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A FRET-Based Biosensor for Imaging SYK Activities in Living Cells

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Abstract

Spleen tyrosine kinase (SYK) is crucial to cellular functions mediated by immunoreceptors and integrins. We have developed and characterized a new genetically-encoded Förster resonance energy transfer (FRET)-based biosensor for studying the dynamics of SYK activities in living cells at a subcellular level. It contains an N-terminal ECFP, SH2 domain, a peptide derived from a SYK substrate VAV2, and a C-terminal YPet. Upon the specific phosphorylation by SYK *in vitro*, the biosensor substrate peptide bound to the intramolecular SH2 domain to reduce the FRET efficiency. Transfection of the biosensor did not affect activation of the endogenous SYK in host cells. Phosphorylation of the biosensor followed the same kinetics as the endogenous VAV2. Using FRET imaging and ratiometric analysis with this SYK biosensor, we visualized and quantified the realtime activation of SYK in K562 cells upon IgG Fc engagement of Fcγ receptor IIA and in mouse embryonic fibroblasts upon stimulation by the platelet derived growth factor. These results demonstrate our biosensor as a powerful tool for studying cellular signaling that involves SYK.

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Keywords

SYK; Immunoreceptor; FRET; Biosensor; Signaling

Introduction

Spleen tyrosine kinase (SYK) is a member of nonreceptor tyrosine kinase family and is expressed in all hematopoietic cells.¹³ It plays a crucial role in mediating signaling induced by immunoreceptors, which include B-cell receptors, activating receptors of natural killer cells and Fc receptors (FcRs).³⁰ Stimulation of immunoreceptors initiates signaling cascades that begin with phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) either within the immunoreceptors themselves or in receptor-associated molecules.¹³ Phosphorylated ITAMs serve as docking sites to recruit SYK, thereby inducing SYK activation. Several molecules, including growth factor receptorbound protein 2 (Grb2), members of the VAV oncogene family (VAVs) and protein tyrosine kinase 2 (PTK2), are implicated in relaying SYK activation to downstream signal transduction to intermediate Ca^{2+} mobilization, protein kinase C (PKC) phosphorylation^{4,13,21,22} and ultimate cytokine production and/or cell proliferation.^{1,33} Recent studies have revealed that SYK is also involved in other cellular functions regulated by receptors independent of the conventional ITAMs, such as integrin mediated cell adhesion,³⁴ platelet activation by collagen¹⁹ and vascular development.² These studies suggest a role of SYK in regulating cell functions beyond the immune responses.

Previous studies have shown that SYK is critical for the $\text{Fc}\gamma\text{R}$ -mediated signal transduction in macrophages and neutrophils.¹¹ Traditionally, kinase activities in signaling cascades have been analyzed biochemically by Western, immunostaining or flow cytometry. These methods have several limitations. Anti-phosphotyrosine antibodies and fluorescentlylabeled kinase substrate peptides usually require fixing and permeabilizing cells and are not quantitative.³⁵ Methods requiring lysing cells have no spatial resolution and poor temporal resolution. To circumvent these limitations, genetically encoded biosensors based on Förster resonance energy transfer (FRET) technology have been developed for monitoring the dynamic signaling events in living cells.^{9,26,31} Here we describe the development and characterization of a SYK biosensor containing an N-terminal enhanced cyan fluorescence protein (ECFP) as the donor, Src homology 2 (SH2) domain, a peptide derived from a SYK substrate VAV2, and a C-terminal YPet (a variant of yellow fluorescence protein YFP) as the acceptor (Fig. 1a). The model is that upon phosphorylation by activated SYK, the substrate peptide within the biosensor would bind to the intramolecular SH2 domain, inducing a conformational change in the biosensor, thereby causing a FRET change (Fig. 1b). With this design, the biosensor should have a SYK-dependent FRET response and, therefore, can be used to monitor the spatial and temporal SYK activity in living cells. Characterization and application of this biosensor revealed that SYK was activated rapidly upon $\text{Fc}\gamma\text{RIIA}$ (CD32A) engagement in K562 cells and upon platelet-derived growth factor (PDGF) stimulation in mouse embryonic fibroblasts (MEFs).

