2 Imaging Protein-Protein Interactions in Whole Cells and Living Animals

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Protein-protein interactions regulate a variety of cellular functions, including cell cycle progression, signal transduction, and metabolic pathways. On a whole organism scale, protein-protein interactions regulate signals that affect overall homeostasis, patterns of development, normal physiology, and disease in living animals (Zhang et al. 1997; Stark et al. 1998; Ogawa et al. 2002). In addition, protein-protein interactions have untapped potential as therapeutic targets (Heldin 2001; Darnell 2002). Evidence is accumulating that pathways of protein interactions in specific tissues produce regional effects that cannot be investigated fully within vitro systems and thus, there is considerable interest in evaluating protein interactions in living animals.

Fundamentally, the detection of physical interaction among two or more proteins can be observed if association between the interactive partners leads to production of a readily observed biological or physical readout (Toby and Golemis 2001). Most strategies for detecting protein-protein interactions in intact cells are based on fusion of the pair of interacting molecules to defined protein elements to

reconstitute a biological or biochemical function. Examples of reconstituted activities include activation of transcription, repression of transcription, activation of signal transduction pathways, and reconstitution of a disrupted enzymatic activity (Toby and Golemis 2001). A variety of these techniques have been established for investigating protein-protein interactions in cultured cells and several are now being validated for use in living animals, including the two-hybrid system and protein fragment complementation. The major features of these two methods and their potential utility for in vivo imaging are described below.

2.1 Two-Hybrid Systems

Two-hybrid systems exploit the modular nature of transcription factors, many of which can be separated into discrete DNA-binding and activation domains (Fields and Song 1989). Proteins of interest are expressed as fusions with either a DNA-binding domain (BD) or activation domain (AD), creating hybrid proteins. If the hybrid proteins bind to each other as a result of interaction between the proteins of interest, then the separate BD and AD of the transcription factor are brought together within the cell nucleus to drive expression of a reporter gene. In the absence of specific interaction between the hybrid proteins, the reporter gene is not expressed because the BD and AD do not associate independently. Two-hybrid assays can detect transient and/or unstable interactions between proteins, and this technique is reported to be independent of expression of endogenous proteins (von Mering et al. 2002). Although the two-hybrid assay originally was developed in yeast, commercial systems (BD Biosciences Clontech, Palo Alto, CA) are now available for studies in bacteria and mammalian cells.

We and other investigators have shown that two-hybrid systems can be used to image protein interactions in living mice with positron emission tomography (PET; Luker et al. 2002, 2003 a–c) or bioluminescence imaging (Ray et al. 2002). For example, to enable noninvasive molecular imaging of protein-protein interactions in vivo by PET and fluorescence imaging, we engineered a fusion reporter gene comprising a mutant herpes simplex virus 1 thymidine kinase

(HSV1-tk) and green fluorescent protein (GFP) for readout of a tetracycline-inducible two-hybrid system in vivo. Using microPET, interactions between p53 tumor suppressor and the large T antigen (TAg) of SV40 virus were visualized in tumor xenografts of HeLa cells stably transfected with the imaging constructs (Luker et al. 2002). However, the two-hybrid method has some limitations. Some types of proteins do not lend themselves to study by the two-hybrid method. For example, because production of signal in the two-hybrid method requires nuclear localization of the hybrid proteins, membrane proteins cannot be studied in their intact state. Also, the time delay associated with both transcriptional activation of the reporter gene and degradation of the reporter protein and mRNA limits its kinetic analysis of protein interactions (Rossi et al. 2000).

2.2 Protein-Fragment Complementation

Protein-fragment complementation (PFC) assays depend on division of a monomeric reporter enzyme into two separate inactive components that can reconstitute function upon association. When these reporter fragments are fused to interacting proteins, the reporter is reactivated upon association of the interacting proteins. PFC strategies based on several enzymes, including β-galactosidase, dihydrofolate reductase (DHFR), β-lactamase, and luciferase have been used to monitor protein-protein interactions in mammalian cells (Rossi et al. 1997; Wehrman et al. 2002; Remy and Michnick 1999; Remy et al. 1999; Galarneau et al. 2002; Ozawa et al. 2001). A fundamental advantage of PFC is that the hybrid proteins directly reconstitute enzymatic activity of the reporter. In principle, therefore, protein interactions may be detected in any subcellular compartment, and assembly of protein complexes may be monitored in real time. A disadvantage of complementation approaches is that reassembly of an enzyme may be susceptible to steric constraints imposed by the interacting proteins. Another potential limitation of PFC for application in living animals is that transient interactions between proteins may produce insufficient amounts of active enzyme to allow noninvasive detection. Nonetheless, because most PFC strategies are based on reconstituting active enzymes, these systems offer the potential bene-

