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Yeast mating for combinatorial Fab library generation and surface display

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Abstract Yeast display of antibody fragments has proven to be an efficient and productive means for directed evolution of single chain Fv antibodies for increased affinity and thermal stability, and more recently for the display and screening of a non-immune library. In this paper, we describe an elegant and simple method for constructing large combinatorial Fab libraries for display on the surface of *Saccharomyces cerevisiae*, from modestly sized, and easily constructed, heavy and light chain libraries. To this end, we have constructed a set of yeast strains and a two vector system for heavy chain and light chain surface display of Fab fragments with free native amino termini. Through yeast mating of the haploid libraries, a very large heterodimeric immune Fab library was displayed on the diploids and high affinity antigen specific Fabs were isolated from the library.
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Key words: Fab; Yeast display; Selection; Library; Antibody; Single chain Fv

1. Introduction

Creating reagents which bind with high specificity and affinity to relevant biomolecules is one of the most critical and challenging tasks facing biologists. Identification and characterization of such reagents can be technically difficult and time consuming. A number of antibody discovery technologies currently employed, from in vivo platforms used to produce polyclonal or monoclonal antibodies to in vitro platforms of ribosomal [1–3], phage [4,5], bacterial display [6,7], and yeast display [8], all identify novel reagents. Compared to other in vitro display technologies, yeast display of non-immune single chain fragment variable (scFv) antibody libraries using the a-agglutinin adhesion receptor complex Aga1 and Aga2 has a number of advantages. Yeast antibody libraries do not appear to suffer the growth mediated diversity loss seen with phage and phagemid antibody libraries [8] and antigen size is not limited as in bacterial display. Yeast and bacterial display both allow selection of well expressing scFv and more precise selection for higher affinity antibodies using fluorescence activated cell sorting (FACS) [8,9]. FACS analysis also allows for more rapid clone characterization including K_D determination, k_{off} measurement, and epitope binning of mutually exclusive clones directly on the surface of yeast [10]. This eliminates the need for purification of scFv protein to perform these characterizations, saving considerable time. If the scFv are of inadequate affinity, yeast display has proven highly effective for affinity maturation [8,11,12].

Non-immune antibody libraries typically contain more than 1 billion members to ensure isolation of a panel of antibodies of adequate affinity; library size is the single most important determinant of antibody diversity and affinity [13]. Creating such large yeast antibody libraries is quite time and labor intensive, due to the lower transformation efficiency of yeast compared to bacteria. The recent successful display of Fab antibody fragments on yeast suggests a simpler approach to large library construction [14]. Since Fab are composed of two distinct polypeptide chains, it is possible to encode the two chains on different vectors in different yeast strains. The two chains can then be brought together in a single diploid yeast by mating, a highly efficient process (Fig. 1). For this work we report the successful construction of yeast vectors and strains for construction of large heterodimeric Fab libraries by mating *Saccharomyces cerevisiae*. We show that this system functions to recreate binding Fab from scFv heavy (V_H) and light (V_k) chain variable region genes and can be used to rapidly construct large Fab libraries from which multiple high affinity Fab can be isolated. This system should greatly simplify construction of large antibody libraries and could also be used for affinity maturation or humanization by chain shuffling [15].

2. Materials and methods

2.1. Yeast strains

The heavy chain library was constructed and displayed in JAR300, which has the following auxotrophic markers, *ura*3-52, *trp*1, *leu*2δ200, *his*3δ200, *pep*4:HIS3, *prbd*1.6R, *can*1, and GAL (a gracious gift from Andrew Rakeshaw, MIT). The strain is based on EBY100 that was derived from BJ5465 and is MATa [11]. The KanM×4 gene, conferring resistance to G418, was inserted through homologous recombination of a polymerase chain reaction (PCR) product encoding the KanM×4 gene flanked by 45 bp of the URA3 gene. The light chain Fab is expressed in YVH10 (Ura−, Trp−, BJ5464, MATα).

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Abbreviations: scFv, single chain fragment variable; mAb, monoclonal antibody; SDCAA, synthetic dextrose plus casein amino acids; EGF, epidermal growth factor; V_H, variable heavy; V_k, variable light

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2.2. Media

YPD and synthetic dextrose plus casein amino acids (SDCAA) were prepared as in Current Protocols, Chapter 13. For induction media, prepare SDCAA medium, substituting 20 g/l each of galactose and raffinose for the dextrose. Uracil and/or tryptophan are added to SDCAA depending on strain requirements. Minus HUT and minus HUL plates were from TekNova (Halfmoon Bay, CA, USA).

2.3. Oligonucleotides

For construction of pPNL30:

StepA for: TCGAAAAAAGAGAGGCTGAAGCTCTCGAGGCGGCCGCG

StepA rev: AATTCCGGCCGCCTCGAGAGCTTCAGCCTCTCTTTTT

Ck1 for: AGCTCTCGAGGCTCGTACGGTGGCTGCACCATCTGTC

Ck1 rev: TAGAATTCCGCCACACTCTCCCCTGTTGAAGCTCTTTG

For construction of pPNL20:

Aga primer 1: ATGAATTCTACTTCATACATTTTCAATTAAGATGAAGGTTTTGATTGTCT

Aga primer 2: CTTTGCCATTGGCTCTAGCTCAACCGGTTATTTCTACTACCGTCGGTTCCGCTGCAGAA

Aga rev 3: GCTAGAGCCAATGGCAAAGCAGCGAAGATAGCCAACAAGACAATCAAAACCTTCTA

Aga rev 4: ATGCGGCCGCGGATCCAGAGTTTCTCTTGTCCAAAGAGCCTTCTGCAGCGGAACCGAC

Aga-18 for: CTCTGGATCCGCGGCCGCCAGGAACTGACAACTATATGCGAG

Aga-18 rev: AATCTCGAGCCTAGCACTAGTAACATACTGTGTGTTTATGGGGC

cmyc stop: CTAGTGAACAAAAACTTATTTCTGAAGAAGATCTGTAATGAC

cmycr stop: TCGAGTCATTACAGATCTTCTTCAGAAATAAGTTTTTGTTCA

CH1 for: CTCTGGATCCGCTAGCACCAAGGGCCCATC

CH1 rev: GTCAGTTCCTGGCGGCCGCTACCTCCGCCACAAGATTTGGGCTCAACTTTCTTGTCC

For cloning V_H and V_k genes into Fab vectors: variable gene specific primers for heavy and light chains containing the following gap repair tails were used to PCR amplify their respective variable regions.

Gap repair tails for V_H:

For: 5′-AAGGCTCTTTGGACAAGAGAAACTCTGGATCC V_H specific forward oligo

Rev: 5′-GTGCCAGGGGGAAGACCGATGGGCCCTTGGTGCTAGC V_H specific reverse oligo

Gap repair tails for V_k:

For: 5′-ATCTCTCGAAAAAAGAGAGGCTGAAGCTCTCGAG V_k specific forward oligo

Rev: 5′-GGCGGGAAGATGAAGACAGATGGTGCAGCCACCGTACG V_k specific reverse oligo

For amplification of human V_H and V_k genes:

