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Mining a Yeast Library for Brain Endothelial Cell-Binding Antibodies

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Abstract

We describe the use of yeast surface display for the identification of antibodies that bind the plasma membranes of living cells. Yeast panning with a nonimmune human single-chain antibody library identified 34 unique lead antibodies that bind ($K_d = 82 \pm 15$ nM) and in some cases internalize into rat brain endothelial cells. In addition, a novel yeast display immunoprecipitation procedure was employed for initial characterization of the cognate antigens.

Main Text

The ability to merge antibody display libraries with intact cells or tissues for the identification of antibodies that bind to cell membranes has the potential to enable a wide range of applications that include membrane proteomics, selective tissue targeting and intracellular delivery of therapeutic payloads. Such cell-based screening of antibody libraries has been successful in identifying antibodies that target tumors¹⁻³, vascular beds⁴, and cellular endocytosis systems^{2, 5}. The vast majority of these selections have employed phage display techniques, while cell-based selections using alternative display platforms have lagged behind⁶. However, we recently demonstrated that yeast display possesses beneficial attributes such as low levels of non-specific interaction and multivalent display that could in principle provide the basis for efficient cell surface selections from synthetic or nonimmune antibody libraries⁷.

To validate the applicability of yeast surface display for cell surface antibody selections, we screened a nonimmune yeast display library consisting of $\sim 10^9$ human single-chain antibody (scFv) clones⁸ for antibodies that bind to the plasma membranes of brain endothelial cells. We chose brain endothelial cells as a relevant cellular target as they comprise the blood-brain barrier (BBB) and act as a selectively permeable interface whose plasma membranes play a particularly important role in separating the circulation from the brain interior. We panned the nonimmune human scFv yeast library against confluent rat brain endothelial cells (RBE4 cell line⁹, Supplementary Methods) for five rounds of binding, washing, clone recovery, and amplification (Supplementary Methods). After four rounds of panning, there was a clear enrichment in the number of binding yeast (Figure 1A, Table 1). The recovery percentages of yeast applied to the RBE4 monolayers increased from 18% after round 4 to 78% after round 5. The totals after round 5 indicate that the recovered pool from round 4 consisted almost exclusively of binding yeast as on average only 70-80% of the yeast applied to the monolayer are actually displaying antibody, primarily as a result of plasmid stability effects¹⁰. Further examination of 12 individual yeast scFv clones recovered from round 4 confirmed the high

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percentage recovery of binding yeast in that all 12 clones bound specifically to RBE4 cells (Figure 1B, Table 1).

In order to analyze scFv clones on a larger scale, and to reduce the characterization of redundant scFv, we employed a high throughput method that led to the identification of 11 unique RBE4-binding scFv out of 66 clones analyzed (Figure 1C, scFvA-K Supplementary Table 1). When performing a screen against multiple cell surface antigens simultaneously, certain scFv clones can dominate the selection as a result of differential antigen abundance, antigenicity, or antibody-antigen affinity characteristics^{1, 3, 11}, thereby masking the diversity of the binding pool. Indeed, two homologous scFv classes (class 1: scFvA, scFvB, scFvC, scFvG, and scFvK and class 2: scFvD and scFvI, Supplementary Table 1) predominated and together represented 61 of the initial 66 clones analyzed, and further mining of the original pool for unique RBE4-binding scFv, although possible, would have been quite laborious. Instead, the VHCDR2 regions for the class 1 and 2 scFv, which were fully conserved within their respective classes, were used as yeast colony Northern blotting targets for rapid, high throughput subtractive prescreening of 2000 clones from the round 4 binding pool (Figure 1C, Supplementary Methods). Then, only those clones not part of classes 1 or 2 were fed through the cell-based screen. The subtractive approach increased the number of unique scFv to 34, and the number of homology-based binding classes was increased to 18 (Supplementary Table 1). In total, 88% of the yeast clones in pool 4 were identified as binders with zero instances of binding that was not scFv-mediated. The selected scFv had germline origins that were largely heavy chains VH3 (6 of 18 classes) and VH6 (6 of 18 classes) and light chains V λ 1 (11 of 18 classes) and V κ III (4 of 18 classes) (Supplementary Table 1), much like that found in the original, non-selected library where VH3, VH6 and V κ III were found at the highest frequency⁸. Similar to the results found when the same library was used for scFv selections against soluble antigens, we recovered V λ 1 at an inordinately high frequency compared with the levels of V λ 1 found in the non-selected library⁸. Thus, the light and heavy chain usage for scFv selected against an array of cell surface antigens mimics that found for selection against soluble antigens, and suggests a similar scFv “fitness” for either form of antigen presentation.

