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Utilizing Yeast Surface Human Proteome Display Libraries to Identify Small Molecule-Protein Interactions

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Abstract

The identification of proteins that interact with small bioactive molecules is a critical but often difficult and time-consuming step in understanding cellular signaling pathways or molecular mechanisms of drug action. Numerous methods for identifying small molecule-interacting proteins have been developed and utilized, including affinity-based purification followed by mass spectrometry analysis, protein microarrays, phage display, and three-hybrid approaches. Although all these methods have been used successfully, there remains a need for additional techniques for analyzing small molecule-protein interactions. A promising method for identifying small molecule-protein interactions is affinity-based selection of yeast surface-displayed human proteome libraries. Large and diverse libraries displaying human protein fragments on the surface of yeast cells have been constructed and subjected to FACS-based enrichment followed by comprehensive exon microarray-based output analysis to identify protein fragments with affinity for small molecule ligands. In a recent example, a proteome-wide search has been successfully carried out to identify cellular proteins binding to the signaling lipids PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃. Known phosphatidylinositide-binding proteins such as pleckstrin homology domains were identified, as well as many novel interactions. Intriguingly, many novel nuclear phosphatidylinositide-binding proteins were discovered. Although the existence of an independent pool of nuclear phosphatidylinositides has been known about for some time, their functions and mechanism of action remain obscure. Thus, the identification and subsequent study of nuclear phosphatidylinositide-binding proteins is expected to bring new insights to this important biological question. Based on the success with phosphatidylinositides, it is expected that the screening of yeast surface-displayed human proteome libraries will be of general use for the discovery of novel small molecule-protein interactions, thus facilitating the study of cellular signaling pathways and mechanisms of drug action or toxicity.

Keywords

Yeast cell surface display; cDNA library; Small molecule-protein interaction; Drug-binding protein; Small signaling molecule-binding protein; Phosphatidylinositides; Chromatin remodeling; Transcription regulation; Homeobox domain-containing protein

1 Methods for Identifying Small Molecule-Interacting Cellular Proteins

Small bioactive molecules are important regulators of many diverse cellular processes and their biological activity is usually dependent on specific interactions with cellular protein targets. Thus, identifying proteins that interact with small molecules is a critical but often challenging and rate-limiting step in understanding cellular signaling pathways or molecular

mechanisms of drug action. This is particularly true in the drug discovery process, where a comprehensive identification of all protein targets is necessary for a thorough understanding of the mechanisms behind a drug's beneficial or potentially deleterious effects.

The most popular approach for identifying small molecule-protein interactions relies on the affinity-based purification of interacting proteins from cellular lysates using an immobilized small molecule target, followed by mass spectrometry analysis [1–4]. For example, this method, termed “chemical proteomics,” has been applied to the identification of cellular targets of the anti-cancer kinase inhibitor drugs gefitinib [5], SU6668 [6], imatinib [7], dasatinib [7], and bosutinib [7], and was recently utilized to identify the E3 ubiquitin ligase complex component CRBN as the primary target of thalidomide teratogenicity [8]. Although this method has been extensively and successfully utilized to identify small molecule-interacting proteins, it is biased towards identifying strong interactions with highly expressed proteins and has difficulty identifying cellular targets expressed at low levels in the queried lysates. It is also less than straightforward using this method to detect transient but meaningful biological interactions. Additionally, mass spectrometry analysis often generates a large number of candidate proteins, including those that do not bind to the bait ligand directly, making validation a challenging task.

Protein microarray technology has great potential for the detection and study of protein-small molecule interactions, in addition to other applications [9–11]. Protein microarrays containing thousands of purified recombinant proteins can be produced and subsequently assayed in a high-throughput manner using fluorescently labeled ligands. The technique has been successfully employed to identify and analyze proteins that bind to several small molecule targets, including phospholipids [12], GTP [13], small molecule inhibitors of rapamycin [14], and a potential small molecule therapeutic agent for Spinal Muscular Atrophy [15]. Although having enormous potential, the application of protein microarrays has been limited by incomplete proteome coverage and the difficulties and high cost of producing high quality protein arrays containing thousands of functional proteins.