HuVH1aBACK: CAGGTGCAGCTGGTGCAGTCTGG

HuVH3aBACK: GAGGTGCAGCTGGTGGAGTCTGG

HuVH4aBACK: CAGGTGCAGCTGCAGGAGTCGGG

HuVH6aBACK: CAGGTACAGCTGCAGCAGTCAGG

HuVH2bBACK: CAGGTCACCTTGAAGGAGTCTGG

HuVH5bBACK: GAGGTGCAGCTGGTGCAGTCTGG

HuVH7aBACK: CAGGTGCAGCTGGTGCAATCTGG

HuJH1–2FOR: TGAGGAGACGGTGACCAGGGTGCC

HuJH3FOR: TGAAGAGACGGTGACCATTGTCCC

HuJH4–5FOR: TGAGGAGACGGTGACCAGGGTTCC

HUJH6FOR: TGAGGAGACGGTGACCGTGGTCCC

HuVK1aBACK: GACATCCAGATGACCCAGTCTCC

HuVK2aBACK: GATGTTGTGATGACTCAGTCTCC

HuVK3aBACK: GAAATTGTGTTGACGCAGTCTCC

HuVK4aBACK: GACATCGTGATGACCCAGTCTCC

HuVK5aBACK: GAAACGACACTCACGCAGTCTCC

HuVK6aBACK: GAAATTGTGCTGACTCAGTCTCC

HuVK2bBACK: GATATTGTGATGACCCAGATCCC

HuJK1FOR: ACGTTTGATTTCCACCTTGGTCCC

HuJK2FOR: ACGTTTGATCTCCAGCTTGGTCCC

HuJK3FOR: ACGTTTGATATCCACTTTGGTCCC

HuJK4FOR: ACGTTTGATCTCCACCTTGGTCCC

HuJK5FOR: ACGTTTAATCTCCAGTCGTGTCCC

2.4. Antigens

C-terminal biotinylated 12 amino acid peptides were used to generate the scFv antibodies 18a, 18-36, 9, and 378 (a gracious gift from Ettore Apella, NCI). Epidermal growth factor (EGF) and calmodulin were biotinylated using the Pierce (Rockford, IL, USA) NHS EZ link kit, and are the antigens bound by EGF and CaM scFv, respectively. Botulinum neurotoxin serotype A was purchased from Metabiologics (Madison, WI, USA).

2.5. Light chain vector pPNL30

The light chain vector was built on a scFv secretion vector pPNL9 which was originally a modified vector pYC2/CT from Invitrogen [16]. pPNL9 was digested with *Xho*I/*Eco*RI to excise the HA epitope tag and linker and replaced with the Kex2 cleavage site. The annealed oligos (stepA for/rev) were cloned into the *Xho*I/*Eco*RI site with a *Not*I site in the middle. The resulting vector was digested with *Xho*I/*Eco*RI and the Ck gene was PCR amplified with primers designed to insert *Xho*I/*Eco*RI restriction enzyme sites (accession number CK P01834). The PCR amplicon was subsequently digested with *Xho*I/*Eco*RI and ligated into the prepped vector to create the pPNL30 light chain Fab vector. Restriction enzyme digestion with *Xho*I/*Bsi*WI linearized and gapped the vector for direct yeast transformation. Cloning of PCR amplified variable light chains with appropriate linkers was achieved by co-transforming (by the method of Gietz and Schiestl [17]) vector and PCR products and utilizing yeast gap repair [18–20]. YVH10 was used for pPNL30+V_k transformation, and the transformants selected on SDCAA+tryptophan.

2.6. Heavy chain vector pPNL20

pCTcon was digested with *Eco*RI/*Xho*I to remove the insert containing the leader sequence, HA epitope tag, and Aga2 [8]. A synthetic prepro region was built based on oligos (Aga primers 1 and 2, Aga rev primers 3 and 4) using the outside primers and PCR to amplify the construct. *Bam*HI and *Not*I restriction sites were included on the oligos for later cloning steps. Aga2 was amplified without the leader sequence but with a 5′-*Not*I and 3′-*Spe*I site for cloning with the primer set Aga primer 1 and Aga-18 Rev. The c-myc tag was built with the oligos *cmyc stop* and *cmycr stop*. This amplicon and the pCTcon vector were digested with *Eco*RI/*Xho*I, ligated together, and the resulting construct was transformed into *Escherichia coli*. Transformants were isolated, sequenced to verify the correct insert, and then prepped for the cloning of the CH1 domain by digesting with *Nhe*I/*Not*I. The CH1 constant domain was PCR amplified with the primers CH1 for/rev, digested with *Nhe*I/*Not*I, and ligated into the pCTcon vector. The resulting vector, pPNL20, was then transformed into *E. coli*. To clone in V_H genes, pPNL20 was digested with *Bam*HI/*Nhe*I to linearize and gap the vector. The cloning of the variable heavy regions through gap repair was accomplished by PCR amplification of V_H genes using V_H family specific primers that have appropriate regions of homology linkers.

The pPNL20 vector was linearized with the restriction enzymes *Bam*HI and *Nhe*I and the pPNL30 vector was linearized with *Xho*I and *Bsi*WI. All linearized vectors were mixed with each V_H or V_k amplicon at a 1:3 ratio. Yeast transformation was performed using standard LIAc TRAFO method [17]. JAR300 was transformed with pPNL20+V_H and the transformants were selected on SDCAA+uracil agar plate. V_H or V_k inserts were verified by PCR before mating.

2.7. Isolation of V_H and V_k from hybridoma cell line

Total RNA from 1×10^8 hybridoma cells (named 9D8, a kind gift of T. Smith and L. Smith, USAMRIID) was isolated using a kit from Promega (RNAgents® Total RNA Isolation System, Cat.# Z5110) following the instructions from the manufacturer. cDNA for V_H or V_k genes was generated using the 5′RACE System (Gibco BRL Cat.# 18374-058) using the primers VHGSP1: GAAATAGCCCTTGACCAG or VLGSP1: CAAGAAGCACACGACTGA. V_H or V_k genes were amplified separately by PCR from the cDNA using the primers VHGSP2: AGATGGGGGTGTCGTTTTGGC or VLGSP2: GATGGATACAGTTGGTGCAGC following the instructions for the 5′RACE system. The PCR amplified V_H and V_k gene products were cloned into pCR®2.1-TOPO vector and transformed into TOP10F′ competent cells using the TOPO TA Cloning kit from Invitrogen (Cat.# 45-0641). Individual transformants were screened by PCR and the correct size PCR products were sequenced. These transformants were subsequently used as templates for variable gene PCR

amplification using V-gene specific primers with gap repair overhangs for cloning into pPNL20 or pPNL30.

2.8. Human immune Fab library construction and selection of antigen specific clones

For the human immune library construction, human antibody V_H or V_k genes were prepared by RT-PCR using the total RNA isolated from 5.0×10^8 peripheral blood lymphocytes, harvested from a human volunteer immunized with pentavalent botulinum toxoid using an equimolar mixture of human VHBack, JHFor, VkBack and JkFor primers. Primers containing the fore mentioned gap repair tails were used to PCR amplify their respective variable regions for cloning following the RT-PCR. A heavy chain library with 3×10^6 unique members in JAR300 and a κ light chain library with 5×10^5 members in YVH10 were prepared by gap repair and LiAc TRAFO transformation method. 3×10^{10} JAR300 yeast cells from heavy chain library were mated with the same amount of YVH10 yeast cells from the kappa light chain library on 20 YPD agar plates (see Section 2.9 for selection of diploids) and a combinatorial immune Fab library containing 3×10^9 diploids were obtained after mating and selection by growth in dual selection SDCAA (Ura− and Trp−) medium. Randomly picked clone sequencing and/or *Bst*NI fingerprinting verified diverse V_H and V_k libraries.

The human Fab library was induced as described below and sorted once with MACS (magnetic bead aided cell sorting, Miltenyi, Germany) and four times with FACS using biotinylated botulinum neurotoxin type A [16]. Five individual clones were picked out after the last round of sorting, both the V_H and V_k gene in each clone were sequenced, and the binding affinities (K_D) with the toxin in solution were measured by FACS [12].

2.9. Mating conditions

For a single Fab construct, fresh cultures of YVH10/pPNL30-LC (MATα strain) and JAR300/pPNL20-HC (MAT "a" strain) were grown in the selectable media, SDCAA+tryptophan or SDCAA+uracil, respectively. 1 OD_{600}/ml (2×10^7 yeast) of each culture were mixed together, pelleted, and resuspend in 200 μl YPD, before placing in the center of a prewarmed 30°C YPD plate without subsequent spreading. The plates were incubated at 30°C for 4–6 h. No mitotic growth is observed presumably due to pheromone release and cell density. The yeast spot were resuspended in SDCAA medium. Appropriate dilutions were plated based on 10% diploid formation and plated on SDCAA agar plate or grown in liquid SDCAA medium at 30°C with shaking. The OD_{600} reading at the start of growth was below 0.1 OD_{600}/ml to allow growth of the diploids to outcompete the non-growing haploids. For Fab library generation, larger numbers of yeast were used and the volumes adjusted accordingly.

