Libraries against libraries for combinatorial selection of replicating antigen–antibody pairs

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Contributed by Richard A. Lerner, December 3, 2008 (sent for review November 15, 2008)

Antibodies are among the most highly selective tight-binding ligands for proteins. Because the human genome project has deciphered the proteome, there is an opportunity to use combinatorial antibody libraries to select high-affinity antibodies to every protein encoded by the genome. However, this is a large task because the selection formats used today for combinatorial antibody libraries are geared toward generating antibodies to one antigen at a time. Here, we describe a method that accelerates the identification of antibodies to a multitude of antigens simultaneously by matching combinatorial antibody libraries against eukaryotic antigen libraries so that replication-competent cognate antigen–antibody pairs can be directly selected. Phage and yeast display systems are used because they each link genotype to phenotype and can be replicated individually. When combined with cell sorting, the two libraries can be selected against each other for recovery of cognate antigen–antibody clones in a single experiment.

antibody libraries | human genome | phage display | yeast display

The generation of antibodies to scientifically and clinically important protein antigens has occupied researchers for the past 25 years and has led to the establishment of combinatorial antibody libraries (1–6). Essentially, these libraries constitute a synthetic immune system. Nowadays, such libraries are routinely prepared and contain antibody collections that exceed the diversity of natural repertoires by many orders of magnitude. These libraries are not restricted by tolerance, they avoid the use of live animals, and have yielded important therapeutic antibodies (6). The libraries are most often formatted in yeast (7, 8) or phage (1, 4) so that single binding events can be replicated and high-affinity antibodies can be selected. However, we have yet to extract the full potential of these powerful library methods because we still select antibodies one antigen at a time (9).

The bottleneck imposed when antibodies are selected to one antigen at a time is illuminated by the opportunities posed by the human and other genome projects. These projects have provided an explosion in the numbers of known proteins, and it would be desirable to generate a set of high-affinity monoclonal antibodies to each of them so that ultimately one has a set of antibodies to every protein in the genome. Because combinatorial antibody libraries are not restricted by immunological tolerance, any self- or nonself-protein can be bound by a member of the antibody library. This means that, with respect to a given antibody library, the human proteome can be considered to be a collection of antigens.

The present article describes a solution to the problem of simultaneous selection of monoclonal antibodies to a large set of antigens rather than to one antigen at a time.

Coselection of cognate antibody–antigen pairs from combinatorial libraries has been attempted by using selectively infective phage (10, 11) or protein fragment complementation (12–14) with only limited success. The central difference in our approach is that we use two different display platforms for the antibody and the antigen libraries. Several considerations dictated the choice of platforms and posed challenges to success. The platforms must allow for the specific interaction of antibody and antigen pairs with minimal background interaction between the platforms themselves. Each partner has to be capable of replication and maintenance of its phenotype–genotype link throughout the selection process, and they must have different growth requirements so they can be replicated separately. Finally, one must be able to disrupt the interaction between the partners in a way that does not abrogate their growth potential so they can be cloned and amplified while still maintaining the information link between the two platforms for identification of the antigen and antibody proteins expressed by the cognate pairs. Although each of these individual requirements seemed achievable, in aggregate they proved to be challenging.

Results

General Strategy. Among the widely used protein display platforms, the yeast and phage systems seemed to best fit the criteria necessary for library-against-library selection of replicating antibody–antigen pairs. In Fig. 1 we show the strategy for combinatorial selection of antigen–antibody pairs by using a yeast–phage system. In initial studies, we found that a scheme such as that described in Fig. 1 was, in principle, workable because yeast were amenable to many typical treatments used for phage panning, including 5% milk protein, 0.05% Tween 20 detergent, incubation at 37 °C, and phage elution by using glycine buffer at pH 2.2 with no loss in either yeast viability or the presence of plasmids.

Nature of Antibody-Mediated Phage–Yeast Interaction. To validate the feasibility of our strategy, we studied the nature of the yeast and phage interactions by fluorescence confocal microscopy (Fig. 2). The displayed Z13e1 single chain antibodies (scFv) are diffusely and evenly distributed on the surface of the yeast cells. When the binding of the soluble form of the small monomeric protein antigen gp41 to the surface of antibody-bearing yeast cells is studied by fluorescent microscopy, the distribution of the antigen appears diffuse and evenly distributed. In contrast, when phage that display the gp41 antigen bind to antibody on the surface of yeast cells, the phage appear to be lying along the surface of the yeast in a punctate distribution with dimensions that are approximately those of M13 phage.

