies (BD Biosciences, San Jose, CA) and guinea pig complement (Rockland Immunobiochemicals, Gilbertsville, PA) to remove T cells, and panned for 1 h to remove monocytes. B cell lymphoma A20 IIA1.6 cells (H-2d, IgG2a$^+$, FcγRIIB$^-$) were cultured in DMEM supplemented with 10% FBS.

2.2. Generation of A20 cells expressing GFP–G-actin and mEos-G-actin

B cell lymphoma A20 IIA1.6 cells were cultured at 37 °C in DMEM supplemented with 10% FBS. The DNA construct encoding the eGFP fusion protein of actin (eGFP–actin) or mEos fusion protein of actin (mEos-actin) was introduced into A20 B cells by electroporation using the Nucleofection kit V from Amaxa (Gaithersburg, MD). Transfected A20 cells were select with G418 (1 mg/ml) in DMEM supplemented with 10% FBS for two weeks and sorted by Flow Aria, and then maintained with G418 (0.5 mg/ml) in DMEM supplemented with 10% FBS.

2.3. Preparation of mono-biotinylated Fab′ antibody

Mono-biotinylated Fab′-anti-mouse IgM+G antibodies (mB-Fab′-anti-Ig) were generated from F(ab′)$_2$ fragments (Jackson ImmunoResearch, West Grove, PA) using a published protocol [17]. The disulfide bond that links the two Fab′ was reduced using 20 mM 2-mercaptoethylamine, and the reduced cysteine was biotinylated by maleimide activated biotin (Thermo Scientific, Odessa, TA). Fab′ was further purified using Amicon Ultra Centrifugal Filter (Millipore, Temecula, CA). Titration of one biotin per Fab′ was confirmed by a biotin quantification kit from Thermo Scientific. Fab′ was labeled with Alexa Fluor 546 using a kit from Invitrogen (Carlsbad, CA).

2.4. Immunofluorescence by confocal microscopy

For live cell imaging, A20 cells that express a GFP fusion of actin were incubated with AF546-mB-Fab′-anti-Ig (10 μg/ml) at room temperature for 10 min to label the BCR, washed, and incubated with streptavidin (1 μg/ml) to activate the BCR. Images were acquired 3 s between each frame using a Zeiss 7 Live-DUO confocal microscope.

2.5. Preparation of antigen-tethered planar lipid bilayers

The planar lipid bilayer was prepared as described previously [18–19]. Liposomes were made by sonication of 1,2-Dioleoyl-sn-Glycero-3-phosphocholine and 1,2-Dioleoyl-sn-Glycero-3-phosphoethanola-mine-cap-biotin (Avanti Polar Lipids, Alabaster, AL) in a 100:1 M ratio in PBS at a lipid concentration of 5 mM. Aggregated liposomes were removed by ultracentrifugation and filtering. Coverslip chambers (Lab-Tek Nalge Nunc, Rochester, NY) were incubated with the liposomes (0.05 mM) for 10 min. After extensive washes, the coated coverslip chambers was incubated with 1 μg/ml streptavidin (Jackson ImmunoResearch), followed by 2 μg/ml AF546-mB-Fab′-anti-Ig and 8 μg/ml mB-Fab′-anti-Ig antibody.

2.6. Total internal reflection microcopy and image analysis

The surface dynamics and organization of the BCR and other molecules were analyzed using a TIRFm (TE2000U, Nikon). Images were acquired using a 100× NA 1.49 Apochromat TIRF objective lens (Nikon). For live cell imaging, time-lapse imaging started upon the addition of B cells onto the lipid bilayers tethered with AF546-mB-anti-Ig and continued for 5–10 min at 37 °C. GFP/AF488, AF546, interference refection images (IRM) were acquired sequentially at each time point. B cell contact area was determined using MATLAB software. Total fluorescence intensities of AF546-mB-anti-Ig in the contact zone and relative fluorescence and IRM intensity along a line across cells were quantified using Andor iQ software (Andor Technology, Belfast, UK). Background fluorescence, such as antigen-tethered lipid bilayers in the absence of B cells was subtracted. For each set of data with statistics, more than 20 individual cells from two or three independent experiments were analyzed.

2.7. Tracking speed and direction of actin flow

Spatio-temporal image correlation spectroscopy [20] was used to detect the directed movement of actin from the analysis of fluorescence image time series. The technique involves calculating the spatial–temporal correlation function of fluorescence intensity fluctuations as a function of the time-lag between successive images. For diffusive motion of the fluorescent particles, the correlation function exhibits a peak that decreases in amplitude and broadens as a function of time-lag. However, for fluorescent proteins exhibiting directional motion, the correlation peak shifts opposite to the direction of motion. The correlation functions were calculated using Fourier transforms and the velocity values were extracted using the STICS algorithm implemented in MATLAB and freely available from the Cell Migration Gateway.

Velocity maps were generated by selecting a grid of overlapping, 16×16 pixel (1.6×1.6 microns) square regions (with an overlap of 8 pixels) that spanned the region of interest. The images were corrected for immobile fraction on each sub-region time stack within this grid. Image series were collected with time intervals of x seconds; which is slower than the diffusive motion of proteins (in the millisecond range).