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A system for reconstructing B cell antigen receptor signaling in the mouse myeloma J558L cell line

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ABSTRACT

B cell antigen receptor (BCR) signaling is positively and negatively regulated by various cell surface receptors such as CD19 and CD45. Functional analysis of these receptors has been performed using gene targeting technology, which is a valid approach to elucidate their functions. However, this type of analysis is restricted when multiple molecules are evaluated simultaneously. From a different perspective, synthetic biology provides a high degree of freedom for analyzing various molecules. Here we developed a system to reconstruct BCR signaling using the J558L myeloma cell line in combination with the protein-based Ca^{2+} indicator YC3.60. BCR-reconstituted J558L cells harboring YC3.60 (J558Lμν11 cells) permitted monitoring of Ca^{2+} mobilization. Reconstituting CD19 in J558Lμν11 cells resulted in detectable BCR-induced Ca^{2+} mobilization but with kinetics different from that of CD45-expressing cells. Furthermore, we evaluated the validity of the J558L system by proteomic analysis of tyrosine-phosphorylated proteins after antigen stimulation. Identification of more than 100 BCR-induced tyrosine-phosphorylated proteins in J558Lμν11 cells revealed a similarity to that observed in B cells, and a novel member, non-receptor protein tyrosine kinase Fer, was found. Thus, this reconstruction system using J558L cells appeared to be valid for comprehensively investigating BCR signaling.

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Introduction

B cell antigen receptor (BCR)[1] signaling plays crucial roles in cell activation, proliferation, differentiation, or apoptosis. BCR signaling is positively or negatively regulated by many cell surface molecules such as CD45, CD19, CD40, and CD22 [1]. Their functional roles in BCR signaling have been analyzed using gene-targeted or overexpressed cells and mouse models. Synthetic biology is also a valid approach and a feasible method particularly to analyze multiple molecules. Synthetic biology has a high flexibility for analyzing BCR signaling using various combinations of components. Till date, the BCR-reconstituted mouse myeloma J558L cell line (J558Lμm3) has been used for BCR signaling analyses [2–6]. J558Lμm3 cells have a nitrophenol (NP)-specific BCR and most of essential intracellular signaling molecules such as Syk, SLP-65/BLNK, and HS1, but lose many cell surface receptors such as CD19, CD22, CD72 and CD45 [5,7]. When used for B cell signaling analyses, this cell line has provided valuable information on the general physiological properties of B cells [4,6,8]. However, Ca^{2+} mobilization, one of the typical BCR signaling events, is almost impaired in this cell line. CD45-reconstituted J558Lμm3 cells have been shown to restore Ca^{2+} mobilization after BCR ligation by an antigen [2]. In addition, further expression of CD19 augmented the Ca^{2+} response [3].

CD45 is a leukocyte specific receptor-like protein tyrosine phosphatase (PTP) [9]. CD45 on B cells lowers the threshold of BCR signaling. However, CD19 is a member of the type 2 complement receptor (CR2) complex found on B cell surfaces [10]. On BCR stimulation, CD19 recruits various signaling molecules, such as Vav and phosphoinositide 3-kinase (PI3K), to the cell surface. Both CD45 and CD19 have been reported to regulate Src-family kinase Lyn and augment the PI3K signaling pathway and Ca^{2+} mobilization induced by BCR engagement [9,10].

BCR-induced Ca^{2+} mobilization has been measured widely using chemical fluorescence indicators, such as Fluo-4 and Indo-1. We previously demonstrated that a protein-based fluorescent Ca^{2+} indicator, YC3.60, could be used to measure BCR-induced Ca^{2+} mobilization [11]. YC3.60 is a fluorescence resonance energy

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[1] *Abbreviations used*: BCR, B cell antigen receptor; PI3K, phosphoinositide 3-kinase; PTP, protein tyrosine phosphatase; FRET, fluorescence resonance energy transfer; Ab, antibody; mAb, monoclonal antibody; SH2, Src-homology domain 2; NP, nitrophenol.