Phage display has found widespread use for identification of antibodies binding specifically to a bait molecule (often a protein). It can be adopted for cDNA library screening based on direct affinity interaction to uncover cellular proteins binding to a bait ligand. In phage display, very large libraries can be generated and selected and since the genotype and phenotype are physically linked, target identification is achieved by sequencing which is rapid and cheap compared to the mass spectrometry and large-scale protein production which are required for chemical proteomics and protein microarray approaches, respectively. Since the early successful identification of cellular proteins that bind the small molecule drugs taxol and doxorubicin [16, 17], a large number of small molecule-interacting cellular proteins have been identified by affinity-based selection from phage cDNA and peptide display libraries [18–31]. Nevertheless, despite these successes, the recovery of some interactions may be limited by potential expression bias against eukaryotic proteins expressed in a prokaryotic host, which could lead to a high rate of both false positive and negative results and the low number of fusion proteins displayed on each phage particle.

An adaptation of the popular eukaryotic yeast two-hybrid system has been developed, termed “three-hybrid,” in which a target small molecule is derivatized with an anchor that allows it to form a complex with the bait fusion protein [32]. Since its development, the three-hybrid method has been successfully used to identify many protein-small molecule interactions [33, 34]. Very recently, the three-hybrid approach has been used to identify proteins that bind to the drugs erlotinib, atorvastatin, and sulfasalazine [35], the glaucoma therapeutic anecortave acetate [36], and a panel of anti-tuberculosis drugs [37]. Although these results are promising, a potential drawback of the three-hybrid technique is the requirement for the production of a derivatized small molecule ligand that must be cell permeable and able to gain access to the nucleus. In addition, like the two-hybrid system, the three-hybrid system also faces issues such as false positives and the need for an independent validation of a direct physical interaction.

An emerging approach for identifying protein-small molecule interactions is yeast surface display, which allows the efficient display of heterologous proteins on the surface of *Saccharomyces cerevisiae* yeast cells [38]. Large and diverse yeast display human cDNA libraries can be easily generated, and when coupled with fluorescence-activated cell sorting (FACS) these libraries can theoretically be used to identify protein fragments that interact with any soluble molecule that can be fluorescently detected. Using FACS, yeast surface-display libraries can be screened at a rate of up to 50,000 cells/s, allowing the full diversity of large (10^8) libraries to be practically screened. In addition, FACS-based selection allows real time monitoring of the selection process and fine discrimination of clones with different binding properties. Target ligand concentrations and other incubation conditions can be adjusted to enrich clones with different affinities or characteristics. These flexibilities are not readily available to other screening methods such as three-hybrid assays and phage display. The selection outputs are observed to be diverse and not excessively dominated by a few high affinity binders. This is in contrast to methods that are based on affinity purification of target proteins directly from cell lysates, which are strongly biased towards the recovery of highly abundant cellular proteins and high affinity interactions.

We have previously described the construction and application of yeast surface-displayed human protein fragment libraries to identify human proteins that interact with various target ligands, including small molecules [39–44]. Below we will summarize highlights of our experiments using yeast surface-displayed human proteome libraries combined with comprehensive exon microarray-based analysis to identify human proteins that interact with phosphatidylinositides [40, 44], a class of cellular lipids that play an important but poorly characterized role in many cellular processes. In particular, we will discuss the potential significance of nuclear phosphatidylinositide-binding proteins we identified and suggest directions for future research efforts.

2 Phosphatidylinositide-Binding Proteins and the Mystery of Nuclear Phosphatidylinositides

Phosphatidylinositides are specialized lipids that make up a small fraction of the total cellular lipid pool and function as important regulators of many processes, including signal