Results

Biosensor Design and In Vitro Validation

A FRET-based Src biosensor was previously developed to visualize the mechanical activation of Src.³¹ The design of our SYK biosensor (Fig. 1a) is similar, except that the YFP was replaced by YPet since our previous study showed a markedly enhanced sensitivity of the ECFP/YPet FRET pair.¹⁶ Furthermore, a natural peptide derived from a SYK substrate, VAV2, was selected as the substrate peptide in the SYK biosensor for reporting SYK activation. The close proximity of the N- and C-termini of the SH2 domain and the flexible linker allows ECFP and YPet to yield high FRET at the rest state. The SH2 domain can bind to the VAV2 peptide upon phosphorylation of its single tyrosine by SYK. Such intramolecular binding is predicted to induce a conformational change that would reduce the proximity of the FRET pair, thereby decreasing the FRET and increasing the ECFP/YPet emission ratio (Fig. 1b).

We tested the validity of our biosensor design *in vitro* using purified biosensor and active SYK proteins. Indeed, phosphorylation of the wild-type(WT) biosensor by SYK enhanced the ECFP/YPet ratio by almost 100% (Fig. 1c), indicating a SYK-induced loss of FRET. Two biosensor mutants were tested as controls to confirm the phosphorylation-dependent FRET response. Substitution of the kinase phosphorylation site Tyr by Phe at residue 172 (Y172F) in the substrate peptide abolished the FRET response. Mutation of Arg to Val at residue 175 (R175V) in the SH2 domain, which impaired substrate binding, also blocked the FRET response (Fig. 1c). These observations are consistent with the previous study³¹ and have validated the phosphorylation-induced FRET response of the SYK biosensor.

Phosphorylation and FRET Response of the Biosensor Depends on SYK Activation in Cells

Cross-linking of CD32A on immune cells can initiate a signaling cascade that involves SYK activation.^{7,24} To verify that our biosensor could be phosphorylated by cellular SYK upon activation, K562 cells transfected with biosensors were stimulated, lysed and subsequently subjected to immunoprecipitation by anti-GFP (green fluorescence protein) and immunoblotting by anti-phospho-tyrosine. Immunoprecipitated WT biosensors were phosphorylated upon cross-linking of CD32A (Fig. 2a, upper panel, and Fig. 2b). Pretreating cells with Piceatannol, a specific inhibitor of SYK,¹⁸ severely suppressed the phosphorylation of the biosensors. In addition, mutations of Y172F or R175V completely abolished phosphorylation of the biosensors. These results indicate that the biosensor phosphorylation depends specifically on SYK activation.

To further examine whether the SYK activation-dependent phosphorylation of biosensor caused FRET response in living cells, emission intensities of ECFP were measured in a plate reader (Fig. 2c). The cross-linking-induced increase in ECFP emission was detected in K562 cells 2 min after stimulation, which was inhibited by pretreatment with Piceatannol (Fig. 2c). These results demonstrated that our biosensor was phosphorylated specifically by activated SYK in mammalian cells and responded with a change in FRET signals.

Biosensor Transfection Does Not Adversely Affect SYK Activation and VAV2 Phosphorylation

To examine whether transfection of the biosensor would perturb the endogenous signaling pathways in cells, K562 cells with and without biosensor transfection were stimulated by cross-linking CD32A, stained with anti-phospho-SYK, and analyzed by flow cytometry. Similar increases in activated SYK were observed in both transfected and untransfected cells upon CD32A cross-linking (Fig. 3a), indicating that introduction of the biosensor did not affect the endogenous SYK activation. We also analyzed the phosphorylation of the transfected biosensor and endogenous VAV2 upon Pervanadate (PV) treatment. The comparable phosphorylation dynamics (Fig. 3b) indicated that activated SYK phosphorylates the biosensor with similar kinetics as it does its endogenous substrate VAV2.