2.10. Inductions

Freshly saturated SDCAA cultures were resuspended in SG/RCAA+0.2% dextrose and grown at 18°C for 24 h with shaking to optimally induce expression of the Fab on the surface of the diploid.

2.11. Flow cytometry

Typically $1–5\times10^6$ yeast were resuspended in 200 μl wash buffer (phosphate-buffered saline+1% bovine serum albumin) to which either 1 μg of anti-c-myc (9E10, Covance, BAbco) or anti-SV5 monoclonal antibody (mAb) was added, and if appropriate, biotinylated antigen was also added. Yeast were incubated at room temperature for 1 h, followed by 10 min on ice. The cells were washed three times with 1 ml of 4°C wash buffer, and then incubated on ice for 30 min with the secondary detection reagents. For the biotinylated antigen, detection was with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA). Goat anti-mouse Alexa-488 or phycoerythrin conjugated antibodies were used to detect the bound anti-c-myc and anti-SV5 antibody. The cells were washed once before analysis by flow cytometry.

2.12. K_D determinations

Quantitative equilibrium binding was determined as described previously [11,12]. To verify the protein–ligand affinity constant (K_D) within the surface display context, we performed flow cytometric analysis of the c-myc normalized antigen binding for scFv clones, and SV5 normalized antigen binding for Fab clones. In general we used 6–10 different antigen concentrations covering 10× antigen concentration above and below the K_D to determine the K_D. Each K_D was determined in triplicate, three separate inductions and measurements.

3. Results

3.1. Construction of vectors and strains

Yeast display of Fab fragments by mating was dependent on the successful construction of appropriate strains and vectors (Fig. 1). In the system developed, the heavy chain is fused to the yeast surface via Aga 1 and 2 and the light chain is secreted. In the haploid yeast, the heavy chain vector is selected using the Trp and Ura auxotrophic markers for selection of heavy and light chain vectors. After mating, maintenance of both plasmids was ensured by selecting for growth in the absence of the amino acid tryptophan and uracil. The heavy chain vector was built on the backbone of the scFv surface display vector, pCTcon [8], and the light chain vector on pYC2/CT from Invitrogen. However, major modifications were required to display the heavy chain of the Fab as a fusion protein to Aga 2 (covered in greater detail in Section 2). In contrast to published scFv and Fab yeast display systems [8,14], the heavy chain was placed at the amino-terminus of the fusion protein and the Aga 2 fusion partner at the carboxy-terminal end of the fusion protein. Thus, the Fab antigen binding pocket faces away from the surface of the yeast and is unhindered by any type of linker. Fig. 1 provides a schematic of the vectors constructed for the display of Fab fragments.

3.2. Testing of expression of Fabs from pPNL20 and pPNL30

Eight antigen specific antibody fragments (seven scFv and one domain antibody (V_H only)) binding seven different antigens were transferred into the Fab vectors using gap repair tailed sequence specific PCR primers, to determine the functionality of the system. Heavy chain expression in haploid strains was verified by flow cytometric detection of the C-terminal c-myc epitope tag indicating complete translation of the V_H, CH1, and Aga2 genes with display on the surface of the yeast of the antibody fragment heavy chains. Examples of six VH-CH1 fusions are shown in Fig. 2A. Of these, two of the eight heavy chains were able to bind antigen in the absence of light chain (Fig. 2B). Light chain gene expression in haploid strains was demonstrated for all antibodies by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting of supernatants from induced cultures with detection of light chain using an anti-SV5 antibody (data not shown).

3.3. Haploid mating and diploid characterization

The haploids were mated as described in Section 2 and diploids selected. Heavy chain (c-myc) and light chain (anti-SV5) expression was measured by flow cytometry after induction (Fig. 2C,D). Light chain can only be detected on the surface of the yeast if bound to the heavy chain, as only the heavy chain forms the covalent attachment to the surface of the yeast through the Aga1 and Aga2 proteins. All eight Fab (AR1 not shown, but data included in Table 1) demonstrated expression and display of both heavy and light chain (Fig. 2C,D). We next determined if the displayed Fab could bind antigen. All four Fab constructed from the V-genes of protein binding scFv bound antigen, however only two of the four Fabs constructed from peptide binding scFv bound antigen (Fig. 2E). The reason for the loss of binding is unknown, but could be related to structural differences between the scFv and Fab forms of the antibody fragment. The binding affinity of

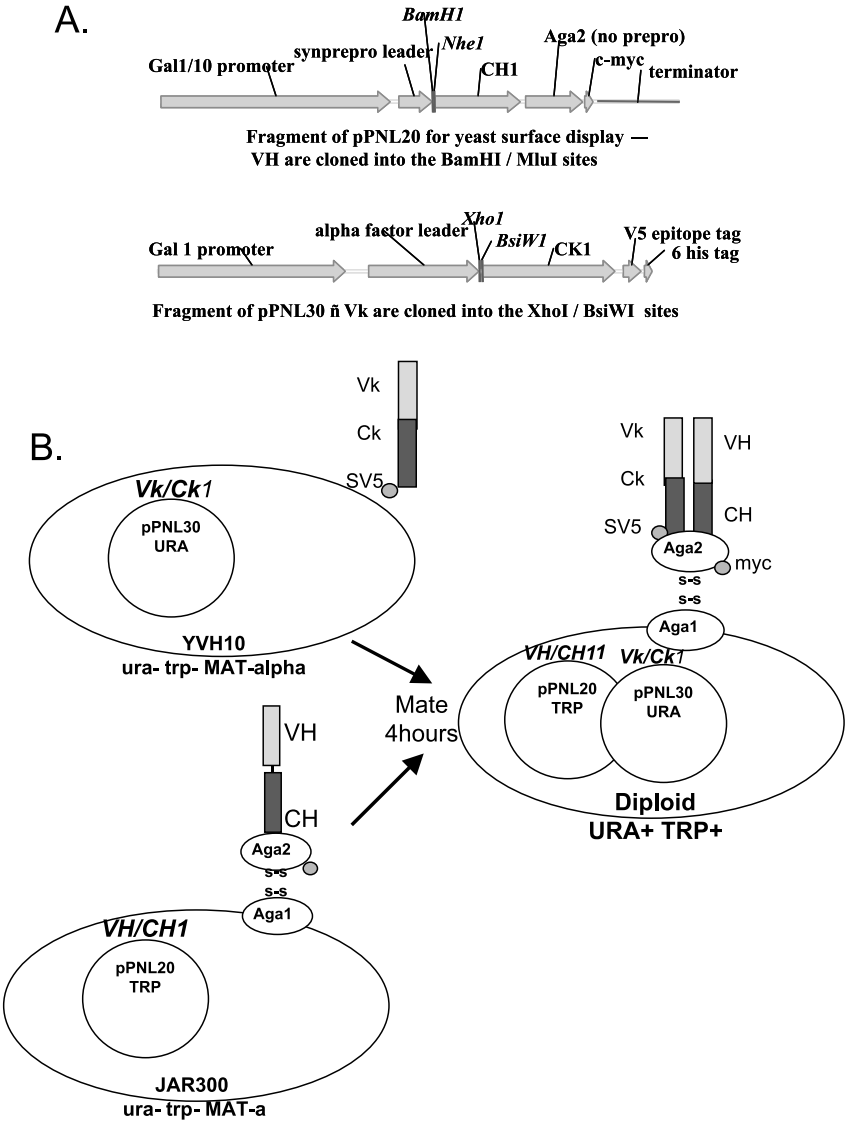

Fig. 1. Vectors used for Fab generation by yeast mating. A: Schematic of vectors used for Fab expression constructs for light chain (pPNL30) and heavy chain (pPNL20). B: Schematic of haploid yeast mating to create heterodimeric Fab display as a heavy chain fusion to Aga2 and the light chain disulfide linked to the heavy chain. The variable heavy chain is cloned into pPNL20 as a translational fusion to Aga2 through the CH1 domain of IgG. The variable light chain is cloned into pPNL30 and is secreted into the medium. After mating of HC::LC haploids, the resulting dual auxotrophic marker selected diploids express light chain associated with the heavy chain. The association presumably occurs in the Golgi, as the Fab is delivered to the surface.

the five antigen binding Fabs was measured in triplicate by equilibrium based measurements using FACS and compared to the affinity of the scFv (Table 1). Three Fabs had similar affinities to the scFv from which they were derived, while the single domain CaM8 clone showed a 10-fold increase in affinity and the 18a peptide binding clone had a decrease in affinity.