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transfer (FRET)-based indicator comprising two fluorescent proteins, CFP and YFP, and two Ca^{2+}-responsive elements, a variant of calmodulin (CaM) and a CaM-binding peptide. The genetically encoded Ca^{2+} indicator YC3.60 exhibits stable fluorescence intensity and localization over time, which conceals the shortcomings of chemical indicators. Thus, YC3.60 is an exquisite probe for analyzing BCR-mediated Ca^{2+} mobilization.

In this study, we developed a reconstruction system using J558L cells to monitor Ca^{2+} mobilization and to analyze multiple signaling components. We evaluated the signaling capacity of J558L cells by proteomic analysis of tyrosine-phosphorylated cellular substrates after BCR ligation. Using this system, we attempted to elucidate the distinct effects of CD19 and CD45 on BCR signaling.

Materials and methods

Plasmids

The retrovirus vectors pMx-CD45 and pMx-CD19 were constructed as follows. A *Xho*I-*Not*I fragment containing mouse CD45B cDNA [12] was inserted into the *Xho*I-*Not*I site of a pMx vector, which resulted in pMx-CD45. Mouse CD19 cDNA was obtained by RT-PCR using a set of primers (5′-ctcgaggctaccatgccatctcctctc-3′ and 5′-gcggccgctcacgtggttcccaagtc-3′), and a *Xho*I-*Not*I fragment containing CD19 cDNA was inserted into the *Xho*I-*Not*I site of a pMx vector, which resulted in pMx-CD19. pMx cameleon was described previously [11]. Mouse Fer cDNA was obtained by RT-PCR using a set of primers (5′-agtcgactacaaaatgggatttgggagtgac-3′ and 5′-agcggccgcctatgtgatcatcttcttgatgac-3′), and a *Sal*I-*Not*I fragment containing Fer cDNA was inserted into the *Xho*I-*Not*I site of pMx vector, which resulted in pMx-Fer.

Cell lines and mice

The mouse myeloma J558Lμm3 cell line and the K46μv and WEHI-231 lymphoma cell lines that expressed cameleon were described previously [11]. To obtain retroviruses, plasmids were transfected into Plat-E cells [13] using a calcium phosphate precipitation method. Cells were infected with a retrovirus expressing CD19 and/or CD45R (B220). To purify cells that expressed these molecules, cells were stained and sorted with an autoMACS (Miltenyi Biotec) or Mo-flo (Beckman Coulter). Spleen B cells from QM mice [14] were purified as described previously [15]. Mice were maintained under specific pathogen-free conditions according to the guidelines of the animal committee of Tokyo Medical and Dental University.

Immunoprecipitation and Western blotting

Cells were stimulated with 0.2 μg/ml NP-BSA (ratio = 15:1) and then lysed in Triton-X 100 lysis buffer [7]. Lysates were immunoprecipitated with rabbit anti-Lyn antibody (Ab), anti-Fer Ab (Santa Cruz Biotechnology), or anti-CD19 Ab together with protein G-Sepharose (Amersham Pharmacia Biotech). Alternatively, cells were stimulated with 0.2 μg/ml NP-BSA or 10 μg/ml anti-IgM Ab (Southern Biotechnology Associates) and then lysed in SDS–PAGE sample buffer. Total cell lysates or immunoprecipitates were separated by SDS–PAGE and transferred to PVDF membranes. Membranes were incubated with anti-phospho-tyrosine monoclonal antibody (mAb) 4G10 (Upstate Biotechnology). Alternatively, membranes were reacted with rabbit anti-Lyn Ab, anti-Fer Ab, anti-phospho-Lyn (Tyr507) Ab, anti-phospho-Akt Ab, anti-phospho-Src family (Tyr416) Ab, anti-phospho-p44/p42 MAPK Ab (the latter four from Cell Signaling Technology), anti-CD19 Ab, or anti- BLNK/SLP-65 Ab [16], followed by peroxidase-conjugated anti-rabbit IgG Ab (New England Biolabs) or anti-β-tubulin mAb TUB 2.1 (Seikagaku Kogyo) and finally reacted with peroxidase-conjugated anti-mouse IgG Ab (Amersham Pharmacia Biotech). Proteins were visualized using Chemi-Lumi One (Nacalai tesque).