Monitoring Immunoreceptor-Triggered SYK Activation Dynamics in Living Cells

We next tested the ability of our biosensor to monitor SYK activity induced by IgG engagement of CD32A on K562 cells. Interactions of Fc γ Rs with Fc portion of IgG initiate various SYK-dependent allergic and inflammatory responses, such as phagocytosis, production of cytokines and changes in cell adhesion.^{1,5,6,10,13,27,30} Biosensor-expressing K562 cells were allowed to settle on an IgG-coated glass-supported lipid bilayer and the emission spectra of ECFP and YPet were measured by confocal microscopy. Binding of cell surface CD32A to IgG Fc resulted in enhanced ECFP emission at the expense of YPet emission (Fig. 4a), which yielded an 8.3% increase in the ECFP/YPet emission ratio (Fig. 4b), indicating a loss of FRET induced by SYK activation. Pretreating cells with Piceatannol markedly inhibited the FRET response (Figs. 4a, 4b). Upon IgG stimulation, a significant ($p < 0.01$) FRET response occurred at 10 min, reached the peak at 30 min, and decreased thereafter (Fig. 4c). This dynamics suggests that SYK activation occurs within 10 min of the interaction between CD32A and IgG Fc coated lipid bilayer. The time period for SYK to reach its maximum activity is consistent with the time scale for Fc γ R-expressing cells to form stable contact areas with IgG-coated lipid bilayer.^{28,32} These data demonstrate that our biosensor can be used to observe the dynamics of SYK activity triggered by engagement of immunoreceptors in living cells.

Visualizing Growth Factor Induced SYK Activation Dynamics in Living Cells

As a final test, we confirmed the ability of our biosensor to visualize SYK activities induced by PDGF stimulation in MEFs. PDGF plays important roles in regulating various cellular functions, including development, proliferation and migration.⁸ Biosensorexpressing MEFs were stimulated with PDGF and observed using fluorescence microscopy. Ratiometric images of a representative MEF at different time points revealed a dynamic change of SYK activation throughout the entire cell upon PDGF stimulation, with a higher activation occurring outside the nucleus (Fig. 5a). A typical emission ratio time course of the biosensor in response to PDGF stimulation in MEFs shows that SYK activation reached the peak at 10 min before a gradual decline thereafter (Fig. 5b). Thus, our FRET biosensor can also report the spatiotemporal pattern of SYK activation.

Discussion

Signals triggered by immunoreceptor engagement with ligands are transduced from the cell membrane to inside the cell to induce gene expression, rearrangement of cytoskeleton and cell surface structures, and ultimate alteration in cell functions, such as differentiation and proliferation.^{3,17,27} The activation of SYK is crucial for signaling processes in cells expressing SYK, as it acts as a signal mediator and amplifier.^{13,27} In addition, SYK is required for integrin signaling in neutrophils, platelets and macrophages. Neutrophils lacking SYK fail to spread on integrin ligands and undergo integrin-dependent functional responses.¹⁴

Given the importance of SYK in cellular signaling, many studies have attempted to determine the precise roles of SYK in signaling events related to immune and inflammatory responses.^{11,29} However, few experiments observed the spatial and temporal distribution of SYK activation or phosphorylation in living cells. This is because the traditional biochemical methods, although are very useful and have provided valuable information, are not sufficiently quantitative, lack spatial and temporal resolution, and often cannot be used in living cells. Studies using SYK-GFP fusion protein have indicated the activation-induced redistribution of the kinase to the plasma membrane.¹² This fluorescent imaging method has provided new insights into the distribution of kinases, but it merely reports the location of the kinase, not its activity or how the phosphorylation is dynamically regulated inside living cells.

A new and more powerful method for visualizing intracellular kinase activities in space and time is genetically-encoded FRET-based biosensors.^{15,16,31} Following the success of the Src biosensor³¹ and focal adhesion kinase biosensor,²³ in this study we developed a SYK biosensor, characterized its properties, and demonstrated its utilities in different signaling pathways. We have shown the use of our FRET-based biosensor for direct visualization of the spatiotemporal activation of SYK in signaling cascades triggered by engagement of an immunoreceptor (CD32A) in K562 cells and a growth factor (PDGF) receptor in MEFs.

Our biosensor uses the VAV2-derived peptide. VAV2 is also a substrate of another member of the SYK family of kinases, the zeta-chain-associated protein kinase 70 (ZAP-70).^{3,13,25} ZAP-70 is expressed on T cells and NK cells.¹³ A previous study reported a FRET-based ZAP-70 biosensor with Grb2 domain and a peptide derived from linker of activated T cells (LAT),²⁰ different substrates from VAV2. In a parallel study, we have shown the use of our biosensor to study the spatiotemporal activation of ZAP-70 upon engagement of the T cell receptor (Xiang *et al.*, unpublished data). Thus, our biosensor has broad utilities in studying signaling pathways involving SYK family of kinases in living cells.

Materials and Methods

Gene Construction and DNA Plasmids

The gene for the SYK biosensor was constructed by polymerase chain reaction (PCR) amplification of the complementary DNA from the c-Src SH2 domain with a sense primer containing a SphI site and a reverse primer containing the gene sequence for a flexible

linker, a SYK substrate peptide from VAV2, and a *SacI* site. The PCR products were fused together with an N-terminal ECFP and a C-terminal YPet.¹⁶ Mutations of R175V and Y172F were conducted with the Quik Change (Stratagene). Constructs were cloned into pRSETB (Invitrogen) using *Bam*HI/*Eco*RI for bacterial expression and into pcDNA3 (Invitrogen) behind a Kozak sequence using *Hind*III/*Eco*RI for mammalian cell expression.

Cell Culture and Transfection

K562 cells were from American Type Culture Collection (ATCC). K562 cells were transfected with the SYK biosensor cDNA by using the lipofectamine method according to protocols provided by the manufacturer (Invitrogen). 24 h after transfection, cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL Penicillin, 100 µg/mL Streptomycin and 2 mM L-glutamine in the presence of 400 µg/mL G418 as selection reagent. Transfected cells were fed and split every 3 days until outgrowth of resistant cells. K562 cells stably expressing SYK biosensor were generated by sorting the YPet positive cells several times with a BD cell sorter.

Mouse embryonic fibroblasts (MEFs) were purchased from ATCC. MEFs were cultured in DMEM supplemented with 10% FBS, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 2 mM L-glutamine. MEFs were transfected with the SYK biosensor cDNA by using the lipofectamine method. 5 h after transfection, MEFs were starved in DMEM supplanted with 0.5% FBS for 36 h before being subjected to imaging.

Reagents

Rabbit anti-GFP polyclonal antibody (ab290) and goat anti-rabbit IgG HRP conjugated polyclonal antibody (ab6721) were from Abcam. Rat anti-DNP monoclonal antibody (mAb) (LO-DNP-2) and NP40 cell lysis buffer were from Invitrogen. Goat anti-human CD32A polyclonal antibody was from R&D System. Fc-specific rabbit anti-goat IgG, goat anti-mouse peroxidase-conjugated IgG, mouse anti-goat IgG and halt phosphatase inhibitor cocktail were from Thermo Scientific. Rabbit anti-phospho-SYK mAb (C87C1) was from Cell Signaling. Goat anti-rabbit Cy5-conjugated IgG was from Chemicon International. Mouse anti-phospho-tyrosine mAb (4G10) was from Millipore. Mouse anti-GFP mAb (JL-8) was from Clontech. Rabbit polyclonal anti-phospho-VAV2 was from Santa Cruz. PITC and DNP-CAP-PE were from Avanti Polar lipids. All general chemicals and other reagents were from Sigma unless otherwise indicated.

Protein Expression and In Vitro Kinase Assay

Biosensors were expressed with N-terminal 6× Histag in *Escherichia coli* and purified by nickel chelation chromatography. Fluorescence emission spectra of the purified biosensors with a final concentration of 1 µM were measured in a 96-well plate with an excitation wavelength of 430 nm by a fluorescence plate reader (TECAN, Sapphire II). For FRET detection of SYK biosensors, emission ratios of donor/acceptor (478 nm/526 nm) were measured at 30 °C before and after the addition of 1 µg/mL active SYK kinase (Calbiochem) in the presence of 1 mM ATP in a kinase buffer (50 mM Tris pH 8, 100 mM NaCl, 10 mM MgCl₂, 2 mM dithiothreitol).

In Vivo Phosphorylation Assays

Experiments of *in vivo* phosphorylation were carried out in 96-well plates. 1×10^6 cells in a total volume of 300 μ L HBSS were added per well. K562 cells stably expressing biosensor were incubated in HBSS (pH 7.4) containing 150 mM NaCl, 20 mM HEPES, 1% human serum albumin (HSA) (MP Biomedicals), 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂ and 3.8 g/L glucose. Cells in some of the wells were pretreated by 80 μ M Piceatannol dissolved in DMSO for 30 min, incubated in plates on ice, and stimulated. To simulate K562 cells by CD32A cross-linking, cells were incubated with 2 μ g/mL goat anti-human CD32A polyclonal antibody for 5 min and then 40 μ g/mL rabbit anti-goat IgG. After loading antibodies into the plates, the ECFP fluorescence intensity was determined by SpectraMax Gemini Plate Reader (Molecular Devices) at 37 °C with excitation at 444 nm and emission at 460 nm. Results were expressed as mean $\pm$ SEM of relative fluorescence units measured in triplicate.

