Finding Partners: Emerging Protein Interaction Technologies Applied to Signaling Networks

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Genome sequencing projects have revealed the massive catalog of genes in various model organisms, from bacteria to human. The scientific community is now faced with a major challenge of how to assign functions to thousands of uncharacterized genes, and how to use this information to identify the proteins that function together to regulate fundamental cellular processes. Besides protein identification and quantification of differences in protein profiles of cells, tissues, and organs, the characterization of physical protein-protein interactions (so-called "interactive proteomics") is being recognized as a key objective of proteomics research (*1*).

Virtually all cellular responses, including growth and differentiation, are stringently controlled by physiological signals in the form of growth factors, hormones, nutrients, and contact with neighboring cells. These various signals are processed by signal transduction mechanisms that ultimately produce an appropriate cellular response. The signal transduction mechanisms include transmembrane and intracellular receptor proteins, protein kinases, protein phosphatases, guanosine triphosphate (GTP)-binding proteins, and many intracellular proteins that function to transmit the signal through specific protein-protein interactions to downstream effector molecules that execute the response (*2*). In addition, important protein-protein interactions in cell signaling are frequently mediated by short, unstructured sequences that specifically interact with peptide binding domains (*3*). Typical examples are the binding of tyrosyl-phosphorylated peptides to proteins containing the Src homology 2 (SH2) domain (*4*) and proline-containing peptides that bind proteins containing the SH3 domain (*5*). Given that specific protein-protein interactions are involved in the execution of all cellular processes, technologies that simplify and facilitate the detection and analysis of the above-mentioned protein-protein interactions are of great importance for understanding signaling pathways (*6*). This Perspective reviews the latest proteomics technologies that are being used for identification of these interactions.

Generally speaking, the approaches for dissecting physical protein-protein interactions can be divided into biochemical and genetic methods. Biochemical methods for identification of protein-protein interactions, such as coimmunoprecipitation and copurification through chromatographic columns, were long considered to be difficult and time consuming because they required harsh treatments for cell disruption and optimization for each protein complex examined. However, this view changed with the rapid advances in protein analytical technologies, including mass spectrometry and sequence databases [reviewed in (*7*)]. Mass spectrometry-based interactive proteomics is rapidly becoming a method of choice for analyzing functional protein complexes (*8*). In support of this notion, two systematic protein complex studies used affinity-tagged proteins expressed in yeast as baits to capture and identify their associated proteins. The composition of the isolated complexes was analyzed by gel electrophoresis and mass spectrometry, and many known and unknown protein interactions were identified (*9, 10*). Although this method is extremely powerful, it remains difficult to distinguish specific from nonspecific interactions and to detect quantitative changes in protein complex abundance and composition.

To address this limitation, the Mann and Aebersold groups have developed two interesting proteomic strategies: stable isotope labeling by amino acids in cell culture (SILAC) (*11*) and the isotope-coded affinity tag (ICAT) (*12*). The ICAT technology has been discussed in detail (*7, 13*) and will not be reviewed here. The SILAC technology (Fig. 1A) has been used to elucidate functional protein-protein interactions in the epidermal growth factor receptor (EGFR) signaling pathway, with the SH2 domain of the adaptor protein Grb2 as bait (*14*). The authors identified 228 proteins that bound the Grb2-SH2 domain, of which 28 were enriched upon EGF stimulation. The Grb2-SH2-interacting proteins characterized in this study included proteins known to be involved in EGFR signaling and cytoskeletal functions; five additional proteins were also linked to EGFR signaling (*14*). The principle of SILAC has also been used in affinity pulldown experiments with a synthetic peptide as bait; in a recent study, a phosphotyrosine-containing peptide of EGFR specifically retrieved the SH2 and SH3 domain-containing Grb2 (*15*). Both SILAC and ICAT approaches provide a powerful new tool for the comprehensive analysis of protein complexes involved not only in cell signaling but also in other cellular processes. The strength of these approaches is that they are unbiased to any particular class of proteins and thus allow not only the identification of novel interacting partners but also the detection of modification-dependent interactions. However, although isotope-tagging methods based on chemical labeling are compatible with almost every protein sample, the application of metabolic labeling is limited to cultured cells. The high cost of isotope labeling is an additional drawback of these methods.

