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Yeast surface display for protein engineering and characterization

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Yeast surface display is being employed to engineer desirable properties into proteins for a broad variety of applications. Labeling with soluble ligands enables rapid and quantitative analysis of yeast-displayed libraries by flow cytometry, while cell-surface selections allow screening of libraries with insoluble or even as-yet-uncharacterized binding targets. In parallel, the utilization of yeast surface display for protein characterization, including in particular the mapping of functional epitopes mediating protein–protein interactions, represents a significant recent advance.

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Introduction

Frequently, improvements in protein function are sought with respect to binding affinity, catalytic activity, and/or structural properties. Given our limited, albeit expanding, understanding of protein sequence/function relationships, achieving the desired improvements through rational protein design is still difficult. However, approaches involving random mutagenesis and directed evolution have been applied with great success for obtaining proteins with defined characteristics.

Yeast surface display is a particularly powerful platform for engineering proteins by directed evolution. Since its introduction 10 years ago [1], yeast surface display has been used to engineer a variety of proteins for improved affinity, specificity, expression, stability, and catalytic activity. A significant feature of the yeast surface display system is its employment of a eukaryotic host possessing the secretory biosynthetic apparatus for promoting efficient oxidative protein folding and N-linked glycosylation. As such, a

diverse assortment of proteins has been successfully displayed on the surface of yeast, enabling their subsequent engineering by yeast surface display. As shown in Figure 1, these include growth factors, antibody fragments, and complex cell-surface receptors such as epidermal growth factor receptor (EGFR), demonstrating the complexity of proteins amenable to engineering by yeast display. However, although well suited to biosynthesis of secreted eukaryotic proteins, the yeast secretory pathway is likely to inefficiently express some cytoplasmic or nuclear proteins due to the presence of multiple reduced cysteines (*e.g.* zinc finger proteins). Other key beneficial attributes of yeast display include: rapid and quantitative library screening by fluorescence-activated cell sorting; minimization of artifacts due to host-expression-bias through concurrent expression labeling; and convenient evaluation of mutant characteristics (*e.g.* affinity, stability) in surface-displayed format without soluble expression and purification of each individual clone [2•].

Equipped with these features, yeast surface display is now a well-established method for protein directed evolution. As illustrated in Figure 2, the number of published studies employing yeast surface display is currently in an exponential growth phase. A recent direct comparison of the yeast and phage display systems, using identical immune antibody libraries and target antigens, also found yeast display to sample the library repertoire 'considerably more fully' while being 'less labor-intensive' [3••]. Notably, of the 12 novel clones identified by yeast display, only five were functional when subcloned and displayed on phage, although 11 could be expressed solubly in functional form in *Escherichia coli* [3••].

Here we review recent applications of yeast surface display, highlighting its role in both protein engineering and characterization. We also discuss recent methodological developments, including new techniques for library screening, that have further expanded the utility of this display platform.

Identifying protein–protein interactions

Recently, several groups used yeast surface display to identify natural protein–protein interactions. For instance, yeast surface display was employed for a proteome-wide search of proteins that interact with either EGFR or focal adhesion kinase, in a tyrosine phosphorylation-dependent manner [4]. By displaying a human cDNA library on the surface of yeast and screening with synthetic phosphopeptides, the authors identified several interactions previously unreported [4]. Renner