Next, we evaluated the class 1, 2, and 3 scFv for their properties as soluble scFv proteins. We subcloned the scFv genes encoding scFvA-K (excluding single domain VH isolates, scFvE and scFvJ) into an scFv expression vector¹² and they were secreted efficiently from yeast (Figure 2A). We then used scFv-containing supernatants and/or purified antibody to immunolabel RBE4 cells, and each scFv possessed cell surface binding capacity (Figure 2D). Class 1 scFv exhibited a clear binding signal with either supernatant or purified material at 3–4 μ g/mL, but class 2 (scFvD) and 3 scFv (scFvF) required approximately 10-fold higher purified concentrations of 20–80 μ g/mL to yield cell surface immunolabeling. Next, we used purified scFv to assess the binding affinity to live RBE4 cells. Class 1 scFvA possessed an affinity of $K_d = 82 \pm 15$ nM, whereas the affinity of class 2 scFvD as a monomeric protein could not be determined using this method (Figure 2B). Instead, we evaluated the binding properties of scFvD after predimerization with an epitope tag antibody (avidity = 2.0 ± 0.1 nM). As a comparison, the affinity of an anti-transferrin receptor scFv isolated using phagemid panning was measured to be 135 nM², of similar affinity to scFvA isolated with our yeast panning system.

To assess the nature of the antigens recognized by the scFv, we developed a novel yeast immunoprecipitation procedure. We used yeast displaying scFv to directly immunoprecipitate the cognate plasma membrane antigens from detergent-solubilized, biotinylated RBE4 lysates (Supplementary Methods). Conveniently, the use of yeast as the immunoprecipitation particle allowed the sizing of antigens without any additional subcloning, production or immobilization of scFv proteins required by traditional immunoprecipitation methods. We assessed the immunoprecipitated products by anti-biotin Western blotting (Figure 2C), and the low amount

of background in such blots was a direct indicator of the specificity of the immunoprecipitation process. The antigens immunoprecipitated by class 1 scFvA (124 kDa-nonreduced, several large molecular weight bands-reduced), class 2 scFvD (104 kDa-nonreduced, 117 kDa-reduced) and class 6 scFvJ (122 kDa-nonreduced, 127 kDa-reduced) were distinct (Figure 2C). As predicted by the homology-based scFv class assignment, other class 1 scFv (scFvB, scFvC) yielded immunoprecipitation products identical to that seen for scFvA, and class 2 scFv I like that observed for class 2 scFvD (data not shown). The multiple bands appearing in the reduced scFvA sample suggest that along with the antigen recognized specifically by scFvA, co-immunoprecipitation of other biotinylated members of a protein complex or possibly multiple specific antigens may be occurring.

Antibodies that target molecular endocytosis systems are often desired because they can be employed for delivering intracellular payloads for a variety of applications. With this in mind, we evaluated several of the class 1, 2, and 3 scFv for their ability to mediate cellular internalization. The scFv were predimerized to provide the bivalency often required for receptor clustering and endocytosis, and subsequently applied to living RBE4 cells (Figure 2D and Supplementary Methods). Class 1 scFvA was rapidly endocytosed into vesicular structures within the RBE4 cells, whereas class 2 scFvD and class 3 scFvF bound the RBE4 surface but did not promote internalization (Figure 2D and data not shown). As a comparison, the anti-transferrin receptor monoclonal antibody (O×26) that is known to endocytose and transcytose across the BBB¹³ was also internalized into RBE4 cells, albeit primarily into single putative endosomal structures (Figure 2D). From the standpoint of downstream antibody applications, endocytosing and transcytosing anti-transferrin and anti-insulin receptor antibodies have been used to overcome the BBB for delivery of therapeutic agents into the brain after intravenous injection¹⁴. Thus, since scFvA was found to target an endocytosis system, we wished to compare the antigen targeted by scFvA with the transferrin and insulin receptors. We used the anti-transferrin receptor O×26 scFv¹⁵ in a surface display format to immunoprecipitate the transferrin receptor, and the immunoprecipitated products were of the expected size (183 kDa-nonreduced, 96 kDa-reduced) and were distinct from those recognized by scFvA (Figure 2C). In addition, Western blotting against the immunoprecipitated scFvA antigen indicated that scFvA targets neither the transferrin nor the insulin receptor (Figure 2C and data not shown). Finally, the antigen recognized by scFvA was shown to be well expressed both by RBE4 cells and by intact brain capillaries (Supplementary Figure 1). Taken together, these data suggest that the scFvA-antigen system represents a novel antibody-BBB transporter combination that may offer promise for brain drug delivery, although further study will be required to validate an antibody for such an application. In conclusion, this investigation demonstrates the utility of yeast surface display-based cell surface selections for the efficient identification of antibodies that bind and internalize into target cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Identification of RBE4-binding scFv clones by cell panning and high throughput scFv analysis. (a) Light microscopic analysis of enrichment after each round of panning against a confluent RBE4 monolayer. Scale bar: 50 µm. Yeast are the small objects (~5 µm) residing on the monolayer. (b) Test for scFv-mediated yeast binding. Rescued scFv-encoding plasmids from the binding yeast clones were retransformed into the EBY100 parent display strain and panned against RBE4 monolayers. Left panel: induced yeast, right panel: uninduced yeast. Scale bar: 25 µm. (c) Schematic of strategy for high-throughput analysis of individual scFv clones.