A new biochemical method for probing physical protein-protein interactions between signaling proteins is called "SH2 profiling" (Fig. 1B) and is based on the well-known Far Western assay (*16*). A central premise of this approach is that different SH2 domains maintain their phosphorylation- and sequence-dependent binding specificities in the Far Western binding assay. SH2 profiling has been tested in human cells expressing either wild-type platelet-derived growth factor (PDGF) β receptor or mutants in which specific SH2 binding sites were eliminated. The SH2 domain detected the wild-type PDGF receptor when the receptor was autophosphorylated in response to PDGF treatment. In contrast, the SH2 domain probe no longer bound the receptor when the target tyrosine residue was mutat-

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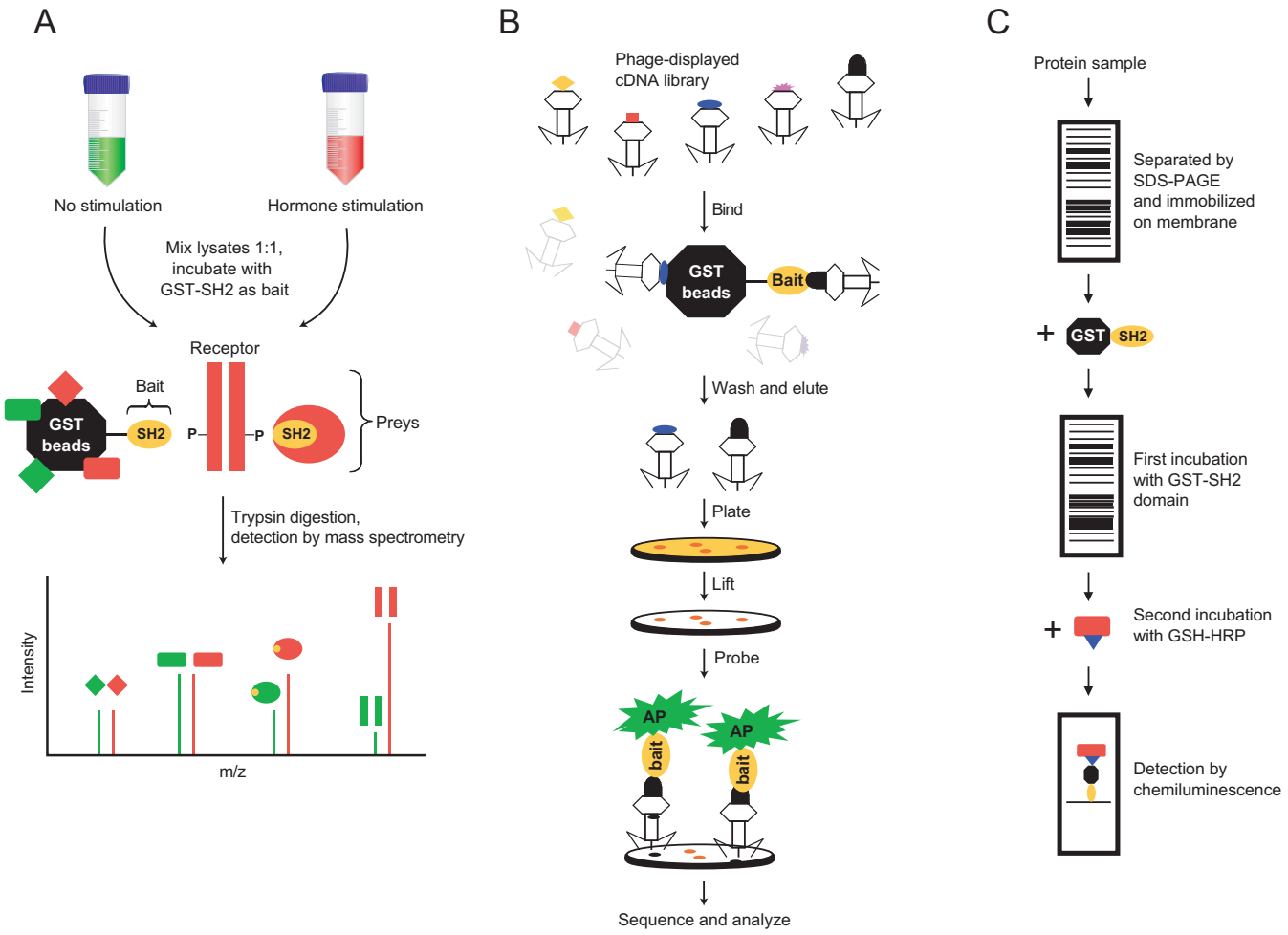