Figure 1
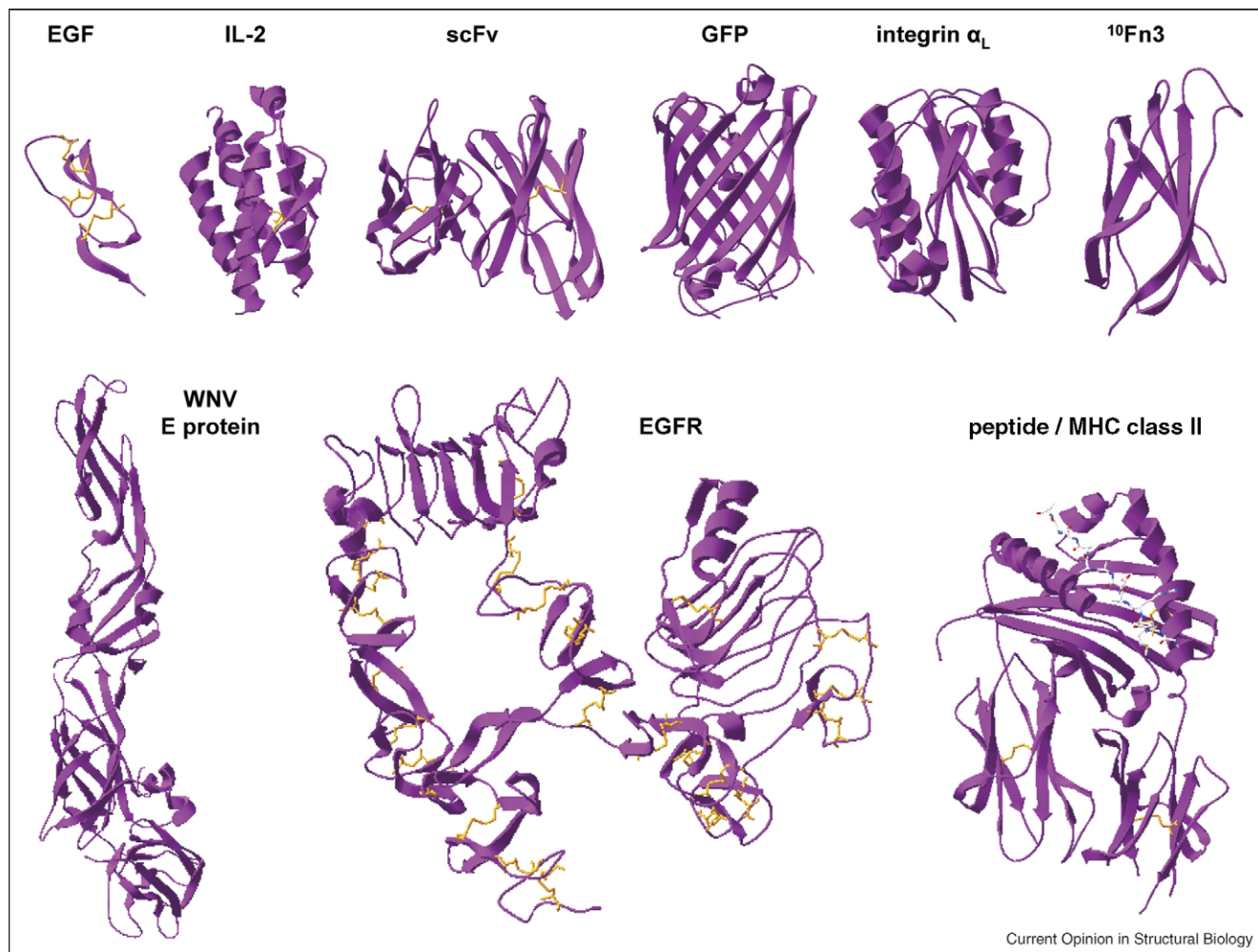
A selection of proteins successfully displayed as Aga2p fusions on the surface of yeast. Top row, left to right: human epidermal growth factor [31••] (1JL9), human interleukin-2 [61] (2B51), single-chain antibody fragment 4m5.3 [62] (1X9Q), green fluorescent protein [63] (1EMA), human α_L integrin inserted domain [19] (1LFA), and human fibronectin [18] (1FNA). Bottom row, left to right: West Nile Virus envelope protein [32•] (2I69), human EGF receptor ectodomain [27] (1NQL), and human MHC class II HLA-DR4αβ in complex with peptide [64] (2SEB). The PDB IDs for the structures shown are noted in parentheses. This figure was generated using Swiss-Pdb Viewer [65].

and colleagues recently used yeast-displayed tumor antigens to assess tumor-specific antibody responses in cancer patients [5,6] and have also screened a yeast-displayed cancer-patient cDNA library for novel tumor antigens [7]. Notably, the screening of yeast-displayed libraries revealed many tumor antigens not previously detected from prokaryote-displayed libraries [7]. However, given the formal possibility that mimotopes could be selected due to expression of frameshifted peptides, confirmation of antisera binding to expressed gene products is necessary following such screens. In addition, several groups have isolated novel lead antibodies binding to a variety of targets, from immune [8] or nonimmune [9–12] human single-chain variable fragment (scFv) libraries.