Conditions for Library-Against-Library Selections. To test the utility of phage and yeast display for library-against-library selection, we studied an antibody library expressed on the surface of yeast

Author contributions: D.R. Bowley, T.M.J., D.R. Burton, and R.A.L. designed research; D.R. Bowley and T.M.J. performed research; D.R. Bowley, T.M.J., D.R. Burton, and R.A.L. analyzed data; and D.R. Bowley, T.M.J., D.R. Burton, and R.A.L. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0812291106/DCSupplemental.

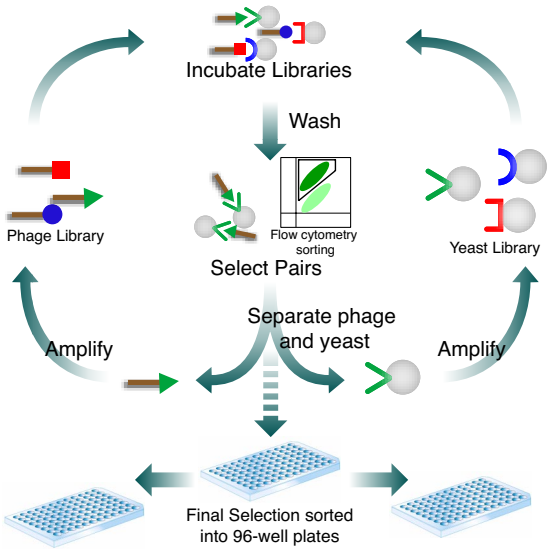


Fig. 1. Dual-display for the identification of antibody–antigen pairs by library-against-library selection. A library of antigens (or antibodies) is displayed on phage, and a library of antibodies (or antigens) is displayed on yeast. The two libraries are mixed, and phage that are not bound to yeast cells are washed away. Phage that are bound to yeast cells are labeled with a fluorescence reagent, and flow cytometry sorting is used to select yeast cells bound to phage. The yeast and phage are separated for amplification, and the selection round is repeated until significant enrichment of pairs has been achieved. During the final round of selection, single cells of phage-positive yeast are sorted into 96-well plates. By eluting the phage from a single yeast cell, the information link between the platforms is maintained, and clonal pairs of antigens and antibodies are isolated.

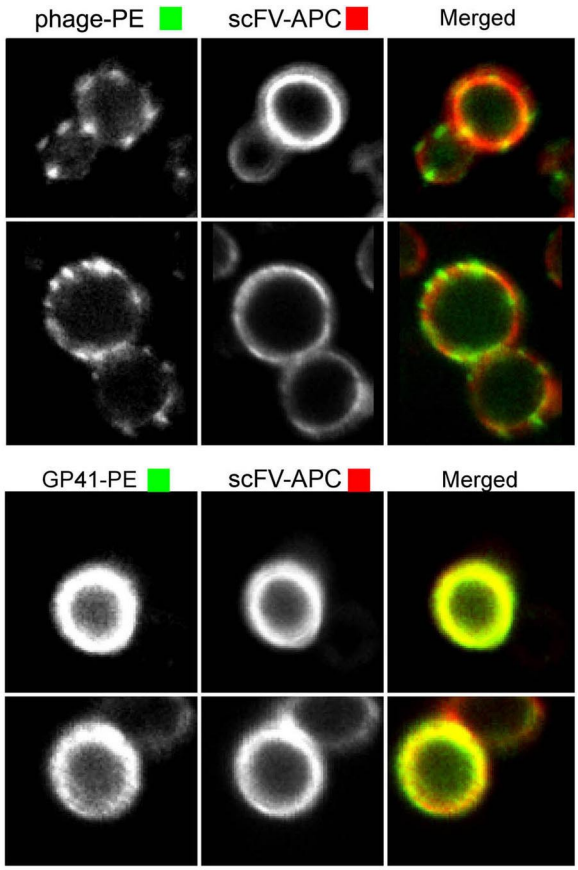


Fig. 2. Confocal microscopy imaging of the yeast–phage interaction. Yeast cells displaying Z13e1 scFv were stained with anti-c-myc-Alexa Fluor 647 to visualize the presence of scFv on the cell surface (scFv-APC pseudocolored red). Binding of TJ1D phage to yeast cells was visualized by using an anti-phage antibody and Zenon-PE (phage-PE, pseudocolored green). For yeast cells binding to monomeric soluble protein, biotinylated HIV gp41 was visualized with streptavidin-PE (gp41-PE, pseudocolored green).

