

Challenges in Structural Investigation of Transient Protein-Protein Interactions

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Introduction

Receptor Tyrosine Kinases (RTKs) are transmembrane proteins that, upon stimulation by their soluble ligands dimerize or multimerize and autophosphorylate themselves on the cytoplasmic side and activate signal transduction pathways, which in turn trigger either nuclear, transcriptional responses or a cellular effect such as cytoskeletal remodeling. However, while the classical RTK pathway is well

understood, the intracellular protein interaction events that lead to these responses are still not yet well understood.

Upon activation of signal transduction pathways, many stable and transient Protein-Protein Interactions (PPIs) occur. However, analyzing PPIs in these signaling pathways is difficult using current methods and any improvement in this regard will help in understanding these pathways and how they function.

Current Methods for Analysis of PPIs

Analysis of PPIs in signaling pathways starts with the Immunoprecipitation (IP) of the receptors followed by identification of their protein interaction partners. In the next step, additional interacting partners may be identified [1-9]. A very common method for studying PPIs is co-Immunoprecipitation (co-IP) [10,11]. However, this method is knowledge-dependent: it may be used only if preliminary data already exists. For example, if two proteins are already known to interact with each other (i.e. interaction of two proteins is already established by a different method, such as two-hybrid screen), co-IP may be confirmative. Therefore, analysis of PPIs has been limited to co-IPs or two hybrid screens leading to the construction of interaction networks. Additional biochemical methods for identification of PPIs include electrophoresis, chromatography or mass spectrometry.

Two electrophoresis methods are of particular interest: native electrophoresis and Blue Native PAGE (BN-PAGE). In native electrophoresis, one may use regular self-made, custom gradient, PAGE, but without SDS. Having no SDS, the proteins do not denature and the PPIs are not compromised. A variation of Native electrophoresis that is used for identification of PPIs is Electrophoresis Mobility Shift Assay (EMSA), where the PPIs can be easily identified based on their shift in electrophoresis mobility. Advantage for this method is that not only PPIs, but also DNA-proteins or RNA-proteins interactions can be investigated and identified.

BN-PAGE is a relatively recently discovered method and is particularly suitable for investigation of PPIs and protein complexes [12-18] (Figure 1). This method separates protein complexes based on their molecular mass, which is induced by addition of Coomassie dye to the protein sample and to the running buffer. The Coomassie dye induces a uniform external charge to the protein complexes, playing a role similar to detergent SDS (induced external charge), but without denaturing the proteins. Separation of protein complexes from the BN-PAGE gel lane