fits of signal amplification to enhance sensitivity for detecting interacting proteins in living animals.

2.3 Optimized Luciferase Fragment Complementation

As discussed, complementation strategies may offer advantages for near real-time applications in living animals, and pilot studies with firefly and *Renilla* luciferases have demonstrated the feasibility of this approach (Ozawa et al. 2001; Paulmurugan and Gambhir 2003). However, the available fragments suffer from considerable constitutive activity of the N-terminus fragments, thereby precluding general use. Thus, no enzyme fragment pair yet has been found that satisfies all criteria for noninvasive analysis of protein-protein interactions and enables interrogation in cell lysates, intact cells, and living animals.

To develop an optimized protein fragment complementation imaging system for broad use in living cells and animals, we screened a combinatorial incremental truncation library for reconstitution of the enzymatic activity of a heterodimeric firefly (*Photinus pyralis*) luciferase (Luker and Piwnica-Worms 2004). This library employed a well-characterized protein interaction system: rapamycin-mediated association of the FRB domain of human mTOR (residues 2024–2113) with FKBP-12 (Remy and Michnick 1999; Galarneau et al. 2002; Chen et al. 1995). Initial fusions of FRB and FKBP with N- and C-terminal fragments of luciferase, respectively, were designed such that the enzymatic activities of the individual overlapping fragments were weak or absent. From these constructs, N- and C-terminal incremental truncation libraries were generated by unidirectional exonuclease digestion and validated essentially as described (Ostermeier et al. 1999). The libraries were coexpressed in *E. coli* and screened in the presence of rapamycin for bioluminescence. From this screen, one could identify an optimal pair of overlapping amino acid sequences for the NLuc fragment and for the CLuc fragment. The optimized combination of fragments produced no signal in the absence of rapamycin and strong bioluminescence in the presence of the dimerizing agent rapamycin.

In live cells and in cell lysates, our optimized complementation system successfully reproduced published apparent K_d values for ra-

pamycin (Remy and Michnick 1999; Galarneau et al. 2002; Luker and Piwnica-Worms 2004; Chen et al. 1995). To test the specificity of the complementation system, we mutated the FRB fragment in the FRB-NLuc construct to a form FRB(S2035I) (Chen et al. 1995), which should be insensitive to rapamycin. We showed that this mutation, even in the presence of rapamycin, produced low bioluminescence signal similar to the optimal pair in the absence of rapamycin. Also, expression of single constructs produced no detectable signal relative to untransfected cells. Thus, our optimized complementation pair eliminated the substantial bioluminescence activity of the N-terminal luciferase fragment that was problematic with previous split-luciferase systems based on simple bisection of luciferase (Paulmurugan et al. 2002).

Furthermore, bioluminescence imaging of animals using charged couple device (CCD) cameras such as the IVIS (Xenogen, Alameda, CA) enabled us to quantify relative expression of the luciferase reporter activity in vivo. In mice bearing implants of cells expressing our FRB-NLuc/CLuc-FKBP fusion pair, repetitive bioluminescence imaging showed dose- and time-dependent luciferase activity induced by rapamycin with a maximal in vivo signal-to-background ratio of greater than 20:1.

2.4 Conclusions

These studies demonstrate that noninvasive molecular imaging of protein-protein interactions may enable investigators to determine how intrinsic binding specificities of proteins are regulated in a wide variety of normal and pathophysiologic conditions. These tools provide a platform for detection of regulated and small molecule-induced protein-protein interactions in intact cells and living animals and should enable a wide range of novel applications in biomedicine, drug discovery, chemical genetics, and proteomics research.

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