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Identification of Posttranslational Modification-Dependent Protein Interactions Using Yeast Surface Displayed Human Proteome Libraries

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Abstract

The identification of proteins that interact specifically with posttranslational modifications such as phosphorylation is often necessary to understand cellular signaling pathways. Numerous methods for identifying proteins that interact with posttranslational modifications have been utilized, including affinity-based purification and analysis, protein microarrays, phage display, and tethered catalysis. Although these techniques have been used successfully, each has limitations. Recently, yeast surface-displayed human proteome libraries have been utilized to identify protein fragments with affinity for various target molecules, including phosphorylated peptides. When coupled with fluorescently activated cell sorting and high throughput methods for the analysis of selection outputs, yeast surface-displayed human proteome libraries can rapidly and efficiently identify protein fragments with affinity for any soluble ligand that can be fluorescently detected, including posttranslational modifications. In this review we compare the use of yeast surface display libraries to other methods for the identification of interactions between proteins and posttranslational modifications and discuss future applications of the technology.

Keywords

Posttranslational modification; Yeast surface display; cDNA library; Phosphopeptide; Src homology 2; Phosphotyrosine binding domains; Plant homeodomain finger; Glycosylation; Lipidation; Sumoylation; Acetylation; Ubiquitination; Methylation

1 Importance of Posttranslational Modification-Dependent Protein Interactions

For many if not most cellular processes, posttranslational modifications (PTMs) play a critical role in signal transmission or functional regulation. More than 200 types of PTM are generated by thousands of cellular enzymes, including, but not limited to phosphorylation, glycosylation, lipidation, sumoylation, acetylation, ubiquitination, and methylation [1]. With recent advances in mass-spectrometry-based proteomics, enormous amounts of data can be rapidly generated. As a result, for several of the most intensively studied PTMs, such as phosphorylation, glycosylation, acetylation, and ubiquitylation, many thousands of modification sites have been accurately mapped [2]. In addition, increasingly sophisticated prediction software continuously generates more putative modification sites [3]. Indeed, current evidence suggests that the majority of eukaryotic proteins are likely to be posttranslationally modified in vivo, and it is almost a certainty that novel forms of

posttranslational modification are yet to be discovered. Thus, the challenge of determining the biological function of each PTM site is vast and will only grow as our ability to identify PTMs outstrips our capacity to analyze their function.

A common mechanism by which PTMs regulate biological functions is by modulating interactions with target proteins, often through specialized PTM-specific binding domains that are present in many types of protein. Protein phosphorylation is by far the most extensively studied PTM and numerous phosphorylation-specific protein-binding domains have been characterized and found to perform critical functions in almost all cellular processes. For example, through specific recognition of phosphotyrosine, SH2 and PTB domains play a crucial role in signal transduction pathways [4]. Similarly, phospho-Ser/Thr-binding domains such as 14-3-3, polo box, FHA, FF, BRCT, WW, WD40, and MH2 are known to regulate cell cycle progression and DNA damage responses [5].

An area of intense study is the regulation of histone function by PTMs, in particular methylation and acetylation, which plays a critical role in cellular processes such as transcription, DNA repair, chromosome segregation, and cell differentiation [6]. Methylation of histone tails at different residues recruits proteins or protein complexes that regulate chromatin activation or inactivation. Several domains that bind to methylated amino acid residues in proteins have been identified, including the plant homeodomain (PHD) finger, the Tudor domain, and the malignant brain tumor (MBT) domain [7].

Another important PTM, ubiquitination, is a critical regulator of cellular signaling pathways that control a wide range of biological processes including protein degradation, endocytosis, DNA repair, autophagy, transcription, immunity, and inflammation [8 – 13]. Ubiquitin signaling is decoded by proteins containing ubiquitin-binding domains (UBDs) and to date more than twenty UBD families have been described, with more continuing to be discovered [14]. It is thought that UBDs facilitate the formation of protein complexes, which might result in signal amplification [14]. In addition to ubiquitination, hundreds of proteins involved in almost all critical cellular functions are subject to posttranslational modification with small ubiquitin-related modifier (SUMO) proteins (termed sumoylation) [15 – 17]. Sumoylated proteins are recognized by proteins containing SUMO-interacting motifs (SIMs) which can promote the formation of protein complexes or modulate the activity or stability of the sumoylated protein [18 – 22]. Despite their importance, relatively few UBDs and SIMs have been identified, suggesting that more work is required in this area to further our understanding of how ubiquitination and sumoylation regulate cellular processes.