3.4. Demonstration of random mating and concomitant chain shuffling

Eight heavy chain and six light chain haploids (Table 1 and Section 2) were mixed to allow random mating to occur creating a mini-library with a total diversity of 48 in order to demonstrate that heavy chain and light chain will associate and recapitulate Fab binding. The expected frequency of any antigen specific Fab would be approximately 2% of the displayed population. After induction of the mini-library, library diploids were stained with either biotinylated phosphopeptide 18p, phosphopeptide 378p, or EGF. For the two phosphopeptide antigens, the frequency of antigen binding yeast was 0.5% and 0.8% of the total population, or 1.7% and 2.8% of the antibody expressing population (29% are SV5 positive) (Fig. 3A–D). This is consistent with the expected 2% positive if mating is random. The difference in mean fluorescent intensity for 18p and 378p antigen binding is due to the differences in affinity of the two Fabs for their antigen (1.5 μM vs. 4 nM). The two 18p and 378p antigen binding populations were sorted by FACS and the diploids plated on −HUT plates. Five clones, of each specificity, were sequenced, confirming the expected heavy and light chain pairings in all 10 clones.

The mini-library was also stained with biotinylated EGF. As noted above, the heavy chain alone binds EGF in the absence of light chain and can also bind EGF in the presence of any of the six light chains. Therefore, the expected antigen

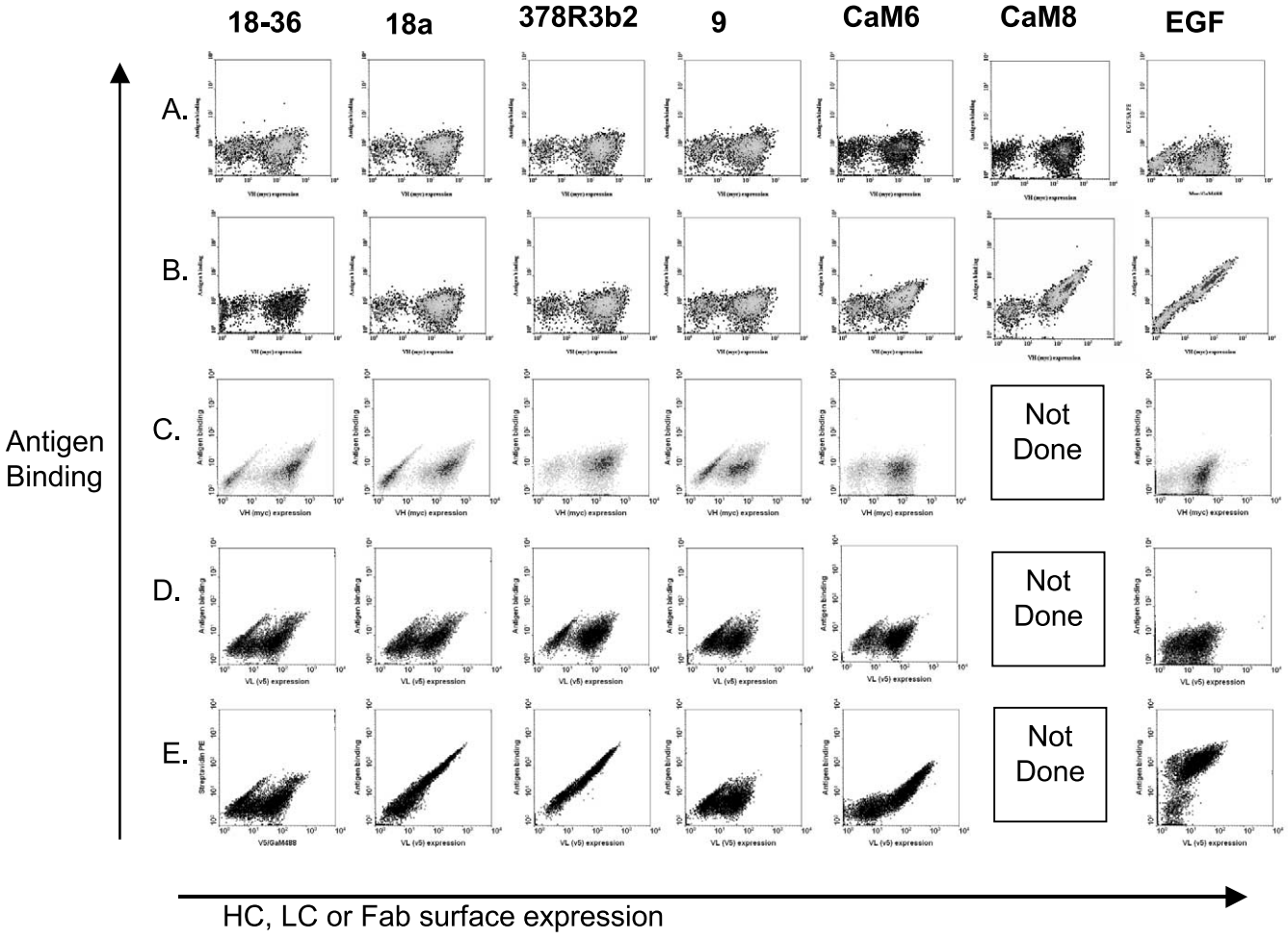

Fig. 2. Heavy and light chain surface display and antigen binding of Fab constructed by yeast mating. Flow cytometric bivariate plots of heavy and light chain surface display (*x*-axis) and antigen binding (*y*-axis) are shown for six Fab constructed from antigen specific scFv (18-36, 18a, 378R, 9, CaM6 and EGF). Heavy chain only display and antigen binding is also shown for a single domain antibody (CaM8). A: Heavy chain surface display in haploid yeast. B: Heavy chain surface display and antigen binding in haploid yeast. C: Heavy chain surface display in diploid yeast. D: Light chain surface display on diploid yeast. E: Light chain display and antigen binding of surface displayed Fab. Heavy chain expression was determined using a C-terminal myc epitope tag and light chain expression determined using a C-terminal SV5 epitope tag and an appropriate secondary antibody and is shown on the *x*-axis. Antigen binding was detected using biotinylated antigen and streptavidin-phycoerythrin (PE) or streptavidin-Alexa633 (SA633) and is shown on the *y*-axis.

binding population in the mini-library was 6/48 or 12.5%, of the Fab expressing population. Experimentally, 11.3% of the Fab expressing population bound EGF (3.3% binding EGF out of the 29% of Fab expressing yeast, Fig. 3E). After sorting the EGF binding population, all 10 colonies analyzed had the correct heavy chain and all six light chains in the mini-library were represented. After EGF staining, we also noted that 0.5% of the population was SV5 negative, myc positive, and EGF positive (Fig. 3E). These clones presumably were heavy chain only with no light chain present. We sorted out these clones and plated them on non-selectable YPD plates and then subsequently replica plated the colonies onto the three selectable plates, −HUT for diploids, −HUL for light chains, and −HU for heavy chains. We found that the SV5 negative, antigen binding positive population represents yeast that had lost the light chain plasmid. The estimated frequency of plasmid loss can be calculated as approximately 15% (0.5%/3.3%) and likely occurs for all clones. While not insignificant, the impact of plasmid loss/clone loss can be overcome by screening and sorting a greater number of yeast than the library size.