transduction, ion channels and transporters, vesicle trafficking, cytoskeletal organization, and motility [45, 46]. The various biological functions of phosphatidylinositides are mediated through association with proteins containing phosphatidylinositide-binding globular domains (such as PH, C1, FYVE, and PX domains) [47], or unstructured regions with clusters of basic and hydrophobic residues [48]. The discovery of additional protein domains or regions that bind to phosphatidylinositides is important for understanding the mechanisms by which they regulate cellular processes. Various techniques have been utilized to identify proteins that bind phosphatidylinositides (reviewed in [49]), including protein microarrays [12], affinity purification [50], neomycin nuclear extraction [51], a yeast growth rescue assay [52], λ gt11-based expression cloning [53], quantitative mass spectrometry [54], and an in vitro transcription/translation-based expression cloning assay [55]. In addition to cytosolic membrane phosphatidylinositides, an independent pool of phosphatidylinositides and enzymes involved in their synthesis exists in the nucleus. Although significantly less is known about the functions, mechanism of action, and physical form of nuclear phosphatidylinositides, they are now known to be involved in the regulation of many important biological processes, such as cell cycle progression, chromatin organization, transcription, DNA repair, and RNA splicing and export (reviewed in [56–59]). To understand the mechanisms by which nuclear phosphatidylinositides regulate cellular processes, it is necessary to identify nuclear phosphatidylinositide-binding proteins. Although the presence of nuclear phosphatidylinositides has been known for many years, only a few nuclear proteins that bind phosphatidylinositides have thus far been identified, including histones H1 and H3 [60], the PHD finger of ING2 [61], the nuclear receptors SF-1 and LRH-1 [62–64], Nucleophosmin/B23 [50], the PDZ domains of syntenin-2 and zonula occludens-1 and -2 [65], SAP30L and SAP30 [66], the transcriptional corepressor BASP1 [67], and RNA polymerase I and the Pol I transcription factor UBF [68]. Recently, a method specifically designed to enrich for nuclear phosphatidylinositide-interacting proteins based on neomycin extraction of intact nuclei was successfully utilized to identify many nuclear phosphatidylinositide-binding proteins, and a direct interaction between phosphatidylinositol 4,5-bisphosphate and DNA Topoisomerase II α was verified [51]. Despite these successes, new methods are required to efficiently and comprehensively identify novel phosphatidylinositide-binding proteins.

3 Comprehensive Analysis of Yeast Display Human Proteome Library Selection Outputs Identifies Novel Phosphatidylinositide-Binding Nuclear Proteins

We utilized biotinylated phosphatidylinositides for FACS-based enrichment of yeast surface-displayed human proteome libraries coupled with comprehensive output analysis by exon microarray to identify phosphatidylinositol-binding proteins [40, 44]. Intriguingly, many of the novel phosphatidylinositide-binding proteins we discovered are nuclear (summarized in Table 1). Four of the nuclear phosphatidylinositide-binding proteins (HOXA5, HOXB6, HOXC6, and HOXD4) contain a homeobox domain (Table 1), which is a sequence-dependent DNA binding domain found in transcription factors [69]. Follow-up experiments demonstrated that phosphatidylinositides and homeobox domain target DNA sequences bind

competitively to homeobox domain-containing protein fragments [44]. We speculate that nuclear phosphatidylinositides may regulate transcription by modulating the interaction between homeobox domain proteins and target DNA sequences (Fig. 1a), similar to a model proposed for the Sin3A corepressor complex proteins SAP30 and SAP30L [66]. Future experiments will be required to define the potential role that nuclear phosphatidylinositides play in the regulation of homeodomain transcription factors. The identification of mutations within the homeobox domain that selectively disrupt phosphatidylinositide binding without affecting DNA binding would be extremely beneficial to this endeavor.

Three of the novel phosphatidylinositide-binding proteins we identified and verified (FAM71B, RNPS1, and SFRS4) and one unverified candidate (SRRP35) localize to nuclear speckles (Table 1) [70–73]. Interestingly, although the exact nature and location of nuclear phosphatidylinositides is currently unclear, evidence suggests that they may at least partially reside in distinct nuclear domains termed “speckles” [74–76], which are subnuclear structures enriched in pre-mRNA splicing factors [77]. The PDZ domains of syntenin-2 and zonula occludens-1 and -2 bind to phosphatidylinositides and regulate their localization to nuclear speckles [65, 78]. Thus, FAM71B, RNPS1, SFRS4, and SRRP35 may similarly be localized to nuclear speckles through binding to phosphatidylinositides.

