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ROZA-XL, an improved FRET based biosensor with an increased dynamic range for visualizing Zeta Associated Protein 70 kD (ZAP-70) tyrosine kinase activity in live T cells

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ABSTRACT

Genetically encoded FRET based biosensors allow one to visualize the spatial and temporal evolution of specific enzyme activities in live cells. We have previously reported the creation of a FRET based biosensor specific for Zeta-Associated Protein −70 kD (ZAP-70) (Randriamampita et al., 2008), a Syk family protein tyrosine kinase. ZAP-70 is essential for early T cell receptor (TCR) signaling events, T lymphocyte development and has also been implicated in integrin mediated T lymphocyte migration. In order to facilitate the study of ZAP-70 kinase activity during dynamic phenomena such as immunological synapse formation or cell migration, we have designed and prepared a second generation of ZAP-70 specific biosensors. Here we describe a novel biosensor named ROZA-XL, that displays a 3–4 times greater dynamic range than its predecessor and possesses a robust baseline FRET value when expressed in the Jurkat human T cell line. We demonstrate that the robust behavior of this biosensor allows for rapid analysis of TCR mediated of ZAP-70 kinase activity at a single cell level, as shown in a simple end point assay in which ROZA-XL expressing cells are allowed to interact with stimulatory anti-CD3epsilon coated coverslips.

1. Introduction

An important challenge to furthering our understanding of mechanisms underlying receptor mediated signal transduction is the ability to follow key signaling phenomena, notably enzymatic events, in real time at a single cell level. One of the more promising approaches available today involves genetically encoded FRET based biosensors that have been designed to report the spatial and temporal evolution of specific enzymatic activities, such as small GTPase activity, or protein kinase activity, in live cells [1]. Single chain FRET based biosensors have been applied to a number of signaling events important to the biology of T lymphocytes, notably migration, adhesion and T cell receptor (TCR) signaling ([2–4] recently reviewed in Ref. [5]). We have previously developed a biosensor for the Syk family tyrosine kinase ZAP-70 [6]. ZAP-70, expressed exclusively in T cells and NK cells, is best known for its critical role in early TCR signaling. TCR engagement leads rapidly to ZAP-70 activation and subsequent ZAP-70 mediated phosphorylation of signaling adaptors such as Linker for Activation of T cells (LAT) and SH2 domain–containing leukocyte phosphoprotein of 76 kDa (SLP-76) [7,8], both important signaling branch points leading to changes in internal [Ca2+], MAP kinase pathway activation and cytoskeletal modifications [9]. ZAP-70 has also been shown to be required for integrin mediated T lymphocyte

Abbreviations: ZAP-70, Zeta Associated Protein 70 kD; SH2, Src homology 2; LAT, Linker for Activation of T cells; PLCγ1, Phospholipase Cγ1; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa.

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migration [10]. Like other protein kinase biosensors, the ZAP-70 biosensor ROZA (Reporter of Zap-70 Activity) was designed to be a surrogate substrate for ZAP-70, and when expressed in T cells, its FRET status reflects the local balance of activated kinases and phosphatases acting on the biosensor [11].

In the majority of biochemical or cellular imaging experiments described in the literature, ZAP-70 tyrosine kinase activity is examined indirectly using methods (i.e. FACS, western blotting or immunofluorescence staining) that rely on immune-detection of the phosphorylation state of ZAP-70 residues known to be involved in regulating the kinase activity, notably the interdomain B tyrosine residue 319. These methods require cell fixation or cell lysis, thus precluding detailed study of the spatial or temporal evolution of the kinase activity. The tyrosine 319 residue and others in the interdomain B are also binding sites for SH2 domain containing signaling partners, and therefore, the immunolabeling is also an indicator of the adaptor function of ZAP-70. In addition, recent reports have demonstrated that tyrosine 319 can be phosphorylated even if the catalytic site is inactivated, emphasizing the continuing need for alternative methods for the study of this important enzyme that are both compatible with live cell imaging and provide a direct read-out of the tyrosine kinase activity [12,13].

We previously employed the ROZA biosensor to examine the spatial-temporal distribution of ZAP-70 kinase activity in primary human T cells during the formation of a super-antigen mediated immunological synapse with Raji B cells [6]. This first generation ROZA gave a ~25% change in normalized ratiometric FRET signal in a ZAP-70-dependent manner, a dynamic range similar to other FRET-based kinase biosensors [6,14]. Further study of the differential ZAP-70 response to more physiological antigenic stimuli, or the subcellular redistribution of activated ZAP-70 during synapse formation, would be facilitated by a biosensor with an increased dynamic range. We report here the elaboration of a second generation of ZAP-70 reporters based on elements of either LAT or SLP-76, from which we identified one, named ROZA-XL, who possesses a dynamic range 3–4 times greater than the first generation ROZA, and who displays a robust background FRET condition when stably expressed in the Jurkat E6 human T cell line. In a simple assay employing coverslips coated with a standard anti-CD3e antibody and epifluorescent imaging, we show that this improved biosensor can be used to rapidly assess ZAP-70 kinase activity upon TCR engagement at a single cell level opening the way to more sophisticated experiments.

