II-G Development of Fluorescent and Bioluminescent Probes for Imaging Biomolecules

Our understanding of biological systems is increasingly dependent upon the ability to quantify and image biomolecules in living animals and plants. To probe the biomolecular functions and dynamics in living organisms, we are exploring a new way for developing fluorescent and bioluminescent reporter proteins based on protein splicing. 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 bio-molecules. We are also currently investigating analytical techniques such as complementary DNA library screenings and proteome analysis.

II-G-1 A Short Peptide Sequence that Targets Fluorescent and Functional Proteins into the Mitochondrial Intermembrane Space

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Protein-based fluorescent and functional probes are widely used for real-time visualization, purification and regulation of a variety of biological molecules. The protein-based probes can generally be targeted into subcellular compartments of eukaryotic cells by a particular short peptide sequence. Little is known, however, about the sequence that targets probes into the mitochondrial intermembrane space (IMS). To identify the IMS-targeting sequence, we developed a simple genetic screening method to discriminate the proteins localized in the IMS from those in the mitochondrial matrix, thereby revealing a sequence sorting into the IMS. An IMS-localized protein, Smac/DIABLO, was randomly mutated and mitochondrial localization of each mutant was analyzed. We found that the four residues of Ala-Val-Pro-Ile are required for the IMS localization, and a sequence of the four residues fused with matrix-targeting signals is sufficient for targeting the Smac/DIABLO in the IMS. The sequence was shown to readily direct multiple proteins of interest to the IMS, which will open avenues to elucidating IMS functions in live cells.

II-G-2 Intein-Mediated Reporter Gene Assay for Detecting Protein-Protein Interactions in Living Mammalian Cells

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For nondestructive analysis of chemical processes in living mammalian cells, we developed a new reporter gene assay for detecting cytosolic protein-protein interactions based on protein splicing of transcription factors with DNA inteins. The protein splicing induces connection of a DNA-binding protein (modified LexA; mLexA) with a transcription activation domain of a herpes simplex virus protein (VP16AD). We thereby circumvented the limitation of earlier methods for monitoring protein-protein interactions, including the two-hybrid systems, protein complementation systems (PCS), and protein reconstitution systems, and rather combined their advantages. To test the applicability of this method, we monitored epidermal growth factor (EGF)-induced interactions on cell membranes of a known partner, an oncogenic product Ras and its target Raf-1. Ras was connected with N-terminal DnaE and mLexA, while Raf-1 was connected with C-terminal DnaE and VP16AD. Upon stimulation with EGF, the interaction between Ras and Raf-1 triggered folding of the DnaEs, thereby inducing protein splicing to form mLexA-VP16AD fusion protein, and transcription of a reporter gene, firefly luciferase. The extent of Ras-Raf-1 interaction was quantified by measuring the luciferase activity. The interaction was not able to be monitored by two-hybrid systems nor by PCS of split firefly luciferases; however, by using the protein splicing elements and the reporter gene, we obtained the bioluminescence signals sufficient for evaluation of the interactions close to cell membranes.

II-G-3 A Genetically Encoded Indicator for Assaying Bioactive Chemicals that Induce Nuclear Transport of Glucocorticoid Receptor

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Glucocorticoids, the adrenal steroid hormones secreted during stress, are essential to homeostasis and metabolism in the human body. An impaired glucocorticoid signaling due to dysfunction of the glucocorticoid receptor (GR) by synthetic chemicals can cause diseases and disruptions of the homeostasis and metabolism. Here we demonstrate the development of a method for screening endocrine-disrupting chemicals and potent risk factors of human diseases based on the nuclear trafficking of the GR. We constructed a new assay using a pair of genetic indicators with the full length of the GR, split Renilla luciferase (RLuc), and split DnaE (a protein splicing element). The GR-containing fusion protein with C-terminal halves of DnaE and RLuc is localized in cytosol due to the cytosolic character of the GR, whereas the fusion protein with N-terminal halves of DnaE and RLuc stays in the nucleus due to the cofused nucleus localization signal. On being stimulated
with a ligand, the GR is translocated into the cellular nucleus. Thus, the protein splicing occurs in the nucleus by an interaction between the splicing junctions of each DnaE fragment. The enzymatic activities from the reconstituted RLuc allow the ligand-dependent luminescence intensities. The feasibility of the method was evaluated by quantifying the hormonal activities of 20 different kinds of steroids and synthetic chemicals using the NIH 3T3 cells carrying the pair of indicators. The hormonal activities of tested ligands are discussed based on the chemical structure-activity relationship. We found that androgens, testosterone, and 19-nortestosterone weakly induce the nuclear transport of the GR. The current assay allows high-throughput screening of risk chemicals and drug candidates influential to a signal transduction pathway of the GR.

II-G-4  A Method for Determining the Activities of Cytokines Based on the Nuclear Transport of Nuclear Factor-κB

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Gene expressions are controlled by regulatory proteins known as transcription factors. One important transcription factor is nuclear factor-κB (NF-κB), which is related to cellular proliferation, survival, differentiation, or apoptosis. We developed a method to evaluate the activities of cytokines based on the nuclear transport of NF-κB. A pair of bioluminescent indicators were made for conferring cytokine sensitivity to cervical carcinoma-derived HeLa cells. The principle is based on reconstitution of split fragments of Renilla luciferase (RLuc) by protein splicing with a DnaE intein. The bioluminescence intensity of thus reconstituted RLuc in the HeLa cells was used as a measure of the activities for cytokines. The present method would be a useful high-throughput assay for determining the activities of potential biomedical inhibitors on NF-κB trafficking.