Flow cytometry

Cells were incubated with biotin-labeled anti-mouse CD22 mAb Cy34.1 (BD Biosciences), followed by reaction with FITC-labeled streptavidin (Dako) or Alexa647-labeled anti-mouse B220 mAb (BioLegend). Labeled cells were analyzed by flow cytometry using a CyAn ADP™ (Beckman Coulter).

Determinations of intracellular Ca^{2+} mobilization

Ca^{2+} mobilization in cameleon-expressing cells was analyzed by flow cytometry using a Flicyme (Mitsui Engineering & Shipbuilding Co., Ltd) and a CyAn ADP™ (Beckman Coulter) equipped with a 408 nm Laser Diode (LD) and 405 nm solid state laser, respectively. At 408 nm or 405 nm excitation, FRET was calculated as the ratio of YFP to CFP intensity [11].

Immunoaffinity enrichment of tyrosine-phosphorylated proteins and mass spectrometry

Tyrosine-phosphorylated proteins in antigen-stimulated cells (1×10^8) were purified using an anti-phospho-tyrosine mAb-coupled Sepharose column as described previously [17]. For protein identification, the concentrated affinity-purified phosphorylated proteins were separated by SDS–PAGE (8% polyacrylamide gel), after which the protein bands were stained with Coomassie Brilliant Blue and excised. The gel pieces were destained, reduced (10 mM dithiothreitol), and alkylated (55 mM idoacetoamide). After washing and drying by vacuum centrifugation, trypsin (sequencing grade; Promega) was added to the gel pieces, which were then incubated overnight at 30 °C. The mixture of extracted peptides was concentrated by vacuum centrifugation.

Analysis of peptide mixtures was performed using an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) and an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). The peptide mixtures were acidified with 0.1% (v/v) trifluoroacetic acid and desalted using C18 ZipTip (Merck Millipore). The mixtures were prepared using the thin layer affinity method with an AnchorChip PAC 96 HCCA (Bruker Daltonics) prespotted with an α-cyano-4-hydroxycinnamic acid matrix. MALDI-TOF spectra were acquired on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer. Tandem mass spectrometry (MS/MS) data were acquired by MALDI postsource decay.

Positively charged ions were analyzed automatically in the reflector mode using FlexControl 3.3 operation software (Bruker Daltonics). Postprocessing of the MS and MS/MS spectra was performed using FlexAnalysis version 3.3 (Bruker Daltonics). Database searches in a primary sequence database were performed using the Mascot Software 2.3.01 (Matrix Science) to identify proteins. Alternatively, the peptide mixtures were analyzed using an LTQ-Orbitrap Velos mass spectrometer connected to Proxeon EASY-nLC (Thermo Fisher Scientific). Each peptide mixture reconstituted in 0.1% formic acid (solvent A) was loaded on a 75 μm inner diameter × 120 mm C18 reverse phase column (NIKKYO, NTCC-360/75-3-125). Peptides were separated using the following gradient: 0–30 min, 14.3%–71.5% solvent B (70% acetonitrile in 0.1% formic acid); 30–31 min, 71.5%–100% solvent B; 31–36 min, 100% solvent B. The flow rate was set at 300 nl/min.

MS data were acquired with an Orbitrap analyzer from m/z 300 to 2000 at a resolution of 60,000. The 10 most abundant ions were selected for fragmentation. Fragmentation methods used were

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