Fig. 1. Emerging biochemical technologies for detection of protein-protein interactions. (**A**) Stable isotope labeling by amino acids in cell culture (SILAC), a method for studying hormone-activated protein complexes. One cell population is grown in normal Arg-containing medium (green Falcon tube) and another population is grown in [^{13}C]Arg-containing medium (red Falcon tube), resulting in labeling of all Arg-containing proteins with this 6-dalton-heavier amino acid (red color). The latter cell population is stimulated with a hormone, lysed, and mixed 1:1 with the lysate of unlabeled, untreated cells (green Falcon tube). The combined cell lysates are then affinity-purified over the glutathione-*S*-transferase (GST) column fused to the domain of interest (for example, SH2), which will specifically interact with an activated (phosphorylated) form of the receptor (for example, EGFR) and its associated interacting proteins (for example, Grb2). Proteins bound to the bait domain of interest are eluted from the beads, digested with trypsin, and detected by mass spectrometry. Arg-containing peptides will occur in doublets, separated by 6 dalton. Tryptic peptides from proteins specifically binding to the bait in a hormone-stimulated manner will have a larger intensity of the ^{13}C-labeled form (red peaks). Unspecific binders will manifest themselves by their 1:1 ratio between stimulated and unstimulated states. (**B**) Target-assisted iterative screening (TAIS). In a first (preselection) step, a bait protein (or a defined protein domain) is immobilized on a solid support and incubated with a phage-displayed cDNA library in solution. The goal of this step is to retain phages displaying on their surfaces the interacting partners of the bait on the solid support. After washing, the retained phages are eluted and plated on a bacterial lawn. In the second step, the preselected cDNA library plaques are transferred onto a nitrocellulose membrane by blotting the bacterial lawn with the membrane. The bait protein tagged with reporter, such as alkaline phosphatase (AP), is then used as a one-step detection reagent to screen for interacting plaques on the membrane. The identities of the displayed polypeptides that interact with the target are deduced by sequencing of the corresponding cDNA inserts of the phages from individual positive plaques. (**C**) SH2 profiling. In this method, total cellular protein extracts are separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto membrane, and then probed with a purified GST-SH2 domain fusion. After extensive washing, the bound GST-SH2 domain is detected in a Far Western procedure by glutathione-conjugated horseradish peroxidase (GSH-HRP) and by chemiluminescence.

ed (*16*). This simple and rapid technology clearly demonstrates the feasibility of SH2 profiling for characterization of signaling between protein tyrosine kinases and their adaptor proteins. Moreover, this method is well suited as a molecular diagnostic tool for the classification of cells based on functional differences in their signaling state, and it is potentially adaptable to other protein interaction motifs (especially those that exhibit binding that is regulated by posttranslational modifications).

One disadvantage of this in vitro method is a relatively high background that can make detection of tyrosine-phosphorylated proteins problematic in cases where phosphorylation levels are low.