Despite the critical importance of PTM-dependent binding proteins in regulating cellular processes, relatively few methods exist that allow efficient and comprehensive identification of proteins or domains that interact with PTMs. Several factors contribute to the challenge of identifying PTM-dependent binding proteins. PTMs are often dynamic and occur on only a small fraction of the cellular protein. This limitation is difficult to overcome as it is challenging to efficiently and specifically direct the incorporation of PTMs into proteins in vivo. In addition, some PTM-dependent protein interactions are weak, making them difficult to identify by existing techniques. As a result, while the rate at which new PTM sites are being discovered is accelerating due to improved proteomic and computational approaches,

our understanding of their detailed biological functions is lagging behind. Thus, there is a critical need for improved methods of identifying PTM-dependent binding proteins. In the following section we discuss and compare various methods that have been utilized for the identification of PTM-dependent binding proteins (summarized in Table 1). We propose that the yeast surface cDNA display approach that we originally described for identification of phosphopeptide binding proteins [23] is highly effective and generally applicable to the identification of novel PTM binding proteins.

2 Affinity Purification from Cellular Lysates Followed by Quantitative Mass Spectrometry

Recently, affinity-based purification using modified peptide probes designed to mimic natural PTMs has been combined with stable isotope labeling with amino acids in cell culture (SILAC) and quantitative mass spectrometry to identify PTM-dependent protein–protein interactions [24, 25]. Peptide probes designed to mimic methylated and phosphorylated histone H3 as well as corresponding unmodified probes were used for affinity-based enrichment of binding proteins from SILAC-labeled nuclear extracts. To improve the recovery of weak interactions, the probes contain a benzophenone moiety, which allows photo-cross-linking. Quantitative mass spectrometry was then used to identify proteins selectively enriched by the modified histone probes. Several known and new proteins that bind methylated and phosphorylated histone H3 were identified [24, 25].

This approach, which the authors termed CLASPI (cross-linking-assisted and stable isotope labeling in cell culture-based protein identification) is flexible, comprehensive and addresses potential sensitivity issues posed by weak interactions with the addition of a cross-linking step. Since new probes mimicking different PTMs can be rapidly generated and tested, the method should be of significant value in identifying new PTM-dependent protein interactions. Disadvantages of the method include possible difficulties in recovering interacting proteins that are low abundance in the cellular lysates, and the inability to distinguish direct from indirect interactions in the primary mass spectrometry data.

3 Protein Microarray-Based Methods

Protein microarrays are a rapidly emerging technology that allows researchers to quickly analyze protein interactions and function in a high-throughput manner while requiring only small amounts of reagent [26 – 28]. Several groups have utilized protein microarrays to identify and characterize PTM-dependent protein interactions. Using protein microarrays constructed with recombinant versions of nearly all human Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains, binding specificity and affinity was analyzed by probing with peptides designed to represent both known and potential sites of tyrosine phosphorylation on several receptor tyrosine kinases, including the four ErbB receptors, FGFR1 and IGF1R [29 – 31]. Using this approach allowed the rapid collection of tens of thousands of binding measurements and resulted in the discovery of many previously unrecognized interactions. In another example of the use of protein microarrays to identify PTM- dependent binding proteins, Kowenz-Leutz et al. identified proteins that bind to the transcription factor C/EBP β in a methylation-dependent manner using array peptide

screening (APS) [32, 33]. Bacterially expressed His-tagged recombinant proteins produced from a cDNA expression library covering one third of the human proteome were immobilized on PVDF membranes and interrogated with methylated and unmethylated control peptides derived from C/EBP β . Numerous interactions were uncovered, confirming the importance of C/EBP β methylation status in modulating interactions with other proteins. Recently, Guzzo et al. used protein microarrays containing ~4,000 human transcription factors to conduct SUMO-binding assays and discovered that MYM-type zinc fingers from the transcription factors ZNF261 and ZNF198 function as SIMs [34].