3.5. Light chain binding to heavy chain is diploid specific

To determine if light chain promiscuity is present, for example secreted LC of a Fab diploid can bind to the HC Fab displayed on the surface of yeast, we performed the following

Table 1
Affinity measurements of scFv and Fab by flow cytometry

Clone	V_H/V_k usage	scFv	Fab
378R	VH1/VK1	3.9 ± 1.9 nM	4.3 ± 1.8 nM
9	VH6/VK1	9 ± 4 nM	No binding
18-36	VH4/VK1	310 ± 100 nM	No binding
18a	VH3/VK1	666 ± 137 nM	>1 μM
CaM6	VH3/VKIII	>2 μM	>2 μM
CaM8	VH3/none	>2 μM	164 ± 56 nM
EGF	VH4/VKIII	8 ± 1 nM	5.1 ± 1.6 nM
AR1	VH3/VKIII	330 ± 70 pM	300 ± 59 pM

The affinity of eight Fab constructed from the V-genes of seven scFv and one domain antibody (V_H only) are shown, The V_H and V_k usage is listed to demonstrate no variable region usage bias in binding characteristics. The measurements were determined in triplicate as in [11,12].

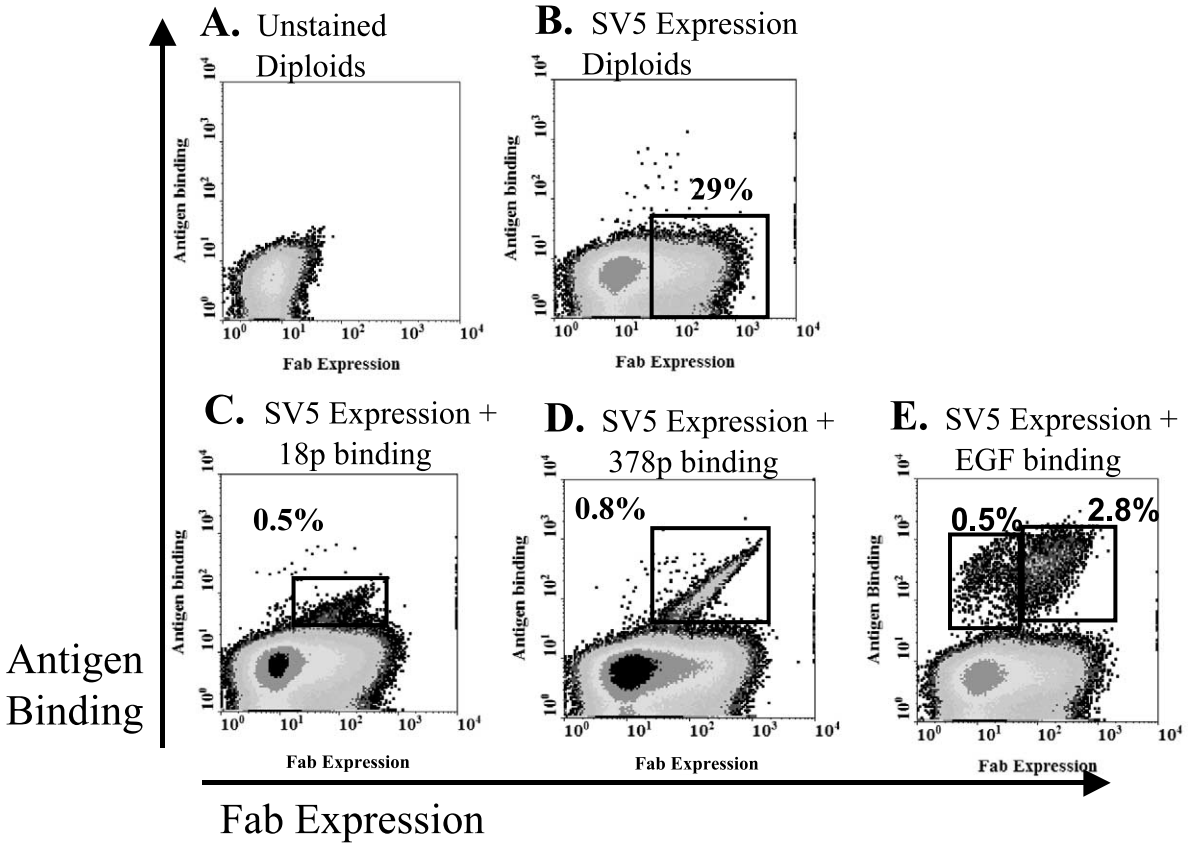

Fig. 3. Recapitulation of heavy and light chain pairing and antigen binding in a mini-library created by yeast mating. A Fab mini-library was created by mating six different heavy chains with eight different light chains and surface display and antigen binding measured and shown as flow cytometric bivariate plots. A: Unstained diploid yeast. B: Fab expression as determined using the light chain SV5 epitope tag (anti-V5 mAb+goat anti-mouse phycoerythrin). C: Fab expression and binding of biotinylated 18p peptide. The sort gate indicates the 0.5% of the population binding the peptide. D: Fab expression and binding of biotinylated 378p peptide. The sort gate indicates the 0.8% of the population binding the peptide. E: Fab expression and binding of biotinylated EGF. The sort gates indicate the 2.8% population binding EGF as a Fab and the 0.5% of the population binding as a heavy chain only (SV5 negative). Antigen binding was detected using streptavidin-phycoerythrin or streptavidin-Alexa633 and is shown on the *y*-axis.

analysis using the fore mentioned haploid HC and LC expressing clones and diploid Fabs, 378 and 9. The HC haploid of a known antigen binding clones, 378 or irrelevant HC 9, was induced to express the HC on the surface. The respective 378 and 9 LC haploids as well as the 378 diploid were also induced to secrete the light chain into the supernatant. The V5 labeled LC secretion was verified by V5 Western of the supernatant (data not shown). The HC expression was verified by c-myc staining. The LC containing supernatants were then incubated with the HC expressing haploid yeast for 8 h at 20°C. The yeast were washed and the ability to label the yeast with anti-V5 mAb was determined by flow cytometry. We examined the ability of secreted LC from the *diploid* 378 expressing Fab to bind to the 378 or 9 HC on the surface of the *haploid* yeast. No V5 epitope label could be detected, therefore no LC is bound to the HC (Fig. 4C,I). In a second experiment, both *haploid* LC supernatants were incubated with each of the 378 and 9 HC *haploid cells*. Interestingly, a small percentage, 6% of the clonal population of both 9 and 378 HC haploids, were positive for V5 labeling with 378 LC, but not 9 LC, supernatant (Fig. 4D,E,J,K). The lack of binding of the LC to the HC may simply be a reflection of the glycosylated homodimeric form of the LC present in the supernatant (unpublished observation). The homodimeric form may be the result of a bifurcation in the secretory pathway at the juncture of plasma membrane delivery and secretory vesicles where differential glycosylation and lack of pairing to HC results in the secreted hyperglycosylated homodimeric form of the LC. Therefore, we conclude that although secreted LC from diploids could potentially bind to other yeast, thus breaking the phenotype–genotype link, we are unable to measure it, or detect this event.

3.6. *Identification of the correct V_H and V_k pairing from a polyclonal antibody*

To further demonstrate the ability of the mating system to recapitulate Fab antigen binding, we used the system to identify the correct V_H and V_k pairing from a hybridoma producing multiple antibodies. The V_H and V_k genes of the botulinum neurotoxin binding murine 'monoclonal' antibody 9D8 were cloned from the hybridoma using 5′RACE. Sequencing of 20 V_H and V_k genes revealed the presence of two different V_H genes and three different V_k genes, indicating the presence of multiple different antibodies in the hybridoma. To determine which V_H and V_k genes were responsible for antigen binding each V_H and V_k gene was cloned into the appropriate yeast vector and then mated to generate the six possible Fabs. After induction, all six unique pairings were displayed on the

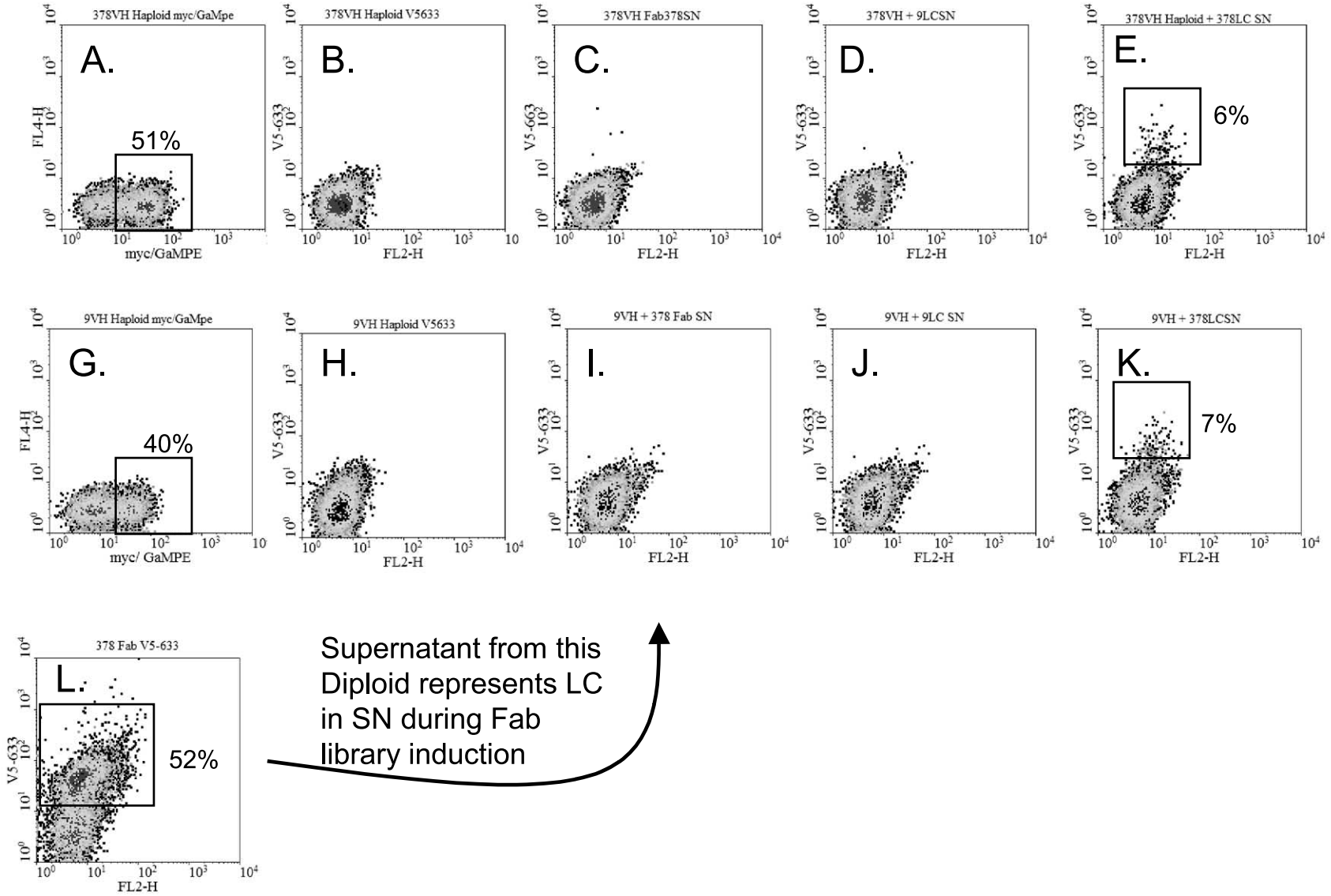

Fig. 4. Secreted light chain from diploids does not bind to heavy chains expressed on haploids. Bivariate plot analysis of LC binding to HC expressing haploids 378 and 9. A,G: Haploids 378 and 9 HC c-myc expression on the *x*-axis. LC binding is detected using anti-V5-633 (*y*-axis). B,H: HC haploids 378 and 9 anti-V5-633 control in the absence of LC. C,I: LC containing supernatant from diploid 378 binding to HC of haploids 378 and 9. D,J: LC containing supernatant from LC haploid 9. E,K: LC containing supernatant from LC haploid 378. L: V5-633 labeling of diploid Fab clone 378 showing LC binding to HC.

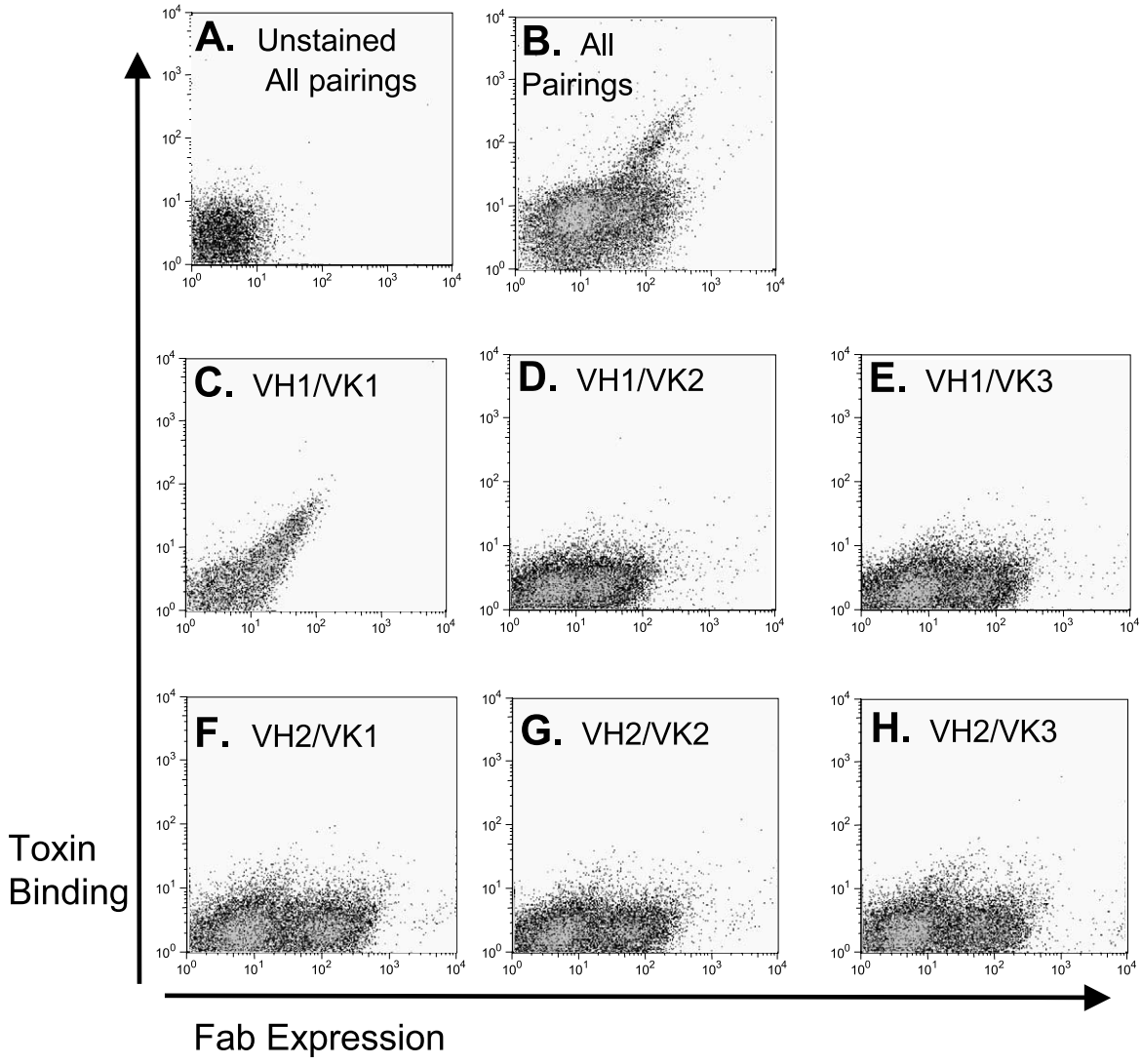

Fig. 5. Identification of correct heavy and light chain pairing from the hybridoma, 9D8, containing multiple heavy and light chains. The two heavy chains and three light chains cloned from the mixed hybridoma 9D8 were cloned into the appropriate yeast vector and the haploid yeast mated to generate the six possible Fabs. While all six possible Fab had Fab surface display, only one combination, VH1/VK1, recapitulated antigen binding. When random mating was allowed to occur, a distinct population of antigen binding clones is observed (All pairings). Fab surface display detected using anti-SV5 antibody. Antigen (toxin) binding detected using biotinylated botulinum neurotoxin type A and streptavidin-phycoerythrin.

