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Development of an endoplasmic reticulum calcium sensor based on fluorescence resonance energy transfer

Tae-Jin Kim[a,b,c,*], Kyung-A Kim[a,b], Sang Hoon Jung[a,b,*]

[a] Natural Products Research Center, Korea Institute of Science and Technology (KIST), Gangneung 25451, Republic of Korea
[b] Department of Biological Chemistry, University of Science and Technology (UST), Daejeon 34113, Republic of Korea
[c] Department of Biological Sciences, Pusan National University, Busan 46241, Republic of Korea

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ABSTRACT

Endoplasmic reticulum (ER) is an organelle critically involved in Ca^{2+} signaling by regulating the intracellular Ca^{2+} gradient. In the present study, we have developed an ER Ca^{2+} sensor based on fluorescence resonance energy transfer (FRET), capable of monitoring dynamic Ca^{2+} signals within the ER. This genetically-encoded ER sensor contains a mutated version of calmodulin (mCaM) and its binding peptide derived from smooth muscle myosin light chain kinase (m-smMLCK) as the Ca^{2+}-responsive elements, thereby enabling an effective ER Ca^{2+} sensing without perturbation by endogenous CaM. The Ca^{2+} sensitivity, including detection limit, of the sensor was evaluated through *in vitro* characterization. In cell-based assay, our biosensor displayed high accuracy to ER Ca^{2+} sensing in response to pharmacological stimuli, indicating that this sensor is reliable for visualization of ER Ca^{2+} signals at high spatiotemporal resolutions in live cells.

1. Introduction

Calcium ion (Ca^{2+}) is a ubiquitous intracellular messenger involved in many cellular signaling processes within eukaryotic cells. For three decades, researchers have attempted to quantitatively measure Ca^{2+} dynamics in *in vitro* and *in vivo* through various experimental tools [1,2]. Among many powerful applications, genetically encoded Ca^{2+} indicators have been found to be indispensable tools in studying the dynamics of Ca^{2+} signaling owing to their advantages over small-molecule fluorescent dyes [3–6]. Although, most fluorescent Ca^{2+} dyes such as Fura-2, Indo-1 and Fluo-4, which exhibit greater sensitivity, rapid response kinetics, and greater convenience for use, are still available, they present limitations in precise control of dyes in individual subcellular locations, and leakage during long-term imaging [7–10].

The endoplasmic reticulum (ER) serves as an internal Ca^{2+} store that continuously releases Ca^{2+} for a variety of cellular processes, including gene regulation, transcription, apoptosis, contraction, and metabolism [11–17]. However, there is a need to understand the biochemical and/or physiological networks between ER Ca^{2+} and Ca^{2+}-dependent signaling reactions through appropriate experimental approach. Thus, direct measurement of ER Ca^{2+} signals, using effective Ca^{2+} indicators, may be crucial for understanding unknown biological questions related to ER Ca^{2+} signals and Ca^{2+} homeostasis.

In previous studies, genetically encoded Ca^{2+} indicators targeting the ER have been developed by a great pioneer of the field, Dr. Tsien, and his co-workers, who introduced calmodulin (CaM), a calcium-binding protein and its binding partner M13 peptide derived from skeletal muscle myosin light chain kinase (skMLCK) as the Ca^{2+}-responsive elements into the sensor's main body [18,19]. Binding of Ca^{2+} can promote interaction between CaM and M13 peptide, which leads to conformational change in the sensor. However, it is possible that native CaM interferes with Ca^{2+} sensitivity, affecting the response of the sensor, which may result in its limited availability. To overcome this issue, complementary mutations in CaM (mCaM) and its binding partner (mM13p) were introduced to create an improved ER Ca^{2+} indicator that would not be disrupted by endogenous CaM. Yet, such an indicator exhibits high affinity that might not be suitable for detecting ER Ca^{2+} [20], and thus needs to be further enhanced for Ca^{2+} sensing within the ER. Thus, the goal of this study was to develop an improved ER Ca^{2+} sensor with the optimal Ca^{2+} sensitivity for ER, and to test whether

* Corresponding authors at: Natural Products Research Center, Korea Institute of Science and Technology (KIST), Gangneung 25451, Republic of Korea., Department of Biological Sciences, Pusan National University, Busan 46241, Republic of Korea.
E-mail addresses: tjkim77@pusan.ac.kr, tjkim@kist.re.kr (T.-J. Kim), shjung@kist.re.kr (S.H. Jung).