Another approach for elucidation of protein-protein interactions is target-assisted iterative screening (TAIS) (*17*). This method has been designed to screen the protein products of a cDNA library displayed on the phage surface with a target protein of interest, thus allowing rapid screening of a proteome for binding partners of the target protein (Fig. 1C). The bait protein is used iteratively within two different screening formats, phage display and expression library screens, hence the name TAIS. Previously, SH3, PDZ, and WW domains of the Abl, Src, Crk, PSD95, and Nedd4 proteins have been used as test baits. In total, 12 novel putative interactions and two previously described interactions have been identified for these well-studied protein interaction modules (*17*). In an effort led by Kurakin *et al.,* TAIS was applied to isolate peptide ligands of the CIN85 SH3 domain and thus allowed accurate mapping of CIN85 SH3 domain binding sites within known CIN85 interactors, c-Cbl, BLNK, Cbl-b, AIP/Alix, SB1, and CD2 proteins (*18*). On the basis of this observation, it is now possible to predict novel CIN85 interacting partners in protein databases. Although not yet applied on a large scale, this approach is scalable to allow identification of novel interactors of many other protein domains, which could be a basis for further functional characterization. However, a limitation of this approach is the fact that certain types of interactions may not occur in bacteria because of the need for eukaryote-specific modifications. In addition, a short polypeptide displayed on the phage surface might exhibit binding activities that are different from the ones it displays as part of a large protein sequence, thus contributing to false positives.

Aside from biochemical approaches, expression systems that are based on the detection of protein-protein interactions in vivo have become a popular tool because they require little individual optimization and are well suited for screenings in a high-throughput format (*19*). The yeast two-hybrid system (YTH), which provides a means to rapidly screen for binary interactions between proteins in the nucleus of a yeast cell (*20*), is one of these expression systems. The YTH method has been efficiently used to identify many novel soluble components of the cell signaling pathways (*21*). Furthermore, genome-wide YTH projects have been conducted to analyze protein-protein interactions at a global level in the human gastric pathogen *Helicobacter pylori* (*22*), the budding yeast *Saccharomyces cerevisiae* (*23, 24*), the nematode *Caenorhabditis elegans* (*25*), and the fruit fly *Drosophila melanogaster* (*26*) and have produced a wealth of new protein interaction data. A major limitation of the traditional YTH method is that interaction between bait and prey occurs in the nucleus. Consequently, transmembrane proteins, such as receptor tyrosine kinases and phosphatases (which represent an important class of signaling molecules), cannot be efficiently studied by the YTH method because they tend to form aggregates when expressed in the nucleus in their full-length form. In addition, interactions between many membrane proteins are dependent on posttranslational modifications, such as glycosylation and disulfide bond formation, that take place within the endoplasmic reticulum but not in the nucleus (*27*). Moreover, the genome-wide screenings mentioned above have shown that the representation of transmembrane domain proteins is poor (*22-24*).

An alternative YTH approach that can circumvent these disadvantages and is applicable to studying membrane proteins is the split-ubiquitin membrane yeast two-hybrid system (MbYTH) (*28*). MbYTH uses the split-ubiquitin approach in which the reconstitution of two ubiquitin halves is mediated by a specific protein-protein interaction (*29*). Thus, interaction between two membrane proteins results in ubiquitin reconstitution and leads to the proteolytic cleavage and subsequent release of a transcription factor that triggers the activation of a yeast reporter gene system, enabling indirect detection of protein interactions (Fig. 2). The MbYTH system has been successfully adapted for prey library screening and has identified three novel