Microarrays have the advantages of being rapid, flexible, and high-throughput. With modified peptide probes, many different PTMs can be rapidly tested and binding affinities and specificity can be at least semi-quantitatively determined. In addition, in contrast with lysate pulldown-based methods, interactions with naturally low abundance protein targets may be more readily identified and only direct interactions are likely to be recovered. Although the initial start up costs are high due to the need to express and purify large numbers of recombinant proteins, the cost diminishes over time since many experiments can be performed with a small amount of material. One significant issue with protein microarrays is quality control. Since a large number, often thousands, of proteins are being produced in parallel, it is impossible to ensure that they are properly folded and active. In addition, the proteins are often produced in a non-native host expression system, increasing the possibility of improper folding or modification. These factors could lead to the generation of both false negative and false positive results in the screening data. Finally, the way that a protein microarray is constructed limits its discovery potential to the annotated gene/protein space.

4 Phage Display

Phage display is a widely used technique for identifying antibodies against a wide variety of target antigens [35, 36]. Using this versatile technique, highly diverse libraries displaying 10^9 unique antibody clones can easily be constructed and utilized to select antibodies with almost any desired binding property [37 – 42]. Kehoe et al. employed a number of selection strategies to isolate an anti-sulfotyrosine antibody from a single chain Fv (scFv) phage display library [43]. After several rounds of selection followed by the screening of almost 8,000 individual clones, a single scFv capable of recognizing sulfotyrosine in a sequence-independent manner was identified. Based on this result, it is likely that phage antibody display could be utilized to identify antibodies that bind specifically to other types of PTM.

Although phage display has been extensively used for antibody discovery, its use for displaying large numbers of diverse protein types (e.g., the human proteome) and application to identification of PTM-binding proteins has been more limited, possibly due to complications that may arise when expressing mammalian proteins in a bacterial host.

5 Yeast and Mammalian Tethered Catalysis

A modified yeast two-hybrid termed “tethered catalysis” was first described by Guo et al. and utilized to identify proteins that bind specifically to acetylated histone tails [44]. To

create the “bait” for the two-hybrid experiment, fusions consisting of various histone N-terminal tail domains, a histone acetyltransferase domain, and a DNA-binding domain were constructed. The physical linkage between the histone acetyltransferase and its substrate results in the constitutive acetylation of the histone tail domain. As a control, versions with a catalytically inactive mutant histone acetyltransferase were also produced. Standard two-hybrid screens were performed using an activation domain library and three clones were identified that exhibit acetylation-dependent binding to the histone tail. Although relatively few interactions were discovered by this method, it served as an important proof of principle demonstrating that PTMs can be generated in a precisely targeted manner in vivo.

Extending the tethered catalysis concept to a mammalian system, Spektor et al. developed a technique termed “mammalian tethered catalysis” and demonstrated its effectiveness by characterizing both known and novel histone H3 methylation-dependent binding proteins [45]. The initial step in mammalian tethered catalysis is similar to the yeast system, beginning with the construction of a bait expression construct consisting of an epitope tag for affinity purification, a histone H3-derived peptide containing the amino acid residue targeted for methylation, and the catalytic domain of histone methyltransferase G9a fused in tandem. This plasmid and a control plasmid containing a catalytically inactive mutant methyltransferase were transfected into mammalian cells and proteins associated with the fusion protein baits were purified from nuclear lysates by immunoprecipitation. SDS/PAGE analysis revealed a single methylation-dependent band which upon analysis by mass spectrometry was determined to be a truncated form of G9a encompassing the C-terminal region.

In contrast to other methods, which generally utilize reagents modified in vitro, the tethered catalysis approach allows PTMs to be enzymatically produced in vivo, which may provide a more natural target as bait for identifying interacting proteins. As with other lysate pulldown-based methods, low abundance PTM-dependent binding proteins may not be recovered and it will be difficult to distinguish between direct and indirect binding interactions.

