Development of Fluorescent and Bioluminescent Proteins for Imaging Biomolecules

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Current focus on biological research is to quantify and visualize biomolecules in living cells and animals. To probe biomolecular functions and dynamics, we are exploring a new way for developing fluorescent and bioluminescent reporter proteins based on protein splicing and complementation techniques. The reporter proteins can be utilized to development of analytical methods for detecting protein–protein interactions, intracellular localization of proteins and their dynamics, enzyme activities, gene expression and production of small biomolecules.

1. Development of Multicolor Bioluminescent Probes for Protein–Protein Interactions in Living Subjects

Bimolecular complementation using luminescent proteins has a potential to perform reversible and multiplex detection of protein–protein interactions in opaque or auto-fluorescent living subjects. We constructed multicolor luciferase fragments, of which sensitivity and signal-to-background ratio were considerably improved by using an engineered carboxy-terminal fragment of a click beetle luciferase and amino-terminal fragments of luciferases with different spectral characteristics. We investigated complementation of split luciferases from firefly (PpyLuc), click beetle in green (ELuc) and click beetle in red (CBRLuc). The N- and C-terminal fragments were fused to FK506-binding protein (FKBP) and FKBP-binding domain (FRB). A pair of the fusion proteins was co-expressed in mammalian cells and the luminescence intensities were examined by a luminometer. Amino- and carboxy-fragments of individual luciferase were found complement enough to recover the bioluminescence upon interaction between FKBP and FRB in the presence of rapamycin. But, no cross complementation was detected when combination of different luciferase fragments was used. To make cross complementation possible, a new C-terminal fragment that has an ability to complement multiple N-terminal fragments was developed by the random mutagenesis. A mutant of the C-terminal fragment including three point mutations (mcLuc1) showed most remarkable properties, which enabled complement to all the N-terminal fragments of PpyLuc, CBR and ELuc. The signal-to-background ratio upon using McLuc1 was improved higher than that of the native one. The competitive inhibitor FK506 prevented rapamycin-induced luciferase activity generated by McLuc1 and N-terminal PpyLuc, confirming that their complementation is reversible. Thus, McLuc1 allowed new complementation partners of all the N-terminal luciferases, which can be widely used for monitoring protein-protein interactions in living cells.

2. Bioluminescence Imaging of Kinase-Induced Interactions Among Bad, 14-3-3, and Bcl-2 in Living Mice

To demonstrate the applicability of the complementation of multicolor split luciferases in mammals, we used three proteins of Bad, 14-3-3 and Bcl-2, which are known to regulate cell survival. In the presence of growth factors, Bad is phos-

phorylated, thereby interacting with the 14-3-3 protein. Upon deprivation of growth factors, Bad is dephosphorylated and consequently, binds to Bcl-2. The Bad protein was fused to McLuc1, while the 14-3-3 and Bcl-2 proteins were fused to N-terminal fragments of ELuc and PpyLuc, respectively. The fusion constructs were co-transfected into COS-7 cells and photon counts were evaluated. A strong intensity of bioluminescence was obtained from the cells including Bad and 14-3-3 proteins, indicating that Bad interacts 14-3-3 endogenously. To confirm that the increase in the luminescence signal was indeed triggered by the interaction of Bad and 14-3-3, we constructed Bad mutants in which several serine residues were replaced by alanine. These serine residues are necessary for interaction of Bad with 14-3-3. The luminescence signals were significantly reduced upon expression of 14-3-3 with these Bad mutants in the cells. We next used the same construct of Bad-McLuc1 for the analysis of interaction with Bcl-2. Strong bioluminescence was obtained upon overexpression of Bad-McLuc1 and Bcl-2 connected with N-terminal PpyLuc in COS-7 cells. Addition of Bcl-2 inhibitors resulted in reducing the luminescence, demonstrating that three fragments of McLuc1, N-terminal ELuc and PpyLuc can be used for detecting interactions of a protein associated with multiple distinct protein partners.

3. Bioluminescence Imaging of Kinase-Induced Interaction of pSmad1-Smad4 in *Xenopus laevis* Embryo

To evaluate usefulness of the reversible and high-sensitive complementation analysis with McLuc1, we used a heteromeric complex between Smad1 and Smad4 involved in cytoplasmic signaling of the bone morphogenetic protein (BMP) in a *Xenopus laevis* embryo. The embryo has a large amount of fluorescent yolk, which often hampers fluorescence imaging. We constructed a set of probes consisting of Smad1 connected with N-terminal PpyLuc and Smad4 connected with McLuc1. The probes were expressed in COS-7 cells containing either constitutive active form of transmembrane receptor (ALK3), or its dominant negative form (DL). The cells expressing ALK3 resulted in a strong luminescence in comparison to the cells expressing DL receptor or mock-transfected cells. A Smad1 mutant lacking phosphorylation sites showed negligible luminescence, indicating that the phosphorylated Smad1 interacted with Smad4, thereby resulting in complement of the luciferase fragments in living cells. Next, we synthesized mRNA from each cDNA construct of the probes and microinjected into the cell embryo. The embryonic development was monitored under the fluorescence microscope equipped with a CCD camera. From an early stage, a weak but significant signal of luminescence started to be detected in the trunk region of neural tube, and thereafter the signal became intense along the entire neural tube from head to tail. The signal was sustained up for a while, but changed faint gradually, indicating dissociation between Smad1 and Smad4. These results demonstrate that bioluminescence complementation imaging using the McLuc1 and N-terminal PpyLuc enabled spatial and temporal imaging of protein-protein interactions acting on the endogenous cellular signaling in living *Xenopus* embryos.

4. A Genetically Encoded Luminescent Indicator for Detecting Intracellular Cyclic GMP in Living Cells and Plants

In order to quantify and image cellular signaling processes in living cells and subjects, we developed a new analytical method using a luciferase. The principle is based on intramolecular complementation of the split fragments of luciferase. The split fragments of luciferase are fused to a cGMP-binding domain of phosphodiesterase (PDE). When concentration of cGMP increases in cytosol, interaction of cGMP with its binding domain of PDE causes a conformational change in the domain structure. The amino- and carboxyl-terminal fragments of luciferase are brought to come together, and full-length luciferase is reconstituted thereafter. After this luminescent indicator for cGMP was transiently expressed in HEK293 cells, a membrane permeable cGMP analog or a chemical (sodium nitroprusside) was added to the cell lysates or the living cells in the presence of luciferin. We found that luminescence intensities increased with increasing concentrations of the chemicals, which were detected by a luminometer or luminescent microscope. When the indicator was transiently expressed in *Arabidopsis* cells, chemicals that induce salt or osmotic stress were added to the cell lysates or the living cells. We also detected increases in the luminescence intensities upon stimulating stress-inducing chemicals. Thus, we demonstrated quantitative detection and imaging of ligand-induced increases of cGMP in living cells and plants.

References

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