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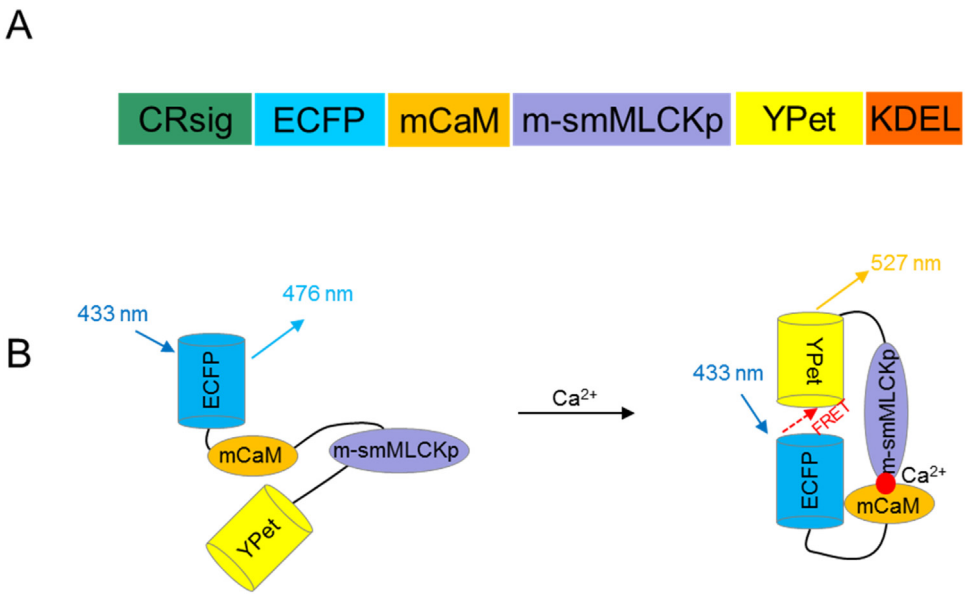

Fig. 1. Schematic representation of the improved Ca^{2+} sensor targeted to the endoplasmic reticulum (ER). (A) The sensor consists of mutated calmodulin (mCaM) and its binding peptide derived from smooth muscle myosin light chain kinase (m-smMLCKp) in conjunction with ECFP and YPet pair. To target the ER, this sensor contains calreticulin signal sequence (CRsig) and ER retention sequence (KDEL). (B) The ER Ca^{2+} sensor shows the conformational change, which leads to an increase in FRET ratio when bound to Ca^{2+}.

such an engineered sensor can offer robust Ca^{2+} responses in a given environment.

2. Materials and methods

2.1. Construction of DNA plasmids

The mCaM and its binding peptide (m-smMLCKp) were digested from D4cpv, using *Sph*I and *Sac*I restriction enzymes [20], and cloned between an enhanced cyan fluorescent protein (ECFP) and yellow fluorescent protein variant (YPet) in the pRSETB vector. For ER-targeted sensor, the calreticulin signal sequence (MLLPVLLLGLLGAAAD) was fused to the N-terminus of ECFP, and an ER retention sequence (KDEL) was added to the C-terminus of YPet [19]. *In vitro* characterization, pRSETB vector containing the sensor was transformed into BL21 strain, and purified using an N-termnial 6xHis tag by nickel chelation chromatography as previously described [21]. Absorbance measurments were performed on UV–vis spectrometer. For expression in mammalian cells, the construct was cloned into pcDNA3.1 (Invitrogen) between the *Hind*III and *Eco*RI sites.

2.2. Cell culture and chemicals

Human mesenchymal stem cells (hMSCs) obtained from Lonza (Lonza Walkersvile, Inc., Walkersvile, MD) were cultured in mesenchymal stem cell growth medium (MSCGM; PT-3001; Lonza) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 95% O_2 and 5% CO_2 at 37 °C. The DNA plasmids (1 μg/μl) were transfected into the cells, using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) reagent according to the product instructions. Dimethyl sulfoxide (DMSO) and ER-tracker Red (Cat no. E34250) were purchased from Thermo Scientific (USA). ATP, thapsigargin (TG), and ionomycin were obtained from Sigma-Aldrich (Sigma, St.Louis, MO), and applied to cells at 10 μM.

2.3. Microscope and live cell imaging

hMSCs were imaged under an Eclipse Ti-E inverted microscope (Nikon, Japan), with a cooled charge-coupled device (CCD) camera (Photometrics, Tucson, AZ), using MetaFluor 6.2 software (Universal Imaging), with a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters controlled by a filter changer (475DF40 for ECFP and 535DF25 for YPet). The emission ratio images were calculated based on the background-subtracted fluorescence intensity, which was shown in the intensity modified display (IMD) mode to represent the FRET efficiency.

2.4. Confocal imaging

Human retinal pigmented epithelial cells (ARPE-19) obtained from American Type Culture Collection (ATCC) were cultured in DEME/F12 containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 95% O_2 and 5% CO_2 at 37 °C. The sensor-transfected ARPE-19 were stained with ER-tracker Red (1 μM) for 1 h, and then the culture medium was replaced with CO_2-independent medium for live cell imaging. Confocal Imaging was conducted on a Nikon A1R confocal system using a 60×/1.4 NA objective, with a 561 nm diode laser and a 600/50 nm emission filter. Images were processed using NIS Elements software.

2.5. Data analysis

Statistical analysis was performed by Student's *t*-test, using Excel (Microsoft), and Graphpad Prism 6.0 software to evaluate the statistical differences between groups. A significant difference was determined by the P-value.

3. Results

3.1. Development of an improved ER Ca^{2+} sensor based on FRET

The typical Ca^{2+} indicators known as Cameleon were mostly comprised of CaM and the CaM-binding peptide (skMLCK or