6 Yeast Displayed Human Proteome Libraries

The key to overcome many of the limitations of the aforementioned methods is to develop a new technique that allows direct affinity capture using modified ligands and ready identification of the binding protein based on a tight linkage between the protein and its encoding gene. A eukaryotic surface display system such as yeast surface display [46, 47] would meet this requirement. We have constructed large ($>2 \times 10^7$) libraries that display human protein fragments (≈ 100 –400 amino acids) on the yeast surface as C-terminal fusions to the yeast α -agglutinin subunit, Aga2p and successfully used them to identify protein fragments that bind to posttranslationally modified phosphorylated peptides [23], small signaling molecule phosphatidylinositides [48, 49], and monoclonal antibodies [50]. To identify protein fragments the major tyrosine auto-phosphorylation sites of the epidermal growth factor receptor or focal adhesion kinase in a phosphorylation-dependent manner, we incubated the induced yeast proteome display libraries with biotinylated, tyrosine-phosphorylated peptides derived from the major autophosphorylation sites of either EGFR

(EGFRpY1173) or FAK (FAKpY397). To compete away non-phosphorylation-dependent binding, corresponding non-phosphorylated, non-biotinylated peptides were added to the incubations in excess. Binding clones were enriched through several rounds of FACS and individual yeast clones were screened by FACS for phosphorylation-dependent binding using the EGFRpY1173 or FAKpY397 peptides. Four unique phosphopeptide-binding clones were identified. Clones expressing fragments encompassing the SH2 domains of adapter protein APS and phosphoinositide 3-kinase regulatory subunit 3 (PIK3R3) were recovered from the EGFRpY1173 sort, while clones expressing the SH2 domains of SH2B and tensin as well as the APS clone were recovered from the FAKpY397 sort. These clones bind the tyrosine-phosphorylated peptides but not the non-phosphorylated control peptides, thereby validating the utility of this approach for identifying PTM-dependent binding proteins.

Yeast human proteome display libraries provide a powerful and flexible tool for identifying protein fragments with affinity for almost any soluble target ligand, and are particularly well suited for the study of PTM-dependent protein interactions. Since yeast protein expression pathways are similar to those found in mammalian cells, human protein fragments displayed by yeast human proteome libraries are likely to be properly folded and functional. As with protein microarrays, interactions with naturally low abundance protein targets may be more readily identified and only direct interactions are recovered. In contrast with protein microarrays, yeast human proteome display libraries can be generated relatively quickly at low cost and are an easily renewable resource [51, 52]. When coupled with a compatible method of high throughput nucleic acid analysis (e.g., exon microarrays or next generation sequencing), selection outputs can be comprehensively screened, allowing the discovery of a greater diversity of interactions [49]. Although expressed in a eukaryotic host, it is possible that some proteins may not be folded or modified properly, which may lead to the generation of false negative or false positive results. False negative results may also result from incomplete library coverage.

7 Future Directions

Given the importance of PTMs in regulating biological processes, and the speed at which new PTMs are being cataloged, it is important to accelerate efforts to identify and characterize PTM-dependent binding proteins in order to understand the underlying biology. Each of the methods described above has strengths and weaknesses, but novel techniques such as yeast surface cDNA display appear to be well suited for this task. Combined with data mining and bioinformatics, these discovery methods should improve our understanding of the role that PTM plays in normal and diseased cells. Technology aside, the choice of the bait ligand (PTM in the context of a biologic) will ultimately impact the significance of any discovery of PTM-binding proteins.

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Table 1

Summary of methods utilized for identifying PTM-dependent protein interactions

Method	Main advantages	Main disadvantages	References
Affinity purification/mass spectrometry	Comprehensive (whole cell lysate is interrogated) Increased ability to detect weak interactions with cross-linking	Difficult to identify low abundance proteins Unable to distinguish between direct and indirect interactions	[24, 25]
Protein microarray	Fast Ability to identify low abundance proteins Direct interactions recovered	High start up cost Protein production quality control issues	[29–34]
Phage display	Large library size Direct interactions recovered	Complications displaying diverse mammalian protein libraries in bacterial expression system	[43]
Tethered catalysis	PTMs generated in vivo may be more natural	Difficult to identify low abundance proteins Unable to distinguish between direct and indirect interactions	[44, 45]
Yeast human proteome display	Ability to identify low abundance proteins Direct interactions recovered Comprehensive high throughput analysis of selection outputs	Potential improper protein folding on the yeast surface Incomplete library coverage	[23, 49, 52]