and an antigen library expressed on phage. The antigen library contains fragments of the HIV-1 gp160 protein (15), and the antibody library contains a collection of scFv molecules from an HIV-1 infected individual (16). To control rigidly for binding specificity, we studied a system where the stringency requirement for antigen–antibody interaction is very high. Antibody scFv Z13e1 binds a linear epitope on HIV-1 envelope protein gp41, and antigens TJ1D or TJ1N are 36-aa-long fragments of the HIV-1 envelope precursor protein gp160. When a single amino acid in the binding epitope in antigen TJ1D is changed from D to N to yield mutated antigen TJ1N, antibody Z13e1 can no longer bind to the modified antigen. The flow cytometry plots for Z13e1 yeast cells binding to phage expressing either the TJ1D or TJ1N antigen are shown in Fig. 3. Because the observed binding can be abolished by a single amino acid change in the antigen, it is clear that the interaction of yeast cells and phage is mediated exclusively by the displayed proteins. In the yeast display system, there are typically at least 15% of cells that do not express antibodies. Like the mutant antigen, those yeast cells that do not express antibodies serve as an important internal control because if phage bound to scFv-negative yeast cells it would indicate that phage are binding nonspecifically to proteins other than the displayed antibody on the surface of the yeast. We note that in this dual display system phage do not bind yeast cells that do not express antibodies.

To determine conditions for selection of cognate antigen–antibody pairs in a library format, both Z13e1 yeast and TJ1D phage were spiked into the yeast and phage libraries at a frequency of $1{:}10^4$, which makes the frequency of the cognate pair $1{:}10^8$ when the libraries are mixed. In establishing the system for coselection of replication-competent antigen–antibody pairs, several problems appeared that related to: (*i*) appearance of growth mutants of phage that overtake the selection, (*ii*) phage concentration requirements, (*iii*) optimum fluorescence visualization of phage, (*iv*) conditions for elution of phage from yeast cells that maintain phage and yeast viability, and (*v*) the need to overcome digestion of phage and/or their expressed antigens by yeast proteases. Ultimately, protocols were developed [see below and supporting information (SI) *Materials and Methods*] that addressed all of these problems.

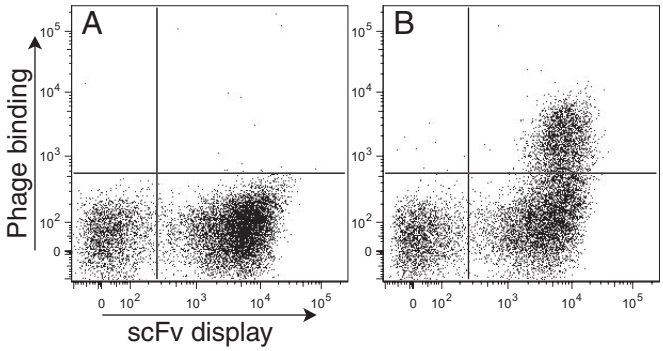
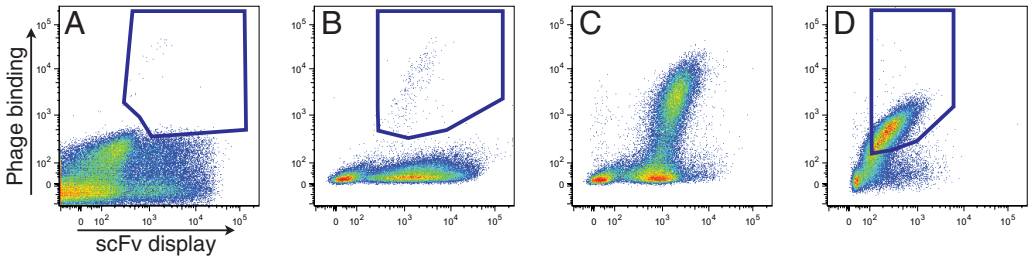


Fig. 3. Interaction of yeast and phage is specific to the displayed proteins. Yeast cells displaying Z13e1 scFv were labeled with anti-c-myc-Alexa Fluor 647 to visualize the presence of scFv on the cell surface (*x* axis). Phage binding to yeast cells was visualized by using an anti-phage antibody and Zenon-PE (*y* axis). (*A*) Phage TJ1N. (*B*) Phage TJ1D.

