博士論文(要約)

Development of genetically encoded antibody-FP fusion FRET probes

(細胞で発現可能な蛍光抗体プローブタンパクの開発)

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This study demonstrated three types of FRET-based probe by fusing fluorescent protein with antibody variable domain or single chain Fv to detect cellular antigens and their modifications.

First, a FRET probe for Osteocalcin, also known as bone Gla protein (BGP) as a model for small molecule detection was established. A probe pair was constructed by tethering a donor fluorescent protein (FP) to the V_H fragment, and an acceptor FP to the V_L fragment. The optimized cyan-yellow FP pair for FRET, CyPet-YPet, was used as a donor and an acceptor, respectively. The two protein fragments, CyPet-V_H (BGP) and YPet-V_L(BGP), interact with each other according to the amount of co-existing antigen, thus the FRET efficiency increased as the antigen concentration increased. This homogeneous assay is called open sandwich fluoroimmunoassay (OS-FIA). This construct will be suitable for small molecule antigens because that large antigen may obstruct energy transfer. This probe also succeeded in detecting the secreted polypeptides from living cells directly. It will provide a simple approach to monitor cellular differentiations by live cell imaging.

In second part, serum albumin (SA) was used as a model for established a probe for large molecule. Anti- Serum albumin scFv obtained from a synthetic phage library and indicated that the interaction between V_H and V_L fragments of this antibody is fairly strong to form a stable complex even in the absence of antigen. Furthermore, the large molecular dimension of SA may hinder the energy transfer when used for OS-FIA probe. To address this problem, a novel immunoassay, open flower fluoroimmunoassay (OF-FIA), was developed. Two fusion proteins, VH-fused donor and VL-fused acceptor were covalently linked by introducing two mutations G44C (VH) and Q100C (VL) in the conserved framework region to make a disulfide-stabilized Fv fragment (dsFv). The FRET efficiency decreased as the concentration of antigen increased due to the disruption of fluorescent protein interaction. Therefore, OF-FIA will be an appropriate approach if the diameter of antigen is more than Förster distance of FP (~5 nm). The sensitivity is greatly improved after introducing a disulfide bond. In the case of SA detection, the ΔFRET index of disulfide stabilized SA probe is much higher than other antibody-based FRET probes due to the dimerization effect of FP. FP_2-dsFv is a single molecular probe that the concentrations of donor and acceptor are the same which can reduce the variation and measure the concentration of antigen by ratiometric FRET quantification. Although the sensitivity of FP_2-dsFv SA probe is modest compared with other scFv-fused FRET probes, the working range is wider. FP_2-dsFv SA probe will provide a

powerful approach in cellular engineering such as monitoring hepatic differentiation of mesenchymal stem cells if the probe is further optimized.

Nowadays, histone modifications are usually detected by immunostaining or histone modification ELISA *in vitro*. These conventional assays cannot provide enough information about temporal and spatial dynamic changes in living cells and organisms. The third probe in this study is an intrabody-based FRET probe which can serve as an important tool in real-time tracking of spatiotemporal dynamics of histone acetylation level. The FRET probe is composed of YFP at the N-terminal of ScFv for acetyl H3K9, and tethered CFP with histone H3 tail at the C-terminus. In nucleus, FRET efficiency increased when the cells were treated with histone deacetylase inhibitor, Trichostatin A (TSA). On the contrary, FRET efficiency was not changed in cytoplasm. It could be inferred that this FRET-based histone modification probe can measure the acetylation level in a single cell. The supposed mechanism is when FRET probes associate to acetylated H3 tails of endogenous histone, they cluster on the histone chains and cause FRET efficiency increase. In addition, when the scFv of probe associates to its own H3 tails, it triggers a conformational change and also causes FRET efficiency increase. This probe is the first demonstration of FP-fused scFv FRET probe which undergoes FRET efficiency change triggered by antigen binding. This study demonstrated that intrabody can be also used as a sensing domain for intramolecular FRET probe. It provides an option for biomolecule detection even if there is no natural sensing domain exists or provides higher affinity and higher specificity.

In summary, genetically encoded antibody-based FRET probes which provide universal approaches to detect different kinds of biomarker in homogeneous condition. The amount of analytes could be estimated by FRET efficiency. These probes will have a great potential as a diagnostic reagent and provide a simple approach to monitor cellular differentiations and epigenetic changes at the single cell level. OS-FIA is suitable for the antibodies whose V_H and V_L can only form a stable complex in the presence of antigen, especially for small molecule antigens. While OF-FIA will be an appropriate approach if the diameter of antigen is more than Förster distance of FP. Intrabody-based FRET probes have great potential to monitor biomolecules or their modifications in cells.