Individual scFv yeast clones were grown in SD-CAA (uninduced, negative control, orange) or SG-CAA (induced, scFv-displaying, blue) in a 96-well plate (1). Yeast cells were then transferred to a 96-well plate with confluent RBE4 cells, incubated at 4 °C for 2 hours (2) and washed as described in the Supplementary Methods. A simple light microscopy test was then applied. If induced yeast were retained after washing steps, but not when they were grown in the absence of galactose, the clone was defined as binding (3 versus 4). The gene encoding the binding scFv clone was then amplified directly from the yeast colony (5) and digested with *Bst*NI (6). ScFv with unique digestion patterns were sequenced. IgBLAST and the Kabat database were used for CDR assignment and human germline classification (7). For subtraction screens, this sequence data was used with yeast colony Northern blotting to create a depleted pool to send through the analysis (8).

Figure 2.

Evaluation of scFv binding and internalization properties. **(a)** Western blotting of full-length, secreted scFv. Batch secretion yields for each clone are listed above the blot. **(b)** Equilibrium binding attributes of scFvA and scFvD. Left panel: binding isotherm for scFvA interaction with live RBE4 cells. The plot shows the fitted monomeric equilibrium binding functions and experimental data from two independent experiments. Right panel: binding isotherm for dimerized scFvD interaction with RBE4 cells. The plot shows the fitted monomeric equilibrium binding functions used to generate an apparent affinity (avidity) and experimental data from two independent experiments. Insets indicate raw flow cytometry histograms that were used to generate the binding curves. **(c)** Yeast display immunoprecipitation of antigens for scFvA (A), scFvD (D), scFvJ (J) and O×26 scFv (O). Irrelevant anti- hen egg lysozyme scFv (N) was used as a negative control. The immunoprecipitation products were resolved by either nonreducing (NR) or reducing (R) gel electrophoresis and were probed with an anti-biotin antibody or an anti-transferrin receptor antibody (O×26). **(d)** Binding and internalization characteristics of scFv. RBE4 cells were labeled with purified, pre-dimerized scFvA, scFvD, irrelevant scFv 4-4-20 or O×26 monoclonal antibody at 4°C for cell surface labeling and then shifted to 37°C to promote cellular trafficking. The cells were then labeled with AlexaFluor 555 conjugated anti-mouse IgG (red) at 4°C followed by AlexaFluor 488 conjugated anti-mouse IgG (green) with or without cell permeabilization by saponin (SAP) treatment. Merged images of the AlexaFluor-labeled images are shown. Scale bar: 20 μm.

Table 1
Summary of panning parameters and enrichment of RBE4-binding yeast.

Round	1	2	3	4	5
Total number of yeast applied	5×10 ⁹	1×10 ⁸	5×10 ⁷	5×10 ⁷	5×10 ⁷
Yeast density (yeast/cm ²)	5×10 ⁷	5×10 ⁶	5×10 ⁶	5×10 ⁶	5×10 ⁶
Number of recovered yeast	ND	8.2×10 ⁴	2.0×10 ⁵	9.0×10 ⁶	3.9×10 ⁷
Recovery %	ND	0.08	0.40	18	78
Number of binders/analyzed yeast	ND	ND	7/12	1760/2000	ND

ND: not determined.