BIOCHEMISTRY



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	Phage Input	Phage Output	Yeast Input	Yeast Output	% positive phage	% positive yeast
Round 1	4×10^{11}	1×10^{5}	1×10^{8}	NA	NA	NA
Round 2	6×10^{11}	6×10^{5}	1×10^{8}	NA	8 %	NA
Round 3	1×10^{13}	NA	5×10^{7}	1.5×10^{5}	NA	0.02 %
Round 4	1×10^{13}	1.5×10^{5}	5×10^{7}	2×10^{4}	48 %	0.82 %
Round 5	1×10^{13}	single cell	5×10^{7}	single cell	NA	52 %

Fig. 4. Flow cytometry selection. (*A* and *B*) Flow cytometry plots from selection rounds 3 and 4 respectively, with the sort gate for selection indicated in blue. (*C* and *D*) Both from round 5 but use different fluorescence markers. (*A–C*) Anti-c-myc-Alexa Fluor 647 was used for scFv visualization and anti-phage/Zenon-PE for phage binding (the phage-negative populations appear different because they were obtained on two separate instruments with different voltage settings). (*D*) Anti-c-myc-Alexa Fluor 488 was used for scFv visualization and anti-HA-Alexa Fluor 647 for phage binding. (*E*) Input and output titers for both phage and yeast for each selection round and the percentage of phage and yeast that are positive for the desired Z13e1–TJ1D antibody–antigen pair. The percentage of positive phage was determined by phage ELISA for 48 clones after each round. The percentage of positive yeast cells was calculated by determining the percentage of scFv-positive yeast cells that bound to phage during the flow cytometry selection; this number actually reflects the percentage of double positive yeast cells that are input into the selection round, not the output from that round.

The problem of phage concentration during selections is of particular note and dictates the selection protocol and the choice of antibody reagents used to detect phage binding to the yeast cells. Typically during yeast display selections by flow cytometry, the number of antigens that bind to the yeast cell and the resultant antigen-binding fluorescence signal are directly proportional to the solution antigen concentration, the affinity of the displayed antibody, and the number of displayed antibody molecules per yeast cell (17). Thus, the concentration of added soluble antigen is adjusted to be greater than or equal to the desired equilibrium-binding constants (K_d) for the yeast-bound antibodies that one wishes to select. The K_d of Z13e1 scFv for its antigen is ≈50 nM. Therefore, to observe strong signals from antigen bound to Z13e1 scFv, the solution antigen concentration should be >50 nM. Standard phage amplification protocols yield a phage concentration of $\approx 10^{12}$ phage per mL. If we assume the typical recombinant protein display percentage of 1–10% for phagemid display systems, the solution antigen concentration would be at most 0.1 nM, and the concentration of TJ1D in the initial spiked library would be 10 fM. At this phage concentration, phage bind to yeast cells but not at sufficient density for the binding to be visualized by flow cytometry. Thus, the first two rounds of selection were designed simply to increase the concentration in the library of phage that specifically bound to the yeast cells. To accomplish this, the yeast and phage libraries were mixed, unbound phage were washed away, and the bound phage were eluted from the yeast cells and amplified. This step enriches the library ≈3 orders of magnitude for phage that bind specifically to yeast cells expressing antibodies.

The second major consideration concerns the nature of the fluorescent anti-phage antibody used to detect phage binding to yeast cells. One has to balance the need to obtain a high signal-to-noise ratio with the requirement that the detecting antibody not neutralize phage infectivity to a point that precludes its recovery. Given that the pComb3X vector used here has a HA epitope tag inserted between the expressed antigen and phage pIII, one could, in principle, use either anti-phage coat or anti-HA antibodies for detection of yeast cells that bound phage. Fluorescent anti-phage antibodies, which have many binding targets per phage, give a much stronger signal than the anti-HA antibody that only has one HA binding target per phage when the pComb3X vector is used. Also, proximity of the HA tag to the displayed protein may cause the HA tag to be partially occluded when phage are bound to yeast cells. However, the anti-phage antibodies significantly reduce the infectivity of the phage and are, therefore, not suitable for single-cell sorting where one needs to recover low numbers of phage by replication. Therefore, two different antibodies are used. Anti-phage antibody is used for the initial selection rounds, and anti-HA antibody is used for the final selection when single cells are sorted.

Finally, the elution conditions that disrupt the antigen–antibody-mediated phage–yeast union were also found to be critical. In the strategy outlined here, the system only becomes clonal when individual yeast cells bound to their cognate phage bound are sorted as single cells during the final selection round. However, it is at this point where the potential for replication of the partners is at greatest risk because of their low numbers. To recover both yeast and phage successfully, we found that it was necessary to sort directly into glycine elution buffer and immediately centrifuge the plate to pellet the individual yeast cell and collect most of the buffer containing phage. Medium is then added to the yeast cells, and *Escherichia coli* are infected with the recovered phage for their respective amplifications (see *SI Materials and Methods*). Using this protocol, we recovered an average of 60% of yeast cells with an average of 18 copies of phage bound to their surface. Eighty-nine percent of the phage recovered by this procedure bind to the antibody that was displayed on the surface of the yeast cell from which they were eluted. If we immediately elute the phage and then store them at 4 °C before infection, we observe an average of 10 copies of viable phage per yeast cell with 98% of the phage binding. At these numbers, both yeast and phage clones were easily recovered.

Recovery and Characterization of Clones from Single-Cell Sorts. When the conditions described above were used, we were able to enrich successfully for the Z13e1–TJ1D pair with only five rounds of sorting. In Fig. 4, the results of a protocol in which successful

selection of the Z13e1–TJ1D pair was accomplished are summarized. In this selection, 100% of yeast clones analyzed were Z13e1, and 88% of the analyzed phage were TJ1D. Although we used an HIV gp160 fragment library and an HIV patient antibody library, we did not isolate any additional pairs. This is not unexpected because it is well-established that the majority of patient antibodies against HIV envelope proteins bind to discontinuous and conformational epitopes. Indeed, previous characterization of the antibody library studied here showed that it contained few, if any, antibodies to linear protein sequences other than those contained in the TJ1D epitope (15, 16, 18, 19).

Given the potential numbers of cognate pairs that could be identified by this selection method, it is critical to have a high throughput means of characterizing the isolated pairs. To accomplish this, we first analyzed the yeast antibodies by BstNI digestion to identify unique clones and verified the nature of the insert by DNA sequencing. Then, the cognate phage were grown, and whole-cell ELISA was used to verify that they bound to the specific scFv–yeast, and representative phage clones were sequenced. All phage that bound Z13e1–yeast by ELISA were confirmed to be TJ1D by sequencing. A small proportion (12%) of phage clones did not bind to Z13e1–yeast and were of unknown origin. These clones could not be sequenced by using any pComb3X sequence primers, perhaps because the vector had recombined but still maintained carbenicilllin (carb) resistance. This analysis can be applied to a large number of clones simultaneously with sequences easily determined within 2 weeks of the final selection round.

Discussion

The technology described here should allow saturation of large proteomes, including that of man, with cognate antibodies. Just in terms of efficiency, it would be useful to the research community if a library of replicating antigen–antibody pairs that saturated the genome were available. The library could be progressively annotated if researchers who received members of the library recorded their results with the antibodies in, for example, a WIKI-type format. But, the real value of such combinatorial antibody libraries may be in the generation of novel therapeutic antibodies. Currently, libraries are most often screened against a single antigen in situations where physiological insight or results from study of the pathogenesis of a disease suggest that antibody binding to the antigen in question might be of therapeutic advantage.

We anticipate that, when practicing the library-against-library methodology, the antigenic library will be displayed on yeast, and the antibody library will be displayed on phage. This format takes advantage of the unique strengths of each system. In general, yeast systems allow display of larger protein fragments than phage and can express mutagenesis libraries that contain entire large proteins (20, 21). Also, unlike proteins expressed in *E. coli*, yeast systems permit glycosylation. These features of yeast expression systems are desirable because one wants to select against domains and/or properly folded large protein fragments to generate antibodies against discontinuous as well as continuous epitopes. Although, in terms of numbers, the yeast library size is limited compared with phage, its capacity exceeds any reasonable estimate of the number of fragments one will need to display. We anticipate that the antibody library should be many orders of magnitude larger than the antigen library because some binding events may be rare. Also, the large size of combinatorial antibody libraries in phage should allow for selection of many different antibodies to each target antigen. This may useful when the exact region of the antigen to which the antibodies bind is important, as might occur, for example, in the search for agonist antibodies. In this context, it is important to realize that because cognate pairs of antigens and antibodies are selected, the region to which the antibodies bind is automatically known if fragmented antigens are used. Up until now, the determination of where antibodies bound to their antigen often required considerable experimentation. Indeed, in many instances the site to which antibodies bound was only knowable when anti-peptide antibodies were used because in this case the specificity of the antibody is predetermined (22, 23).

One can imagine alternative strategies and/or formats for library-against-library screening. For example, once one has enriched populations of phage and yeast, single-cell sorting is not absolutely necessary, and other methods such as a checkerboard analysis could be used to identify the cognate pairs. However, a checkerboard analysis approach becomes extremely inefficient with increasing numbers of unique sequences. Also, completely different formats such as phage-against-phage might be used where, for example, filamentous phage-bearing antibodies could be screened against phage lambda plaques expressing antigens (1).

A powerful format for the selection of important therapeutic antibodies could entail a screening mode in which collections of antibodies, annotated for their cognate antigen, are screened for binding and/or physiological effects and the nature of the antigen(s) are de-convoluted latter. Thus, each member of the antibody library could contain a unique nucleotide sequence marker that identifies its antigen so that the antigen can be revealed when the genes of the clones selected from a collection are sequenced. These libraries should complement the substantial efforts already under way aimed at pairing each antigen in the genome with a polyclonal antibody to be able to create immunohistochemical distribution maps for each human protein (24, 25).

Finally, combinatorial antibody libraries obviate the need for live animals and allow construction of immune systems in vitro, thereby bypassing the time-honored process of immunization (26, 27). We now anticipate that all members of these libraries will be known in terms of the antigens to which they bind.

Materials and Methods

Cell Lines, Media, and Vectors. *E. coli* XL1-Blue was used for cloning and preparation of plasmid DNA and was grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride). *E. coli* ER2738 cells (New England Biolabs) were used for phage library propagation in super broth (SB) medium [10 g/L Mops, 30 g/L tryptone, 20 g/L yeast extract (pH 7.0)] with VCSM13 helper phage. The phagemid display vector was pComb3X.

Yeast strain EBY100 was maintained in YPD broth (Difco). Transfection of EBY100 with the vector pYDscFv was completed by using the lithium acetate method and maintained in SD-CAA medium (pH 4.8) (6.7 g/L yeast nitrogen base, 5 g/L casamino acids, and 20 g/L dextrose, 14.7 g/L sodium citrate, and 4.29 g/L citric acid monohydrate] and on SD-CAA plates (SD-CAA + 17 g/L agar). After selections, the medium is supplemented with 0.25 mg/mL ketoconazole to ensure no growth of contaminating yeast. Yeast surface expression of scFv was induced by transferring to SG/R-CAA medium (6.7 g/L yeast nitrogen base, 5 g/L casamino acids, 20 g/L galactose, 20 g/L raffinose, 1 g/L dextrose, 9.67 g/L $NaH_2PO_4{\cdot}2H_2O$, and 10.19 g/L $Na_2HPO_4{\cdot}7H_2O$). The yeast display vector was pYDscFv that was derived from pPNL200 (28) to include SfiI sites and a homologous recombination site for improved transformation efficiency.

Antibodies and Fluorescence Reagents. The anti-phage antibody [unconjugated and horseradish peroxidase (HRP)-conjugated] was purchased from GE Healthcare. Anti-HA-HRP (3F10) was purchased from Roche, and Zenon (IgG2a)-PE was purchased from Invitrogen. Succinimidyl-ester Alexa Fluor 647 and Alexa Fluor 488 were purchased from Invitrogen, and anti-HA and anti-c-myc were labeled according to the manufacturer's directions.

Generation of Antibody and Antigen Libraries. The phage gp160 fragment library in pComb3X was panned by using antibody 2F5 to isolate antigen clone TJ1N and QuikChange mutagenesis (Stratagene) was used to create antigen clone TJ1D. Antibody Z13e1 was reformatted as an scFv from the Fab fragment by overlap PCR, cloned into pYDscFv, and transformed into EBY100 yeast cells. The gp160 antigen phage library and TJ1D were amplified separately, and TJ1D was added to the library at a frequency of $1{:}10^4$ based on volume (assuming phage concentrations were approximately the same). Similarly,

yeast–Z13e1 and the FDA2 scFv library were amplified and induced separately, and Z13e1 was spiked into FDA2 at a frequency of 1:10^4 based on yeast cell concentration.

Confocal Microscopy and Flow Cytometry Staining. Yeast cells (10^6) were stained in 50 μL of wash buffer (WB; 0.5% BSA, 2 mM EDTA/PBS) with 10 μg/mL anti-c-myc-Alexa Fluor 647 for 30 min at room temperature, then 100 μL of precipitated phage (in 1% BSA/PBS), or biotinylated gp41, was added and incubated for 1 h at room temperature. Cells were washed three times with WB then incubated with anti-phage/Zenon-PE (or streptavidin-PE for gp41) in 50 μL of WB for 30 min at 4 °C. Cells were washed three times, and then cells were resuspended in 500 μL of WB for flow cytometry or 10 μL of antifade for confocal microscopy.

Library-Against-Library Library Selections. For the first round of selection, the phage libraries were transformed and amplified by using *E. coli* XL1-Blue in SB with 2% glucose. Tetracycline (tet) was added at 10 μg/mL the 1st h after transformation. Carb (20 μg/mL) was added after the 1st h and subsequently increased to 50 μg/mL for the 2nd h. After an additional hour the culture was expanded to 100 mL, and the cells were superinfected with VCSM13 (6×10^{11} pfu) for 30 min at 37 °C without shaking followed by 90 min at 37 °C at 300 rpm. Cells were centrifuged to remove the glucose-containing medium and resuspended in 100 mL of SB with carb, tet, and kanamycin (kan; 70 μg/mL) and incubated by shaking 16 h at 30 °C. Phage were precipitated on ice for 30 min with 4% PEG and 3% NaCl and resuspended in 2 mL of 1% BSA/PBS. After selection, the phage were infected into ER cells for 15 min, and phage-infected cells were plated on LB agar containing 0.5% glucose and incubated at 30 °C overnight. Cells were scraped from the agar plates into 5 mL of SB and added to 100 mL of SB (plus carb, tet, and glucose) to an A_{600} of 0.1. The culture was incubated at 37 °C until the A_{600} reached 0.8. VCSM13 was added, and all subsequent steps were completed as described above. For the later selection rounds, cells were scraped into 1 L instead of 100 mL, and all steps were scaled up accordingly, except after precipitation, phage were resuspended in 1 mL of PBS.

The yeast libraries were grown as described in ref. 8. Typically, yeast were grown in SD-CAA for ≈8–16 h at 30 °C (depending on starting cell density) and then transferred to SG/R-CAA to induce scFv expression for ≈16–20 h at 20 °C in culture volumes appropriate for the size of the library.

In the initial selection rounds, 10^{12} freshly precipitated phage were panned against 10^8 freshly induced yeast cells by incubating phage and yeast in 1% BSA/PBS buffer for at least 2 h at 37 °C. Unbound phage were washed away by pelleting the yeast cells, resuspending cells in 2 mL of PBST (0.05% Tween 20/PBS), and transferring cells to a new tube. For the first round, yeast cells were washed five times. In the second round, cells were washed 10 times. Any phage still bound to yeast cells were eluted with 200 μL of glycine elution buffer [200 mM glycine, 1 mg/mL BSA, 0.05% Tween 20 (pH 2.2)]. The buffer containing phage was neutralized with 12 μL of 2 M Tris, after which ER cells were infected for phage amplification as described above.