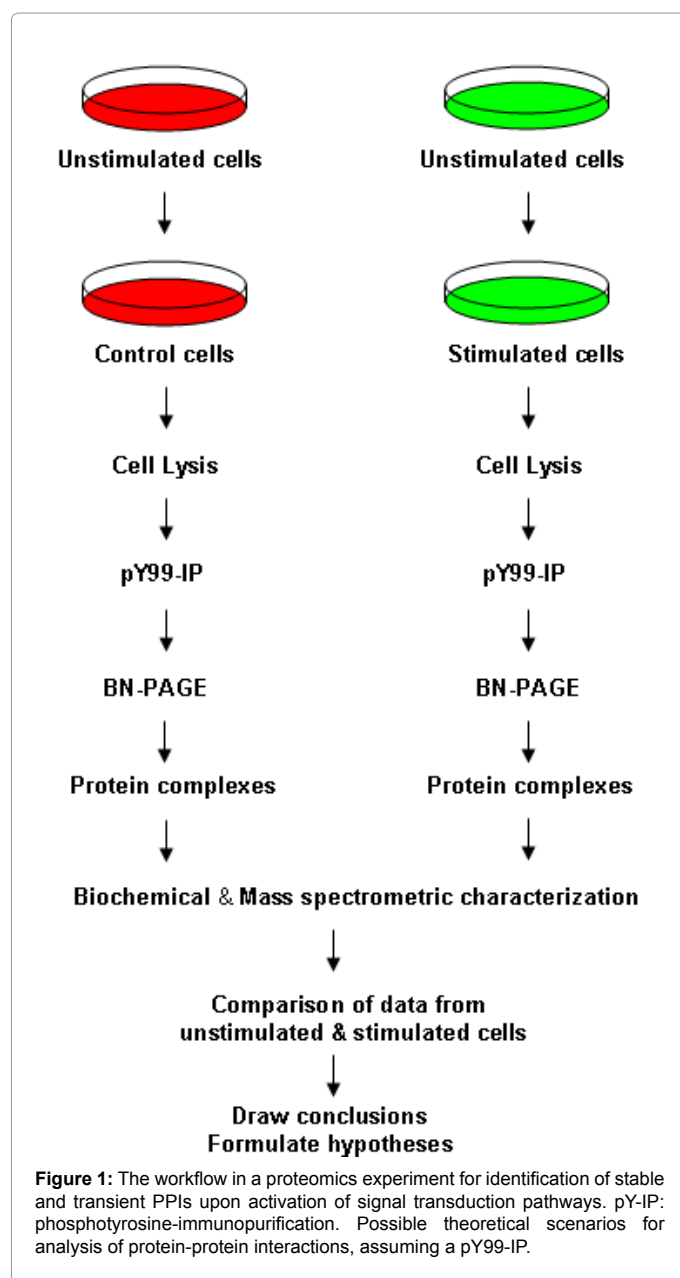


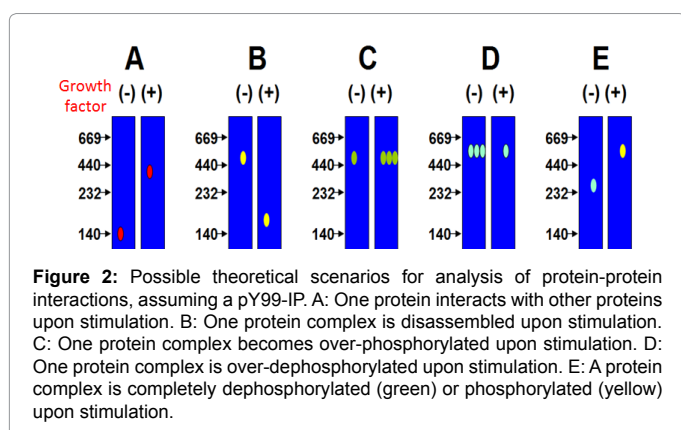
Figure 1: The workflow in a proteomics experiment for identification of stable and transient PPIs upon activation of signal transduction pathways. pY-IP: phosphotyrosine-immunoprecipitation. Possible theoretical scenarios for analysis of protein-protein interactions, assuming a pY99-IP.

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separated in the first dimension (1D) in a second dimension (2D) SDS-PAGE followed by Coomassie staining or by Western Blotting (WB) may reveal the subunit composition of a particular protein complex, as well as the interacting partners of a particular protein. Additional protein complexes may also be identified (Figure 2). A variant of BN-PAGE is Colorless Native PAGE or Clear Native PAGE (CN-PAGE), in which the proteins are separated just like in BN-PAGE, except that the Coomassie dye is not included. As a consequence, the protein complexes are separated according to their internal charge and independent of their molecular mass. A particularly useful combination includes CN-PAGE with SDS-PAGE in 2D-PAGE followed by SDS-PAGE and Coomassie staining or SDS-PAGE and Western blotting, similar to BN-PAGE described earlier. In addition, combination of CN-PAGE and BN-PAGE is very useful and unique for the separation of protein complexes with identical molecular mass, but different internal charge. For example, when two protein complexes with identical mass are separated by BN-PAGE, their subunit composition cannot be identified, unless the subunit composition of the protein complexes investigated is known. However, if not known, the protein complexes can be first separated by CN-PAGE (according to their internal charge) and then by BN-PAGE (according to their molecular mass). Therefore, the end result is complete separation of two protein complexes with identical mass, but different internal charge.

