Affinity maturation of Fab antibody fragments by fluorescent activated cell sorting of yeast-displayed libraries

Twan van den Beucken[a], Henk Pieters[a], Mieke Steukers[a], Marcel van der Vaart[b],
Robert Charles Ladner[c], Hennie R. Hoogenboom[a], Simon E. Hufton[a,*]

[a]*Dyax s.a., Boulevard du Rectorat 27^B, Sart Tilman, 4000 Liège 1, Belgium*
[b]*Unilever Research Vlaardingen, O.V. Noortlaan 120, 3133 AT Vlaardingen, The Netherlands*
[c]*Dyax Corp., 300 Technology Square, Cambridge, MA 02139, USA*

Abstract We report for the first time the affinity maturation of Fab antibody fragments using fluorescent-activated cell sorting (FACS) of yeast-displayed repertoires. A single yeast display vector which enables the inducible expression of an anchored heavy chain and a soluble light chain has been constructed. The assembly and functional display on the yeast cell surface of Fab antibodies specific for different protein targets has been demonstrated by flow cytometry and immunofluorescence microscopy. We have affinity matured a Fab antibody specific for the tetravalent antigen streptavidin using FACS of yeast-displayed repertoires diversified by error-prone polymerase chain reaction. A panel of variants with up to 10.7-fold improvement in affinity was obtained after selection. Two leading clones, R2H10 (3.2 nM) and R3B1 (5.5 nM), had mutations in light chain complementarity determining region 1 LC-CDR1 (H34R) and LC-CDR3 (Y96H or Y96F) and gave a 10.7-fold and 6.3-fold affinity improvement over the starting antibody, respectively. The ability to efficiently affinity mature Fab antibodies is an important component of the antibody development pipeline and we have shown that yeast display is an efficient method for this purpose.
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Key words: Yeast display; Fab antibody; Affinity maturation

1. Introduction

The past decade has seen recombinant antibodies become an increasingly important group of drugs, which currently represent over 30% of biopharmaceuticals in clinical trials. As such, their rapid isolation, characterisation and optimisation are of critical importance. The advent of phage display has done much to accelerate antibody development [1] and large 'single pot' phage-displayed human scFv [2] or Fab [3] antibody libraries have been used for rapid isolation of binders to a broad range of disease-associated targets.

Although phage display is a very powerful technology for the isolation of lead antibodies, these antibodies often require affinity optimisation prior to clinical development, as improved binding generally correlates with improved clinical efficacy. For example, it has been shown that increasing the affinity of antibodies improves selective tumour delivery, which is likely to increase their utility in tumour imaging and therapy [4]. It is, however, difficult to predict what affinity threshold is required for any given therapeutic application since this may not be the only determinant of biological potency. Parameters such as antigen density, antigen location, antibody specificity, antibody stability, antibody clearance and immune effector functions may also be important. However, these parameters are not as easily manipulated as antibody affinity [5]. The antigen location and concentration may be of particular significance. For example, the affinity required to bind and neutralise a soluble target may be much higher than that which is required for specific binding to a cell surface-bound target. This is illustrated by the therapeutic affinities of two very different clinically approved antibodies, Remicade™ for the treatment of rheumatoid arthritis and Herceptin™ for the treatment of breast cancer. Remicade™ is specific for soluble tumour necrosis factor α and has an apparent affinity of 100 pM [6], whereas Herceptin™ binds to the cell surface HER-2/neu proto-oncogene and has a significantly lower affinity, of 5 nM [7]. Generally antibodies that bind with low nM (1–10 nM) monovalent binding affinities to cell surface targets can be expected to be a minimum requirement for clinical development. For cell surface targets, the affinity of lead antibodies from phage display libraries sometimes falls within this range; however, for applications such as cytokine neutralisation it can be expected that affinity improvements of at least 10-fold will generally be required. Thus there is a clear need for affinity maturation methods that can give reliable improvements in affinity quickly.

Affinity maturation requires the generation of secondary diversified libraries, from which isolates with improved affinity are then selected. Various methods of antibody gene diversification have been described, ranging from non-directed approaches (i.e. mutator strains, V-gene chain shuffling, error-prone polymerase chain reaction (PCR) or DNA shuffling), to approaches where mutations are targeted to the antibody's complementarity determining region (CDR) (i.e. parsimonious mutagenesis, CDR randomisation or hot-spot mutagenesis [8]). The ability to quantitatively select these secondary libraries for improved variants is of critical importance and has

*Corresponding author.
E-mail address:* shufton@dyax.com (S.E. