Mechanotransduction in Endothelial Cells Studied with Fluorescence Imaging

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ABSTRACT
Mechanotransduction involves the conversion of mechanical stimuli to intracellular signaling to modulate gene and protein expressions and hence cellular functions in endothelial cells, thus playing important roles in the regulation of homeostasis in health and disease. The aim of this paper is to investigate the dynamics of mechanotransduction in endothelial cells by the use of fluorescent resonance energy transfer (FRET) to study the temporal and spatial activation of Src kinase and focal adhesion kinase, both of which play critical roles in many cellular processes. The results have contributed to the elucidation of the roles of these two important signaling molecules and their interactions in mediating mechanotransduction.

INTRODUCTION
Mechanical stresses resulting from blood flow and pressure can modulate cellular functions by activating sequentially mechano-sensors, signaling pathways, and gene and protein expressions (1). Most studies on these signaling processes have been conducted by using biochemical or molecular procedures to study a large number of cells subjected to experimental conditions. With the increasing recognition of spatial and temporal variations in mechanotransduction, it is necessary to have technologies that would enable investigations on single cells and subcellular components in 3D space and with time-dependent dynamics. To this end, fluorescent resonance energy transfer (FRET) has been used to study the temporal and spatial activation of Src kinase and focal adhesion kinase (FAK), which are known to play critical roles in many cellular processes, including motility/migration, mechanotransduction, and disease states such as cancer. We developed genetically encoded single-molecule Src biosensor (2) and FAK biosensor (3), which enable the imaging and quantification of temporal-spatial activation of Src and FAK activities in live cells in response to mechanical and chemical stimuli. These biosensors have enabled monitoring key signaling transduction cascades and mechanobiology in live cells with temporal-spatial characterization. We have also developed FRET technology to monitor FAK and Src activities at lipid rafts and non-raft regions of the plasma membrane in live cells (4), thus elucidating the dynamics and interplay of these molecules in mediating mechanotransduction.

THE SRC AND FAK FRET BIOSENSORS
Design of the Src and FAK FRET Biosensors
We designed FRET-based biosensors for Src (2) and FAK (3). The Src biosensor is
composed of a Src SH2 domain, a flexible linker peptide, and a Src substrate peptide encompassing Tyr 662 and 664, which were originally concatenated between CFP and YFP, but in its current version between s ECFP and YPet to improve the sensitivity and range of the biosensor (4). The FAK biosensor specifically detects the autophosphorylation of Tyr397 and its subsequent binding to the Src SH2 domain, which are the crucial molecular events in the FAK activation and represent the FAK activity. The FAK biosensor contains the SH2 domain derived from c-Src, a flexible linker peptide, and a specific substrate sequence from FAK encompassing Tyr397, which are concatenated between ECFP and YPet. The active FAK can phosphorylate the Tyr397 in the substrate peptide of the biosensor (trans-activation), and the phosphorylated substrate can then bind to the intramolecular SH2 domain to cause an increase in the ECFP/YPet emission ratio, which represents an enhanced FAK activity.

Incubation of the purified FAK biosensor with active FAK led to an increase of the ECFP/YPet emission ratio, reflecting the phosphorylation of the biosensor by FAK. Src kinase, which caused significant FRET changes of the Src biosensor, had no effect on FRET change of the FAK biosensor, indicating the specificity of the FAK biosensor.

Characterization of the FAK Biosensor in Mammalian Cells

We examined this ECFP/YPet FAK biosensor in mouse embryonic fibroblasts (MEF). The ECFP/YPet emission ratios of the FAK biosensor were significantly higher in MEF cells adhered on fibronectin than those in suspension (Fig. 2A), consistent with the knowledge that the integrin-mediated cell adhesion process can induce FAK activation. In FAK knockout (FAK-/-) MEFs, transfection with the constitutively active FAK (FAK Δ1-375) and wild-type FAK (FAK WT) caused significantly higher ECFP/YPet emission ratios than the negative mutant of FAK (FAK NT), the kinase-dead FAK (FAK KD), or an empty vector. The expression of wild-type Src (Src WT) did not cause a significant FRET change of the FAK biosensor in FAK-/- cells, indicating that the FAK biosensor can detect FAK activity with a high specificity in mammalian cells.

The FRET response in FAK-/- cells expressing the active FAK (FAK Δ1-375) was eliminated after the mutation of Tyr397 in the substrate peptide to Phe or the mutation of Arg175 in the SH2 domain to Val, indicating that the FRET response of the FAK biosensor upon FAK activation is attributable to the intramolecular interaction between the phosphorylated substrate (Tyr397) and the SH2 domain (Arg175).

FAK ACTIVITIES AT DIFFERENT MICRODOMAINS OF THE PLASMA MEMBRANE

FAK plays important roles in regulating the subcellular localization of lipid rafts and the associated signaling events. A Lyn-FAK biosensor was constructed by fusing a raft-targeting motif derived from the Lyn kinase at the N-terminus of the cytosolic FAK (Cyto-FAK) biosensor to monitor the FAK activity at membrane rafts. As a control, a non-raft targeting sequence from KRas was fused at the C-terminus of the
Cyto-FAK biosensor for the generation of KRas-FAK biosensor.

FAK ACTIVATION BY PDGF

The PDGF-induced FAK Activation at Lipid rafts

PDGF caused significant FRET change of the Lyn-FAK biosensor, but not the KRas-FAK biosensor, indicating that the phosphorylation at Tyr 397 is responsible for the PDGF-induced FRET change of the Lyn-FAK biosensor, which targets the rafts. The FAK inhibitor PF228 reversed the PDGF-induced FRET response of Lyn-FAK biosensor. The PDGF-induced FRET signal of the Lyn-FAK biosensor was suppressed after cholesterol extraction and raft disruption by methyl-β-cyclodextrin (MβCD), verifying the localization and functionality of the biosensor at rafts.

The PDGF-induced FAK Activation Is Dependent on Src

The Src kinase inhibitor PP1 completely blocked the PDGF-induced FRET response of the Lyn-FAK biosensor, indicating that the FAK activation at rafts upon PDGF stimulation is dependent on Src.

To test whether FAK also regulates Src activation in response to PDGF, we examined the effect of the FAK inhibitor PF228 on FRET response of the ECFP/YPet Lyn-Src biosensor targeted at rafts. PF228 partially inhibited, but did not prevent, the PDGF-induced FRET response of the Lyn-Src biosensor, suggesting that FAK activity is not essential for the PDGF-induced Src activation.

FAK ACTIVATION BY CELL ADHESION

FAK Activity Is Essential for the Src Activation during Cell Adhesion

In contrast to the PDGF-induced FAK activation, the adhesion-induced FAK activation was not inhibited by blockage of Src activity with PP1. The inhibition of FAK kinase activity by PF228 completely blocked the adhesion-induced Src activation. Therefore, in contrast to the PDGF-induced signaling, the adhesion-mediated FAK activation acts upstream to Src.

SUMMARY AND CONCLUSIONS

The FAK biosensors that can target the lipid rafts and non-raft regions of the plasma membrane have allowed the elucidation of the subcellular regulation of FAK activities. The FAK activity at rafts was found to be significantly higher than that at non-raft regions in response to either PDGF stimulation or integrin-mediated adhesion. These newly developed biosensors also allowed the deciphering of the hierarchical interactions between FAK and Src in response to different modes of stimulation. In response to PDGF, Src activation is upstream to FAK. In contrast, in response to adhesion, FAK activation is upstream to Src.

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targeting the rafts and non-raft regions.

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