surface of yeast, however only one of these pairings bound antigen (Fig. 5). Random mating of yeast containing the two V_H genes and the three V_k genes yielded a frequency of antigen binding Fab (6.25%) which approximated the expected frequency of antigen binding (1/6 of the 40% of the yeast that expressed Fab) (Fig. 5), indicating that mating was random. Sorting and sequencing of the antigen binding population revealed that 100% of the Fab were of the expected pairing determined by analysis of the six possible monoclonal Fabs (VH1/VK1). Fab affinity determined by flow cytometry (0.83 nM, Fig. 6A,B) was comparable to the affinity of the purified IgG (0.7 nM) determined by BIAcore.

3.7. Construction, selection and characterization of a large immune yeast displayed human Fab library

V_H and V_k genes were amplified by PCR from RNA prepared from a human volunteer immunized with pentavalent botulinum toxoid. The PCR products were used to create haploid V_H and V_k libraries in the appropriate yeast strain using gap repair. The V_H library in JAR300 contained 3×10^6 members and was diverse by PCR fingerprinting and DNA sequencing. The V_k library in YVH10 contained 5×10^5 members and was diverse by PCR fingerprinting and DNA sequencing. Mating the two yeast libraries created a large combinatorial library containing 3×10^9 unique members. Twenty random yeast colonies analyzed by PCR contained both heavy and light chain genes, which were diverse by PCR fingerprinting. The resulting library was stringently selected using MACS and FACS and decreasing concentrations of biotinylated botulinum neurotoxin (Fig. 7). These conditions select for the highest affinity and best expressing clones, with a concomitant reduction in clonal diversity. After five rounds of selection, five random clones were sequenced. These clones represented five unique Fab consisting of three unique V_H genes, resulting from the same V-D-J rearrangement, and five unique light chains derived from four different germ line

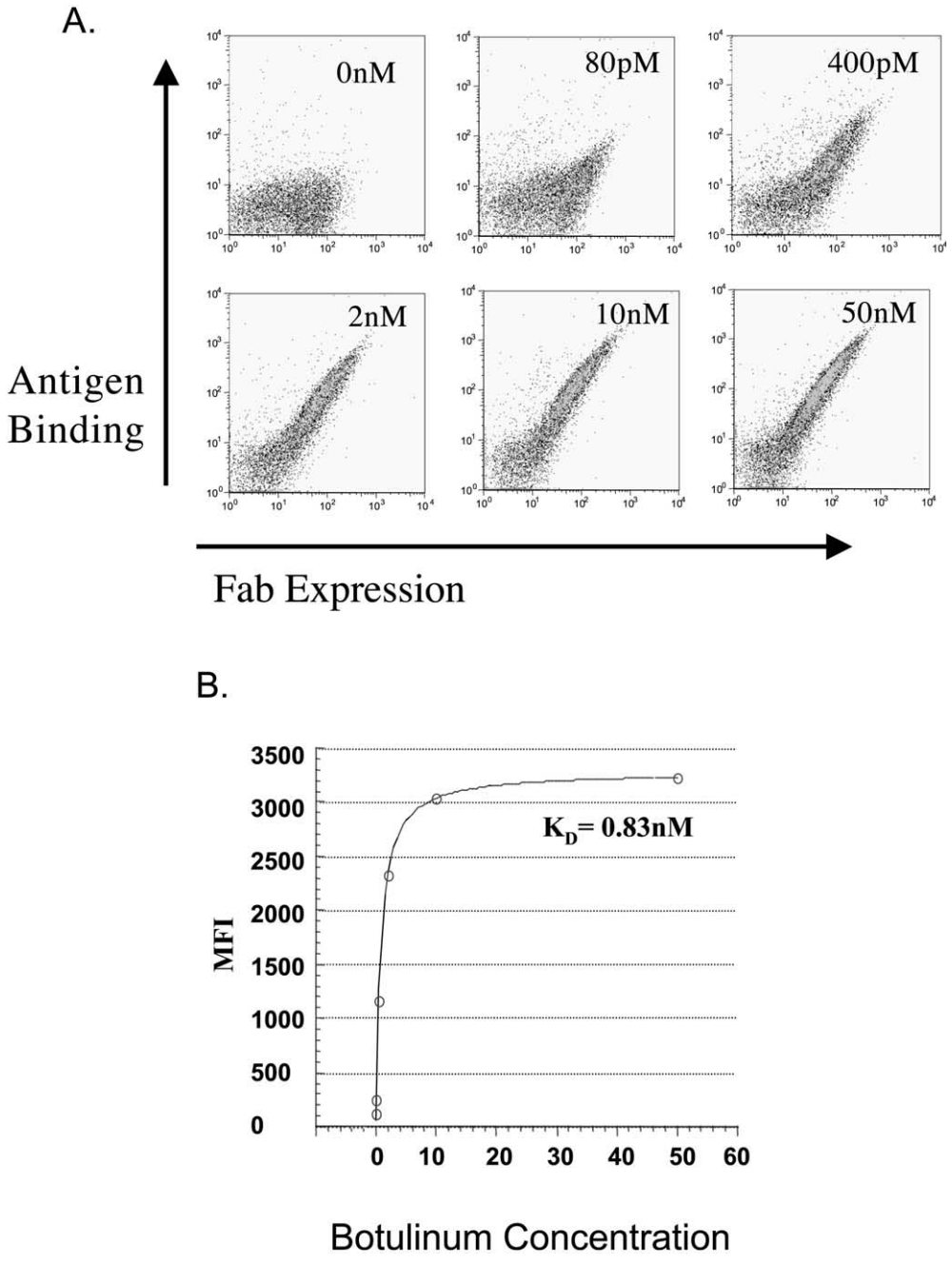

Fig. 6. Equilibrium based K_D determination of surface expressed Fab 9D8. A: Bivariate plot of Fab surface display and antigen (botulinum neurotoxin) binding at the listed antigen concentrations. Fab display was quantified using SV5 antibody. B: The mean fluorescence intensity (MFI) of the Fab antigen binding population is used in conjunction with a non-linear least squares fit to fit the following equation to determine the K_D. $y = m1 + m2 \times m0/(m3 + m0)$ where y = MFI at given antigen concentration, $m0$ = antigen concentration, $m1$ = MFI of no antigen control, $m2$ = MFI at saturation, and $m3 = K_D$.

genes (Table 2). The affinities of these Fabs for botulinum toxin ranged from 0.8 nM to 2.1 nM (Table 2).

4. Discussion

Antibody gene diversity libraries and display technologies have become popular tools to generate reagent, diagnostic and therapeutic antibodies. In considering the choice of display format, important factors include the ability of the library to generate panels of antibodies of high affinity, the ease of antibody characterization, including affinity and epitope, and the stability of the library and ease of amplification by serial culture. Advantages of yeast display compared to other display technologies include the ability to rapidly measure affinity and epitope by flow cytometry while still in the display format, obviating the need for antibody fragment purification. Yeast libraries have also proven stable upon sequential amplification. The lower transformation efficiency of yeast compared to bacteria, however, limits library size. This is important since the single most important factor affecting the number of antibodies generated is library size and diversity.