Mass spectrometry (MS) is one of the most powerful tools for identifying proteins [2,19-34] and PPIs [1,3,4,10,14,35,36], simply because it can be easily combined with IP, SDS-PAGE, chromatography, native PAGE, BN-PAGE CN-PAGE, and allows one to identify many potential interacting partners of a protein by analyzing the proteins or protein complexes (isolated and/or purified by one of the previously described methods) by Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) or by liquid chromatography-tandem mass spectrometry (LC-MS/MS), in an approach referred to as proteomics [3-5,14,29,30,36-42]. As such, most PPIs are indirectly identified through digestion of the protein complexes purified through one of the previously described methods (i.e. IP) followed by MS analysis, which leads to identification of the peptides that are part of proteins that can interact with each other. This approach is also called bottom up proteomics and may also be called bottom-up PPIs proteomics. The opposite method for bottom-up PPIs proteomics is top-down PPIs proteomics, where intact protein complexes are analyzed by MS without prior digestion.

Technical Limitations

Current methodology used for identification of the PPIs could definitely use some improvements. For example, in experiments

in which a particular protein is identified as a participant in signal transduction pathways, no functional assignment is made to it unless its post-translational modifications (such as phosphorylation) are dramatically increased or decreased. Furthermore, even when the interacting partners of the proteins involved in signaling are identified and characterized, the functional significance of newly formed PPIs via ligand stimulation is still missed. Therefore, any optimization of the current technology will be a great improvement. Analysis of protein complexes from signal transduction pathways in terms of size, composition, post-translation modifications, multimerization level, and abundance would also benefit from the introduction of new methods.

Affinity or immunoaffinity purification of protein complexes followed by SDS-PAGE and MALDI-MS or LC-MS/MS is definitely the most frequently used method for identification of PPIs. However, the biggest problem with this approach is that it cannot be adapted for large scale identification of PPIs. Therefore, this approach is not eligible for high throughput identification of PPIs (hopefully in the near future).

BN-PAGE is a good alternative option for simultaneous identification of protein complexes and PPIs. However, although it is a useful method, BN-PAGE has many downsides. A potential problem for BN-PAGE is that protein complexes that have identical molecular mass may co-migrate and subunits from two different protein complexes may be falsely identified as being part of the same protein complex. This pitfall may be solved by adding for those particular protein complexes a pre-purification step to BN-PAGE, known as CN-PAGE. Separation of the gel lane that results from the second dimension in a third dimension (SDS-PAGE) will reveal the subunit composition of each protein complex. Another potential problem includes difficulties in the identification of a protein that is part of a protein complex in BN-PAGE (1D) using Western blotting experiments. This problem may occur because many commercial antibodies are made against only one epitope of a protein and if the epitope is buried inside the protein or protein complex, it may not be identified, thus leading to false negative data. This may happen mainly because the proteins and protein complexes are separated under native conditions that preserve their normal conformation.

MALDI-MS and LC-MS/MS are a good option for identification of the PPIs and scientists from many labs succeeded in analyzing both stable and transient PPIs by their direct analysis. Both MALDI-MS and LC-MS/MS (or electrospray ionization mass spectrometry or ESI-MS, a method that does not use HPLC combined with a mass spectrometer) have been used successfully to analyze and identify individual PPIs. However, we already have the instrumentation required for analysis of PPIs (both MALDI-MS and ESI-MS-based instrumentation), these methods have not yet been used in large scale, high-throughput analysis of PPIs for two reasons: 1) the protein subunits of these PPIs have functional post-translational modifications (PTMs) such as phosphorylation and one modification gives the PPIs a function, while removal of the phosphoryl group will give the PPIs a different function. 2) the second and the most important reason is the still weak bioinformatic support in analysis of PPIs. Even if we have the instruments and expertise to investigate PPIs, we still do not have yet the bioinformatics power to successfully identify the PPIs on a large scale and in high throughput.

Perspectives

Developing new functional proteomics methodology which, in addition to identifying and quantifying proteins, also gives us information about the protein complexes that contain a particular protein and about the size and subunit composition of that particular

protein complex will be a great advancement. Successful establishment of such a method for functional proteomics will hopefully also provide a powerful new approach for studying other key signal transduction pathways. The method could be used to build a comprehensive PPI network involved not only in RTKs pathways, but also in other pathways and stable and transient PPIs. This method is yet to come.

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