Improving affinity and engineering specificity

Affinity and specificity are key parameters governing a protein's function as a diagnostic or therapeutic agent, and yeast surface display has been widely applied for improving or altering these binding properties. Recently, the affinity maturation of several scFvs [13,14] was reported. More notably, yeast surface display has been employed to selectively expand or restrict binding specificity. For example, after affinity maturing several scFvs against botulinum neurotoxin type A1 [15], Marks and colleagues were able to broaden the specificity of the most potent clone, using a dual-selection strategy, to achieve high-affinity binding to type A2 while retaining high-affinity binding to type A1 [16••]. Conversely, Weaver-Feldhaus et al. engineered conformational specificity
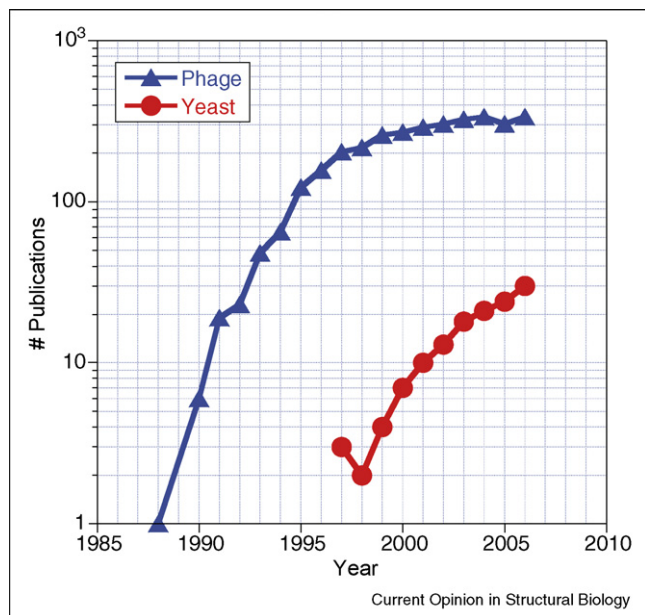
Comparison of numbers of studies employing phage or yeast display.

into calmodulin-binding scFvs, obtaining clones that recognize only one of the Ca^{2+}-free or Ca^{2+}-bound calmodulin forms [10].

In addition to antibody fragments, yeast surface display has been used to affinity mature proteins with a variety of other folds. Two groups recently reported engineering the 10th human fibronectin type III domain (^{10}Fn3) alternative scaffold, for high-affinity binding to model antigens such as maltose-binding protein [17$^{••}$] and lysozyme [18]. Jin *et al.* have also applied yeast surface display to evolve the α_L integrin inserted domain, achieving a 200,000-fold increase in ligand-binding affinity [19]. Aside from reporting a remarkable affinity improvement, this study demonstrates the utility of yeast surface display for probing protein allostery, as the mutations responsible for improving affinity locate to a region proposed to control protein conformation [19]. Other molecules recently engineered for improved affinity by yeast display include EGF [20] and single-chain T-cell receptors (scTCRs) [21,22$^{••}$,23$^{•}$].

A notable advantage to engineering affinity by yeast surface display is the ability to characterize isolated mutants directly in display format. This feature eliminates the need for soluble expression and purification of individual clones and becomes especially significant when many clones require characterization. Importantly, as shown in Figure 3, the equilibrium dissociation constants measured through titration of proteins on yeast have, to date, shown consistency with those measured by a variety of other methods.

Increasing stability and expression

Thermal stability and soluble expression level are often critical parameters determining a protein's practical utility. Protein stability affects shelf life and suitability for applications at elevated temperatures. Concurrently, a protein's expression level strongly influences its cost of production.

The display level of a protein on the surface of yeast, as a fusion to the yeast agglutinin protein Aga2p, has been shown to correlate with both thermal stability as well as soluble expression level [24], enabling engineering of protein stability and expression by yeast surface display. Several groups have employed the procedure of random mutagenesis, yeast display induction, and high-display screening to improve the stability and expression of proteins, including scTCRs [21,22$^{••}$], major histocompatability complex (MHC) class I molecule H-2L^d [25], tumor antigen NY-ESO-1 [26], and the ectodomain of EGFR [27]. In all cases, stability engineering enabled subsequent soluble expression in bacteria [21,22$^{••}$,25] or yeast [26,27], where efforts for the wild-type version had been unsuccessful. However, this stability engineering approach may not be suitable in all cases, as display levels did not correlate with stability for artificial proteins of particularly high thermal stability [28].

