Development of Fluorescent and Bioluminescent Proteins for Imaging Biomolecules

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Current focus on biological research is to quantify and image 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 applied 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. Imaging Dynamics of Endogenous Mitochondrial RNA in Single Living Cells

Location of cytoplasmic mRNA directs proteins to particular intracellular compartments, thereby controlling local cellular functions. Distinct localization of mitochondrial RNA (mtRNA) and the molecular mechanism are, however, poorly understood. We developed genetically-encoded RNA probes for characterizing localization and dynamics of mtRNA in single living cells. The probes consist of two RNA-binding domains of PUMILIO1, each connected with split fragments of a fluorescent protein capable of reconstituting upon binding to a target RNA. We designed the probes to specifically recognize a 16-base sequence of mtRNA encoding NADH dehydrogenase subunit 6 (ND6) and to be targeted into mitochondrial matrix, which allowed real-time imaging of ND6 mtRNA localization in living cells. We showed that ND6 mtRNA is localized within mitochondria and concentrated particularly on mtDNA. Movement of the ND6 mtRNA is restricted but oxidative stress with H_2O_2 induces the mtRNA to diffuse in mitochondria, and the mtRNA gradually decomposed thereafter. The present observation of mtRNA demonstrates that the RNA probes provide a means to understand mtRNA dynamics controlled both temporally and spatially in intracellular compartments in living cells.

2. A Genetically Encoded Optical Probe for Detecting Release of Proteins from Mitochondria toward Cytosol in Living Cells and Animals

We developed a genetically encoded bioluminescence indicator for monitoring the release of proteins from mitochondria in living cells. The principle of this method is based on reconstitution of split *Renilla reniformis* luciferase (Rluc) fragments by protein splicing with an Ssp DnaE intein. A target mitochondrial protein connected with an N-terminal fragment of Rluc and an N-terminal fragment of DnaE is expressed in mammalian cells. If the target protein is released from the mitochondria toward the cytosol upon stimulation with a specific chemical, the N-terminal Rluc meets the C-terminal Rluc connected with C-terminal DnaE in the cytosol, and thereby, the full-length Rluc is reconstituted by protein splicing. The extent of release of the target fusion protein is evaluated by measuring activities of the reconstituted Rluc. To test the feasibility of this method, we monitored the release of a Smac/DIABLO protein from mitochondria during apoptosis in living cells and mice. The present method allowed high-throughput screening of an apoptosis-inducing reagent, staurosporine, and imaging of the Smac/DIABLO release in cells and in living mice. This rapid analysis can be used for

screening and assaying chemicals that would increase or inhibit the release of mitochondrial proteins in living cells and animals.

3. Nongenomic Activity of Ligands in the Association of Androgen Receptor with Src

Androgen receptor (AR) induces cell proliferation by increasing the kinase activity of Src. We developed an approach for discriminating agonist and antagonist in a nongenomic steroid-signaling pathway using an association of AR with Src. We constructed a pair of genetically encoded indicators, where N- and C-terminal fragments of split firefly luciferase (FLuc) were fused to AR and Src, respectively. The fusion proteins with AR and Src are localized in the cytoplasm and on the plasma membrane, respectively. Upon being activated with androgen, AR undergoes an intramolecular conformational change and binds with Src. The association causes the complementation of the split FLuc and recovery of FLuc activity. The resulting luminescence intensities were taken as a measure of the rapid hormonal activity of steroids in the nongenomic AR signaling. Ten minutes were required for the AR-Src association by 5alpha-dihydroxytestosterone (DHT), which was completely inhibited by an antagonist, cyproterone acetate. The activities of ligands in the nongenomic pathway of AR were compared with those in the genomic pathway obtained on the basis of the nuclear trafficking of AR in mammalian cells. The comparison revealed that DHT and testosterone activate both genomic and nongenomic pathways of AR. 17beta-Estradiol and progesterone were found to be specific activators only for the genomic signaling pathway of AR. On the other hand, procymidone exhibited a specific activity only for the nongenomic signaling pathway of AR. The present approach is the first example addressing the agonistic and antagonistic activities of ligands in a nongenomic pathway of AR.

4. Cyclic Luciferase for Real-Time Sensing of Caspase-3 Activities in Living Mammals

Programmed cell death (apoptosis) is a crucial process involved in pathogenesis and progression of diseases, which is executed by Cysteine Aspartyl Proteases (caspases). The caspase activities in living subjects and their regulation with small chemical compounds are of great interest for screening drug candidates or pathological agents. We developed a genetically encoded bioluminescent indicator for high-throughput sensing and noninvasive real-time imaging of caspase activities in living cells and animals. Firefly luciferase connected with a substrate sequence of caspase-3 (Asp-Glu-Val-Asp) is cyclized by an intein DnaE (a catalytic subunit of DNA polymerase III). When the cyclic luciferase is expressed in living cells, the luciferase activity greatly decreases due to a steric effect. If caspase-3 is activated in the cells, it cleaves the substrate sequence embedded in the cyclic luciferase and the luciferase activity is restored. We demonstrated quantitative sensing of caspase-3 activities in living cells upon extracellular stimuli. Furthermore, the indicator enabled noninvasive imaging of the time-dependent caspase-3 activities in living mice. This cyclic luciferase indicator provides a general means for understanding the mechanism of physiological proteolytic processes and for screening novel pharmacological chemicals among candidates in living subjects.

References

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