2. Materials and methods

2.1. Cells and reagents

Jurkat clone E6 was from ATCC; ZAP-70-deficient clone P116 was a generous gift of Hai-Tao He, CIML, Marseille, France. YFP variant YPET was a kind gift of P Daugherty, UC Davis, USA. The following mAbs were used: anti-CD3 epsilon clone OKT3 for stimulations and FACs analysis was from BD Bioscience (Franklin Lakes NJ, USA), anti-phospho-ZAP-70 Y319 was purchased from Cell Signaling Technology (NEB (UK) Ltd); anti-ZAP-70 (2F3.2) was from Upstate (Lake Placid NY,USA); anti-actin (MAB1501R) was from Millipore (Billerica, MA USA); anti-phospho-LAT 191 from Biosource/Life Technologies (Saint Aubin France) and secondary HRP-conjugated goat anti-mouse antibodies were from BioRad (Hercules, CA, USA). DNA sequences coding for the human VAV1-SH2 domain (residues 671–769) coupled to SLP76 peptide sequences (residues 110–120 or 124–135) were custom synthesized by Eurofins MWG Biotech (Ebersberg, Germany). Linker sequences were chosen as per the published ROZA biosensor. Other ROZA variants were prepared and cloned into pCDNA3.1 as described previously [6]. Cells were cultured at the Platforme de Culture Cellulaire, INSERM, Marseille, France. The complete nucleotide sequence of ROZA-XL has been deposited in Genebank (Accession number KM979358). Expression vectors coding for ROZA-XL and ROZA-XL YF will be available from Addgene.

2.2. Cell transfections

Wild type E6 and ZAP-70-deficient Jurkat cell lines were nucleofected with the Amaxa electroporation system using Solution V, program S-018 (Lonza, Basel, Switzerland). Cells were used for stimulation and imaging experiments 24–48 h after transfection. Stable transfectants were created by culturing the cells in the presence of 1.2 μg/mL G-418 (Gibco, Carlsbad CA, USA), sorting for YPET positive cells, and cloning by limiting dilution. All Jurkat clones were evaluated for TCR expression by flow cytometry before use and clones with similar biosensor and TCR CD3ε expression were selected for study.

2.3. FRET image acquisition

Biosensor-expressing Jurkat cells were imaged in custom-made microwells cut from polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer Kit; Dow Corning, Midland, MI USA) and sealed to glass coverslips via O_2-plasma oxidation. Transfected cells were rinsed into HBSS (Gibco) supplemented with 2% FCS (Lonza), 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 5 mM Hepes pH 7.4 and allowed to rest for 15 min at 37 °C before imaging. For coverslip stimulation experiments, the wells were treated with either 0.01% poly-L-lysine in H_20 (Sigma) for 10 min at room temp or 10 ug/mL anti-CD3ε (OKT3) in PBS, 4 °C overnight, after rinsing, wells were filled with 150 uls warm imaging buffer and placed on the pre-warmed microscopy stage. At time 0, cells were added in an equal volume of imaging buffer and FRET sequence was acquired at 5 min.

2.4. Fluorescence microscopy

All FRET experiments were performed at 37 °C using a Zeiss Z1 (Carl Zeiss, Oberkachen, Germany), automated microscope equipped with a CoolSnap HQ CCD camera (Photometrics, Tucson, AZ), a Sutter Lambda 3 emission filter wheel and piloted by μManager [15]. Brightfield and fluorescent images were collected at either 40x/1.3 or 63x/1.3 magnification (Zeiss Plan-Neofluar). For ratiometric FRET imaging, three images were collected consecutively at each time point, brightfield (10 ms), CFP (excitation 436/20 nm, dclp 455, emission 480/40 nm, 100 ms) and FRET (excitation at 436/20 nm, dclp 455, emission 535/30 nm, 100 ms). For time lapse imaging Jurkat cells expressing biosensors, all three channels were collected every 15 s for no more than 20 min to minimize photobleaching.

2.5. Image analysis

An image processing program was developed in house using MATLAB software in order to automatically detect and evaluate the average cell fluorescence intensity of each channel (CFP and FRET) in an image sequence (Mathworks, Natick, MA, USA). The principal steps of the program include background subtraction, thresholding and binarisation of the images to distinguish cells from the background and to create a mask for fluorescence intensity calculations. These steps are followed by identification and numbering of cells in the first image and following them in the next image. In subsequent steps, the fluorescence intensity for each cell identified in each image of the series is calculated and written to output. The FRET ratio was calculated as CFP/FRET intensity for each cell. In time