Improvement of soluble expression levels can also be achieved through engineering the expression host instead of the protein itself. Here, yeast display allows the establishment of the critical link between the desirable high-secretion phenotype and its causative genotype. Wentz and Shusta recently employed yeast surface display to screen a yeast cDNA library for yeast genes whose over-expression improved the yield of scFvs and scTCRs [29]. The authors found Aga2p fusion to alter the secretory processing of the heterologous protein, necessitating the performance of screens under conditions where the dominant determinant of display level is the heterologous protein and not Aga2p [29]. Nonetheless, they identified several genes whose over-expression enhanced secretion even in the absence of Aga2p. An approach that eliminates the potential artifacts resulting from Aga2p fusion but that still maintains the link between secretory phenotype and genotype has been reported by Rakestraw *et al.* [30]. Here, yeast are first tagged with the target protein's binding partner, which subsequently captures the protein of interest as it is secreted by the cell [30]. Mathematical modeling and experimental observations indicate that this selection method is capable of distinguishing subtle differences in secretion level and also possesses a sizable time window for library screening [30]. In addition, since the binding partner is used as bait, only clones secreting well-folded proteins are selected.

Figure 3
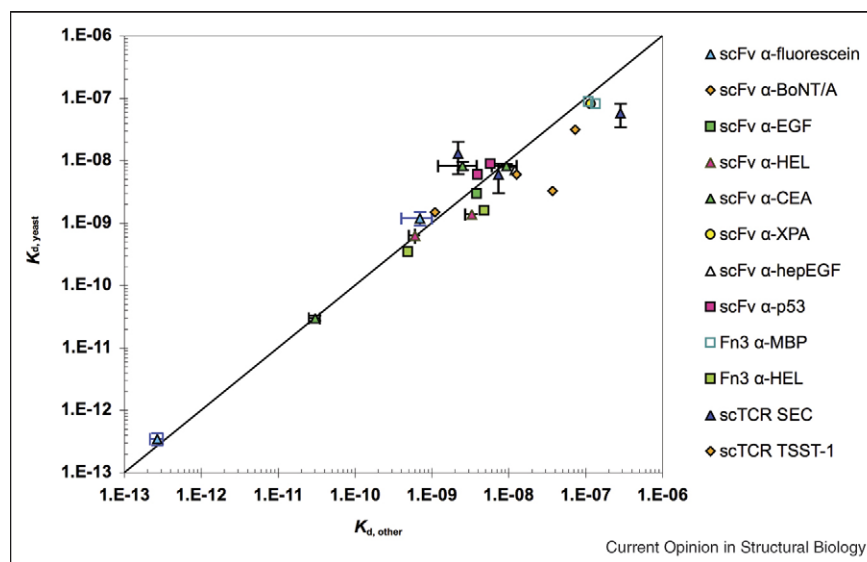
Comparison of dissociation constants determined by yeast surface display, $K_{d\ YSD}$, and other methods, $K_{d\ other}$. scFv binding to fluorescein; $K_{d\ other}$ determined by fluorescence quenching. scFv binding botulinum neurotoxin type A; $K_{d\ other}$ determined by surface plasmon resonance (SPR) [15]. scFv binding to carcinoembryonic antigen (CEA); $K_{d\ other}$ determined by titration of soluble scFv against mammalian-cell-surface-expressed CEA (Michael M Schmidt, unpublished results). scFv binding to hen egg white lysozyme (HEL); $K_{d\ other}$ determined by fluorescence quench titration. scFv binding to p53 peptides; $K_{d\ other}$ determined by SPR. scFv binding to EGF; $K_{d\ other}$ determined by SPR. scFv binding to heparin-binding EGF; $K_{d\ other}$ determined by SPR. scFv binding to xeroderma pigmentosum-complementing protein group A; $K_{d\ other}$ determined by SPR. Fibronectin binding to HEL; $K_{d\ other}$ determined by equilibrium competition titration using purified fibronectin mutants [18]. Fibronection binding to maltose-binding protein; $K_{d\ other}$ determined by SPR [17••]. scTCR binding staphylococcal enterotoxin C3; $K_{d\ other}$ determined by SPR. scTCR binding toxic shock syndrome toxin-1; $K_{d\ other}$ determined by SPR [21]. Figure modified from Figure 8 of Lipovsek et al. [18].

Mapping functional protein epitopes

Identifying the key residues that mediate protein–protein interactions provides insight into biological processes and can also facilitate protein design. Recently, the yeast surface display platform was adapted for identifying such residues in a systematic and high-throughput manner. Specifically, as demonstrated by Chao et al. for EGFR and several anti-EGFR antibodies, screening yeast-displayed libraries of the antigen yields epitope maps with residue-level resolution [31••]. Notably, this technique enables the identification of discontinuous and conformational epitopes. Also, it interrogates protein–protein interactions more comprehensively than alanine scanning. Indeed, Chao et al. noted several energetically important residues for which alanine scanning would have yielded false negative results [31••].