For flow cytometry selections, 10^8 yeast cells were incubated with 20 μg/mL anti-c-myc-Alexa Fluor 647 in 100 μL of WB for 15 min at room temperature. Freshly precipitated phage from a 1-L culture were preblocked with 300 μL of 20% milk for 15 min at room temperature and then added to the yeast cells. Yeast and phage were incubated at room temperature for 2 h followed by 10 min at 4 °C. All remaining steps were carried out at 4 °C. Yeast cells were washed five times with 2 mL of WBT (0.05% Tween 20/wash buffer). Yeast cells were transferred to a new tube with each wash and then incubated with freshly labeled anti-phage/Zenon-PE at 15 μg/mL for 1 h. The anti-phage/Zenon-PE was prepared according to the manufacturer's directions. The cells were washed three times with 2 mL of WB, and then selections were performed by using a FACS Aria (BD Bioscience). Sort gates were determined to select the desired double positive cells. After the first flow cytometry selection, only the yeast cells were amplified. For subsequent rounds, the collected yeast cells were split in half, with half of the yeast cells amplified and the other half mixed with 50 μL of triethylamine (TEA; 100 mM, freshly prepared) for 1 min. Yeast cells were centrifuged, and the TEA was removed and neutralized with 25 μL of 1 M Tris·HCl (pH 7.6) before infecting ER cells to amplify the phage.

For the final selection, round yeast cells were sorted into 96-well plates containing 50 μL of glycine elution buffer. The plates were immediately centrifuged, and ≈45 μL of buffer was removed with care so as to not pipette the single yeast cell, and the solution was neutralized with 4 μL of 2 M Tris. The phage can either be mixed with ER cells immediately or stored at 4 °C before infection. Phage from each well were plated onto separate agar plates and grown overnight at 37 °C after which the plates were stored at 4 °C.

SD-CAA medium (100 μL) was added to the yeast cells, and they were grown at 30 °C for 2 days. After the single yeast cells had replicated, the cells from each well were grown in 1 mL of SD-CAA medium overnight at 30 °C. The vector contained in the yeast cells was isolated by using a Zymoprep yeast miniprep kit (Zymo Research). Each scFv sequence was amplified by PCR, unique clones were identified by BstNI digestion, and their sequence was determined. Once unique yeast clones were identified, the cognate phage were retrieved from the stored plates and grown overnight at 37 °C in 500 μL of SB/carb/VCSM13. The phage-containing supernatant was used to analyze specific phage-yeast binding by whole-cell ELISA.

Phage ELISA. ELISA plates were coated overnight at 4 °C with 25 μL of PBS containing 4 μg/mL of the coating antibody (Z13e1 IgG, 2F5 IgG, or anti-HA). Wells were washed twice with PBST and blocked with 50 μL of 5% milk for 30 min at 37 °C. Phage supernatant (25 μL) or 2-fold dilution series of precipitated phage (25 μL) was added to each well and incubated for 2 h at 37 °C. Wells were washed four times with PBST, and then 25 μL of anti-phage-HRP (diluted 1:1,000 in 5% milk) was added to each well and incubated for 1 h at 37 °C. Wells were again washed four times with PBST and 50 μL of ABTS developer [450 μg/mL ABTS, 0.01% H_20_2, 100 mM citrate buffer (pH 4.0)] was added. After 30 min at room temperature, the A_{405} was measured on a microplate reader (Molecular Devices).

Whole-Cell ELISA. Yeast cells (2×10^6) were mixed with 100 μL of phage supernatant for 2 h at 37 °C. Yeast cells were washed three times with WBT and then incubated with 50 μL of anti-HA-HRP (1:1,000 dilution in 5% milk/PBS) for 1 h at 37 °C. Yeast cells were washed again three times and moved into new tubes for the final wash and then resuspended in 200 μL of ABTS developer. After 30 min at room temperature, cells were centrifuged, and 50 μL of supernatant was transferred to ELISA plate, and the A_{405} was measured.

ACKNOWLEDGMENTS. We thank Michael B. Zwick (the Scripps Research Institute) for reagents and helpful discussion, Diana I. Ruiz for experimental assistance, Laura M. Walker (the Scripps Research Institute) for vector pYDscFv, the Scripps Research Institute Flow Cytometry core facility for assistance with sorting experiments, and William B. Kiosses for assistance with confocal microscopy.

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