One of the phosphatidylinositide-binding proteins we identified is the DNA polymerase POLS (Table 1). Trf4p, the yeast homolog of POLS, is important for sister chromatid cohesion [79] and DNA double-strand break repair [80]. Notably, the phosphoinositide 3-kinase catalytic subunit p110 β , its substrate, PtdIns(4,5), and its product, PtdIns(3,4,5), have been shown to localize to DNA double-strand breaks [81]. Thus, it is possible that phosphatidylinositides at DNA damage sites recruit POLS to facilitate DNA repair or directly regulate the enzymatic activity of POLS (Fig. 1b).

Another phosphatidylinositide-binding protein is HMGN2 (Table 1), a member of the nonhistone chromosomal HMGN protein family. HMGN proteins bind to nucleosome core particles without any DNA sequence specificity [82] and cause unfolding of higher-order chromatin structure to facilitate transcription [83–85] and replication [86]. Since phosphoinositide-mediated modulation of protein-chromatin interactions has been proposed as a general biological mechanism for transcriptional regulation [66], it is possible that phosphatidylinositide binding to HMGN2 contributes to this regulation.

Proteins and lipids are some of the most abundant components in a cell, and current experimental data support the hypothesis that many biological events taking place in a living cell are mediated by protein-lipid interactions. The further identification and characterization of nuclear phosphatidylinositide-binding proteins will greatly advance our understanding of the molecular mechanisms by which nuclear phosphatidylinositols carry out their many important functions. In addition, targeted functional and biochemical studies are needed to understand the mechanisms by which phosphatidylinositides regulate important functions such as transcription and DNA repair through protein-phospholipid interactions.

4 Future Directions

While there are many techniques that have found use in identifying cellular proteins binding to small molecule ligands, yeast surface human cDNA display technology has emerged as a highly effective method for identifying novel interactions as it overcomes many limitations associated with other approaches. When coupled to FACS-based selection, the yeast display method can identify specific protein-small molecule interactions across varying affinity ranges. Comprehensive interrogation of selection outputs by either exon array or deep sequencing allows rapid identification of candidate binding proteins whose binding to the small molecule target ligand can be easily verified. The source of the cDNA used for yeast display library construction can also be expanded to increase proteome representation to further improve the chance of identifying rare but meaningful protein-small molecule ligand interactions. The challenge now is how to use the knowledge of these binding interactions to construct a biological understanding of cell signaling and drug actions.

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Fig. 1. Models for regulation of nuclear functions by phosphatidylinositides. **(a)** Because of the demonstrated binding of phosphatidylinositides to the homeodomain [44], nuclear phosphatidylinositides have the potential to prevent binding of transcription factors such as homeodomain proteins to homeobox target sequences (*blue box*) or displace bound homeodomain proteins from DNA, leading to altered gene expression. Homeodomain proteins have the potential to either positively or negatively regulate target genes—the drawing only depicts one of the scenarios (positive regulation). **(b)** Phosphatidylinositides accumulated at sites of double-strand DNA breaks act to recruit POLS, which participates in repairing the break. The figure was adapted from the original drawing presented in [44]

Table 1

Nuclear phospholipid-binding protein fragments identified by our yeast surface display human cDNA library screening [44]

Protein	Accession #	Subcellular location	Binding verified
ATN1	EAW88707	C, N	+
FAM71B	NP_570969	N, NS	+
HMG2	CAG46919	C, N	+
HOXA5	CAG47073	N	+
HOXB6	AAH14651	N	+
HOXC6	CAG33235	N	+
HOXD4	EAX11086	N	+
NUCKS1	Q9H1E3	N	+
PNPLA7	CAI14582	ER, IM, L, N	+
POLS	Q5XG87	N	+
RNPS1	AAC39791	N, NS, C	+
SBF1	AAH87612	N, IM	+
SFRS4	CAI14326	NS	+
HCFC1	NP_005325	C, N	
SRRP35	EAW48561	N, NS	
PARK7	NP_009193	C, N	
SART1	NP_005137	N	
SLC9A3R2	NP_004776	M, C, N	

Accession # is based on NCBI Entrez protein database. The observed or predicted subcellular localization of the protein is indicated. *ER* endoplasmic reticulum, *IM* integral membrane, *M* membrane, *L* lysosome, *N* nucleus, *C* cytoplasm, *NS* nuclear speckle. *aa* amino acids. The table was adapted from tables originally presented in [44]