Flow Cytometry Assay

For analysis of SYK phosphorylation upon stimulation, K562 cells stably expressing biosensor were washed and re-suspended in HBSS. Stimulation was performed as described above. After stimulation, cells were fixed with cold 4% paraformaldehyde in PBS for 10 min at room temperature, washed and re-suspended in permeabilization buffer (HBSS, 0.1% saponin, 0.05% NaN₃), then stained for 1 h with 10 μ g/mL rabbit anti-Phospho-SYK antibody. After washing, secondary goat anti-rabbit Cy5-conjugated IgG was added for 30 min. The samples were processed using a FACS calibur (Becton-Dickinson BD) and the data were analyzed using FlowJo software (Stanford University-Tree Star).

Immunoprecipitation and Immunoblotting

3×10^7 K562 cells expressing various SYK biosensors were harvested from growth medium, washed and re-suspended in 200 μ L HBSS, then stimulated or kept as a control before being lysed. Some of the cells were pretreated with Piceatannol and simulated by cross-linking of CD32A as described above or by Pervanadate (PV) following an established protocol.⁹ Cells were resuspended in 1 mM PV for the indicated time at room temperature. After stimulation, cells were washed twice, and lysed with 300 μ L ice-cold NP 40 lysis buffer (supplemented with 1 mM PMSF, 1 $\times$ protease inhibitor cocktail and 1 $\times$ phosphatase inhibitor cocktail) for 30 min. Lysates were clarified by centrifuging at 14,000 g for 10 min at 4 °C. Post nuclear supernatants were mixed with 2 $\times$ Laemmli sample loading buffer (Bio-Rad) and boiled or subjected to immunoprecipitation with an anti-GFP antibody coated on Dynabeads Protein G (Invitrogen). 40 μ L beads coated with 2 μ L anti-GFP antibody were used to pull down the biosensors in 3×10^7 cells. The mixtures were incubated for 2 h at 4 °C. Immunoprecipitants were washed and resuspended in 2 $\times$ sample buffer containing β ME and boiled at 95 °C for 5 min. Whole cell lysates and eluted immunoprecipitants were separated by 10% SDS-polyacrylamide gel and analyzed by immunoblotting. Membrane were probed with an anti-phosphotyrosine antibody or anti-phospho-VAV2 antibody to display the tyrosine phosphorylation level, or an anti-GFP antibody (JL-8) to show the expressed protein level, followed by peroxidase conjugated secondary antibody. Images

were revealed by ECL. The molecular weights of the SYK biosensor and phosphor-VAV2 are about 70 and 100 kDa, respectively.

Microscopy, Image Acquisition and Analysis

1×10^6 K562 cells transiently transfected with biosensors were harvested, washed twice and resuspended in 300 μ L HBSS, or pretreated with 80 μ M Piceatannol in HBSS for 30 min at room temperature. DNP lipid bilayers were prepared as previously described on the surface of coverglass,²⁸ which formed at the bottom of a Focht Chamber System 2 (FCS2; Biophtechs). The chamber was kept at 37 °C and placed on the stage of a LSM 510 META Carl Zeiss laser scanning microscope (Jena, Germany). Cells were injected into the chamber and images were acquired at indicated times with a tunable Chameleon laser (Coherent Chameleon, Coherent, Santa Clara, CA) from 710 to 950 nm attached to the in-port of the scan head. Two-photon images with a wide range of spectra were collected in Lambda mode by using the LSM 510 software with an 840 nm Chameleon laser for ECFP excitation. A HFT KP 650 Beam splitter was placed in the detection light path to prevent laser bleed-through into the detector. An emission filter with spanning range from 440 to 580 nm was selected for biosensor emission. Spectrum information was acquired by choosing individual cell as ROI (Region of Interest) in one field of view. Emission intensity of individual wavelength was normalized to the average of different wavelength intensity for a single cell. Data were collected from 3 experiments, each containing no less than 20 cells.