Several other groups have applied this technique for the characterization of antibody–antigen interactions. For instance, Diamond and colleagues used this approach to determine the antigenic epitopes recognized by various antibodies and scFvs capable of neutralizing West Nile Virus [32•,33–36]. Importantly, for antibody E16, whose structure in complex with its ligand was determined, the results of yeast-display epitope mapping studies [32•,33] were validated by crystallographic data [33]. Other anti-

bodies recently analyzed by this method include those against botulinum neurotoxin [37], the B and T lymphocyte attenuator [38], the nucleocapsid protein of severe acute respiratory syndrome coronavirus [39], and, in a case of serendipity, NY-ESO-1 [26]. While the examples cited here all represent antibody–antigen interactions, this technique should be applicable for dissecting protein–protein interactions in general. However, a potential limitation is its requirement for an independent means of verifying proper antigen folding.

Engineering proteins against insoluble or unknown targets

One of the key advantages to engineering and characterizing proteins by yeast surface display is the ability to analyze large populations rapidly and quantitatively by flow cytometry. However, this approach requires a soluble ligand that is not always available. Recently, this limitation was addressed by two new screening methods, both employing intact mammalian cells [40••,41••].

First, Wang and Shusta reported screening yeast-displayed libraries against integral membrane targets by monolayer panning [40••]. Here, desirable clones become selectively enriched by virtue of their affinity for the cell-surface target, while low-affinity clones are washed away

from the monolayer [40$^{\bullet\bullet}$]. Several selection conditions, such as wash stringency and ligand density, can affect the success of this approach and were thoroughly investigated for a model system [40$^{\bullet\bullet}$]. This approach enabled the isolation from a nonimmune human scFv library of clones that recognize antigens expressed on the surface of brain endothelial cells [11]. Notably, the identities of the cognate antigens were not defined or known at the outset of selection. However, the isolated scFvs, in yeast-display format, allowed these antigens to be immunoprecipitated from cell lysates for further characterization [11]. Belcher and colleagues have employed an analogous panning approach to screen libraries of yeast-displayed scFvs and peptides for ones possessing high affinity and specificity for inorganic materials such as cadmium sulfide [12,42] and sapphire [43].

Independently, Kranz and colleagues have developed a cell-surface screening method using density centrifugation [41$^{\bullet\bullet}$]. This approach takes advantage of the differential sedimentation characteristics of yeast and mammalian cells, which, upon centrifugation, sediment through or settle above the density medium Ficoll-Paque, respectively. As yeast expressing high-affinity variants can form conjugates with mammalian cells, they are selectively enriched through retention in the upper layer [41$^{\bullet\bullet}$]. This method has enabled the isolation of high-affinity TCR mutants specific for either class I or class II MHC, which are particularly difficult to solubilize [41$^{\bullet\bullet}$].

Immobilized proteins and enzymes

The recent literature also includes reports of protein immobilization by yeast surface display. Analogous to covalent linkage of proteins to solid beads, such immobilization offers proteins a physical support that often improves stability and facilitates reusability. However, unlike bead conjugation, yeast display does not require additional steps of protein purification and immobilization.

Recent examples of yeast surface immobilized proteins include metal-binding metallothioneins, which sequester toxic cadmium ions [44], as well as streptavidin [45] and protein A [46], which enable capture of desired soluble proteins. Mutants of the integrin α_L inserted domain have also been displayed on the surface of yeast, for investigating the effects of ligand-binding affinity on cell adhesion and rolling [47]. In addition, several groups have proposed using antigen-displaying yeast as preventative or therapeutic vaccines [48–50].

A host of enzymes have been functionally displayed on the surface of yeast, including lipase [51,52], biotin ligase [53], organophosphorous hydrolase [54], carboxylesterase [55], epimerase [56], cyclodextrin glucanotransferase [57], and neurolysin [58]. While efforts to engineer mutants with improved catalytic activity have been reported

[51,52], systematic and high-throughput examples of catalysis engineering have yet to be described. The use of yeast surface display for engineering enzymes and their substrates will be an interesting direction for the future.

Conclusion

In summary, yeast surface display facilitates efforts to engineer proteins with defined characteristics, and can also provide valuable quantitative information regarding protein–protein interactions. The eukaryotic nature of the yeast secretory pathway has enabled the study and manipulation of even complex proteins by this method. However, yeast and mammalian glycosylation structures differ, prompting development of human cell display [59]. Nonetheless, yeast display remains suitable for most proteins of interest; for situations where glycosylation differences matter, yeast strains possessing the human glycosylation pathway may also serve as an alternative [60].

Acknowledgements

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