We have shown that by utilizing yeast mating it is possible

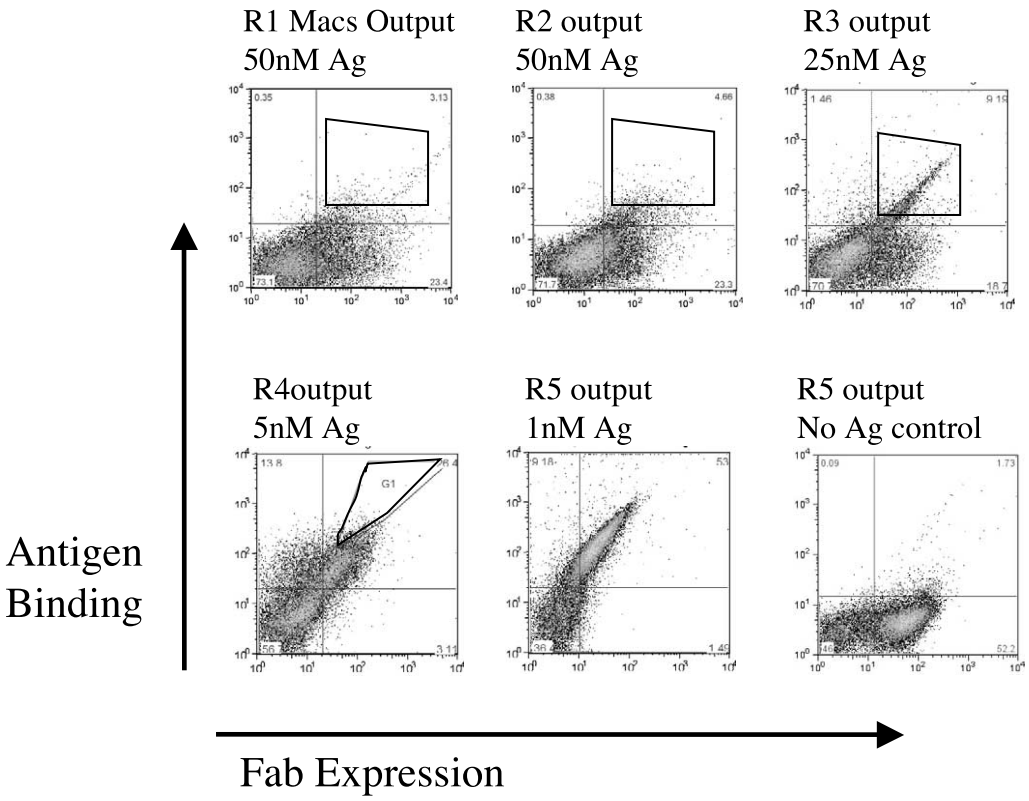

Fig. 7. Bivariate plots of antigen specific Fab selections from an immune Fab yeast display library. Fab surface display and antigen binding are shown for the first (R1) through fifth (R5) round of selection. Plots are shown for yeast induced after the indicated round of selection, with the first round being performed using MACS and subsequent rounds using FACS. The antigen concentration used for selection is indicated above the plots. The approximate sort gates are indicated on the bivariate plots.

to generate very large yeast Fab libraries from easily constructed small libraries of heavy and light chains. By taking advantage of yeast homologous recombination (gap repair), construction of the heavy and light chain libraries is further simplified, eliminating the need to first construct libraries in *E. coli* and shuttle them to yeast. The modular nature of the Fab heterodimer is amenable to this type of combinatorial approach. Making small (10^7 diverse) libraries of the heavy and light chains in yeast, in conjunction with yeast mating, creates combinatorial libraries of 10^{14} theoretical diversity. Realistically, only about 10^{11} yeast, approximately a 15 ml cell pellet, can currently be screened effectively for antigen reactive clones. Utilizing highly parallel magnetic sorting in conjunction with flow cytometry, it is possible to effectively screen a library of 10^{10} clones [10].

Combinatorial Fab libraries have been previously generated in bacteria using infection to introduce phage displayed heavy chains into bacteria already containing light chain genes on a separate plasmid [22,23]. These libraries have turned out to relatively unstable, due to the use of phage and the reversibility of the recombination [22,23]. However, a single vector recombination system that relies on high multiplicity of infection to introduce multiple phagemids carrying different VH and Vk genes into a single bacteria has proven to BE more robust, and has been used to make large functional phage antibody libraries [21]. Although combinatorial yeast two hybrid libraries have been used for years, we are not aware of any prior description of mating to construct large Fab libraries [24–27]. Recently an error prone PCR Fab library was displayed on yeast, however combinatorial mating was not employed and the variable heavy and light chains resided on a single plasmid [14]. The system described here differs by utilizing a two vector, two strain system to display the Fab on the surface of yeast, with the two vectors brought together by mating. Furthermore, the heavy chain is expressed as an amino-terminal fusion to Aga2, thus enabling a nearly native

Table 2
Affinity, V-gene usage and number of mutations from germ line of five Fab binding botulinum neurotoxin type A

Clone name	V_H			V_k			K_D (nM)
	Family	Germ line	Mutations	Family	Germ line	Mutations	
A1	VH3	HHG4	33	VK1	L12a/PCRdi16-5	10	2.1 ± 1.1
A6	VH3	HHG4	25	VK1	DPK9/O12	40	1.1 ± 0.5
A7	VH3	HHG4	26	VK1	DPK5/Vb	11	1.0 ± 0.3
A8	VH3	HHG4	30	VK1	DPK1/O18	54	1.8 ± 1.0
A18	VH3	HHG4	26	VK1	L12a/PCRdi16-5	24	0.8 ± 0.1

The germ line V was determined using the V BASE database of germ line genes (www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu = 90) and DNAPLOT.

and free amino-termini of the heavy chain, which eliminates the potential adverse binding affects a linker could have on antigen interaction.

An ongoing controversy in the antibody engineering field is the format for the displayed antibody fragment, scFv or Fab. As we and others [23] have shown, one potential advantage of the Fab format lies in ease of diversity generation by encoding the two chains on different vectors. In addition, it is likely that Fab are more stable than scFv, due to an additional domain–domain interface resulting from the CH1–CL association. Such stability is critical for applications such as antibody arrays and other proteomic uses, and can result in higher affinity of purified antibody fragment, due to a higher functional concentration of antibody. For example, several IgGs constructed from scFv were found to have significantly higher affinity and faster association rate constants due to increased stability [28]. The results presented here also suggest that antibody affinity increases when the same V-genes are expressed as Fab compared to scFv. As a result, we propose that a Fab library would provide, on average, higher affinity clones when compared to a comparably sized scFv library. Consideration of antibody format is also important when the display technology is merely a route to antibody V-genes that will be converted to another form, most commonly conversion to IgG, the most preferred format for therapeutic antibodies. Since we observed that two scFv lost antigen binding when converted to Fab, it is likely that a similar loss of antigen binding would occur if converted to IgG. Use of the Fab display in the antibody discovery process should reduce the frequency with which antigen binding clones are lost upon conversion to IgG.

In conclusion, we have demonstrated the ability to display the heterodimeric Fab fragment on the yeast surface as an amino-terminal fusion to the Aga1 protein where the two chains are brought together by mating. We have shown that mating is random and can be used to generate large diverse Fab antibody libraries from which high affinity antigen specific Fab can be isolated. Construction of non-immune Fab libraries using this approach should provide a rapid route to high affinity Fab which retain antigen binding properties upon production as a soluble monovalent Fab fragment or IgG. The system should also permit rapid affinity maturation or humanization by chain shuffling. This approach should significantly facilitate the generation of reagent, diagnostic, and therapeutic antibodies.

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