For the PDGF-induced FRET response, glass-bottom dishes (Cell E&G) were coated with 40 μ g/mL fibronectin (Sigma) overnight at 37 °C. Transfected MEFs were plated on fibronectin-coated glass-bottom dishes the night before imaging in DMEM containing 0.5% FBS. During imaging, cells were starved and maintained in CO₂-independent medium (Invitrogen) with 0.5% FBS at 37 °C. 50 ng/mL PDGF (Sigma) was used to stimulate starved MEFs. Images were obtained by a Zeiss Axiovert inverted microscope equipped with a cooled CCD camera (Cascade 512B, Photometrics) using MetaFluor 6.2 software (Universal Imaging). The following filter sets (Chroma) were used in our experiments: dichroic mirror (450 nm), excitation filter 420/20 nm, ECFP emission filter 475/40 nm, FRET emission filter 535/25 nm. The emission ratio images were computed and generated by the MetaFluor software to represent the FRET efficiency before they were subjected to quantification and analysis by Excel (Microsoft).

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Figure 1.

Biosensor design and in vitro characterization. (a) Domain diagram. The SYK kinase biosensor consists of ECFP, the SH2 domain, a flexible linker, the SYK kinase substrate peptide, and YPet. (b) Schematics showing the principle of reporting SYK activity by the FRET-based biosensor. Phosphorylation of the substrate peptide induces its binding to the SH2 domain, resulting in a conformation unfavorable for FRET. (c) Emission ratio time courses of the purified wild-type (WT) or mutant biosensors in response to addition of active SYK at time 0. The two negative mutants are Y172F (YF) that replaced the tyrosine in the substrate peptide and R175V (RV) that replaced the substrate binding residue in the SH2 domain.

Figure 2.

Biosensor responses specifically to SYK activation in live cells. (a) K562 cells transfected without (N) or with WT or mutant (RV and YF) biosensors were pretreated with or without 80 IM Piceatannol and stimulated with or without cross-linking CD32A. Cells were lysed, immunoprecipitated with anti-GFP, analyzed by western blot with anti-phosphotyrosine (upper panel), and stripped and re-blotted with anti- was determined as described in inGFP (lower panel). (b) Densitometry measurement of the western blot intensities in (a). Signal intensity in each band was determined as described in “Materials and Methods” and expressed as percent of maximal phosphorylation. (c) K562 cells expressing biosensor in 96-well plates were pretreated with or without Piceatannol and stimulated by cross-linking CD32A. The emission intensity of ECFP was measured with a fluorescence reader as a function of time upon stimulation. Data were presented in mean $\pm$ SEM of triplicate wells.

Figure 3.

Expression of biosensor does not interrupt normal signaling in live cells. (a) Flow cytometry analysis of SYK phosphorylation in biosensor-expressing cells upon stimulation. K562 cells without (N) or with biosensor transfection were stimulated by cross-linking CD32A, followed by Cy5 staining for phospho-SYK (abscissa). FITC fluorescent channel was chosen to report the biosensor expression (ordinate). (b) In biosensor-expressing K562 cells, Pervanadate (PV) induced phosphorylation of the biosensor and endogenous VAV2 in a comparable time scale. K562 cells stably expressing SYK biosensor were stimulated by PV at time 0. The biosensor was immunoprecipitated from the cell lysate by an anti-GFP antibody and detected by western blot using antiphosphotyrosine antibody at the indicated time points (upper panel). Endogenous phosphor-VAV2 was analyzed by western blot of the whole cell lysate without immunoprecipitation (lower panel).

Figure 4.

Monitoring the IgG-induced SYK activation by FRET-based biosensor. (a) Spectra of normalized emission intensity in response to binding to IgG-coated lipid bilayer. K562 cells transfected with SYK biosensor were pretreated with or without Piceatannol, placed on a glass-supported DNP lipid bilayer coated with or without anti-DNP IgG for 30 min, and fixed. Emission spectrum was measured by two-photon confocal microscopy. Error bars represent standard error of the mean from 3 experiments with each containing 20 or more cells. (b) Emission ratio of ECFP/YPet in response to IgG stimulation. Emission ratio of 463 nm/506 nm in a single cell was calculated. Error bars represent SEM from 3 experiments with each containing 20 or more cells. “**” indicates a significant difference between indicated groups ($p < 0.01$, ANOVA). (c) Emission ratio time courses showing the IgG-induced temporal activation of SYK. Error bars represent SEM from 3 experiments with each containing 8 or more cells.

Figure 5. PDGF can induce the SYK activation in mouse embryonic fibroblasts (MEFs). (a) Representative images of FRET change showing PDGF-induced SYK activation in MEFs. (b) A typical emission ratio time course of the biosensor in response to PDGF stimulation in MEFs.