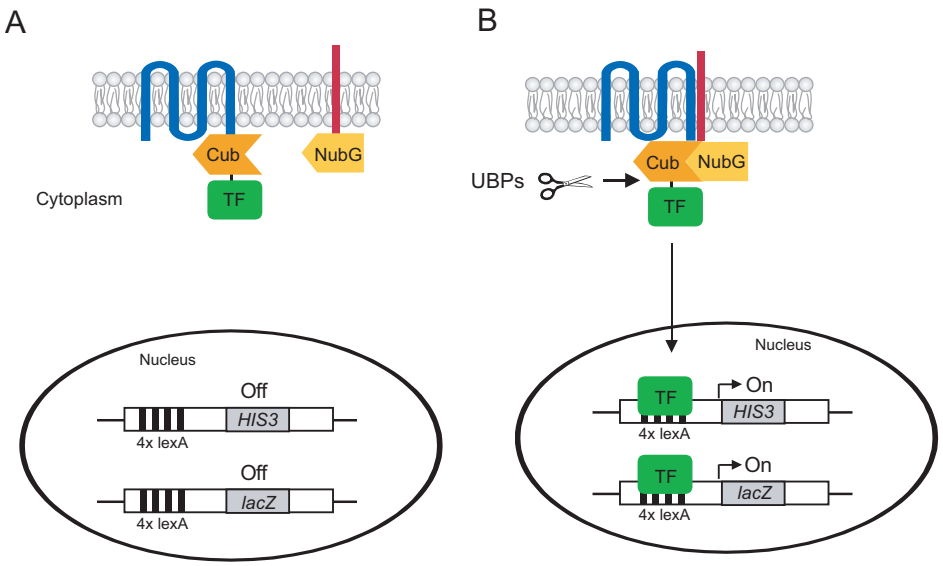

Fig. 2. The split-ubiquitin membrane yeast two-hybrid system (MbYTH), a novel genetic assay for characterization of membrane protein interactions. Here, the membrane bait protein of interest (blue) is fused to the C-terminal domain of ubiquitin (Cub) followed by the artificial transcription factor TF, and the potential interacting prey protein (red) is fused to the mutated N-terminal domain of ubiquitin (NubG) . Note that the prey protein can also be a cytosolic protein [see (*30*) for details]. (**A**) If the two membrane proteins do not interact, then the TF is not cleaved from Cub and remains associated with the membrane. In this case, reporter genes *HIS3* and *lacZ* remain silent, which renders cells unable to grow on medium lacking the amino acid histidine and colorless when exposed to X-gal. (**B**) If the two membrane proteins interact, the reassociated NubG and Cub are recognized and cleaved by the ubiquitin-specific proteases (UBPs) (scissors), liberating the TF, which is transported into the nucleus. This in turn activates the *lacZ* and *HIS3* reporter genes, resulting in β-galactosidase activity and histidine synthesis, respectively.

interacting partners of mammalian ErbB3, a receptor tyrosine kinase involved in regulation of proliferation and differentiation of many tissue types (*30*), and BAP31, a human polytopic integral membrane protein of the endoplasmic reticulum involved in apoptosis (*31*). Thus, MbYTH allows rapid and sensitive characterization of proteins associated with a particular full-length transmembrane protein of interest and is generally applicable to most transmembrane signaling proteins. The major disadvantage of MbYTH is that it identifies a large number of false positives, presumably through interactions between proteins that do not normally occur in vivo. In addition, this technology cannot be applied to study protein complexes, as it detects mostly binary interactions.

In recent years, the scientific community has witnessed the development of several emerging proteomic technologies that are capable of tackling protein-protein interactions between signaling proteins [reviewed in (*6*)]. These technologies have helped to identify novel components of signaling pathways and represent an important step toward the elucidation of those pathways. The future progress of interactive proteomics will involve refinement of the methods discussed here, as well as their application in a high-throughput manner. However, the limitations of these technologies must be accounted for and complemented by additional proteomic techniques. Thus, the best approach to identify an interactor(s) of a particular protein is likely to be a combinatorial approach. In this way, techniques such as protein chips (*32*, *33*), proteome-scale protein localization studies (*34*, *35*), and computational methods for prediction of physical protein-protein interactions (*36*), along with the above-mentioned technologies, should help to create an accurate and comprehensive network of eukaryotic signaling pathways. Clearly, knowledge of signal transduction cascades will be highly instructive in understanding the pathways underlying many human diseases and will likely reveal new targets for their therapy. One thing is certain: The field of interactive proteomics is as exciting as ever.

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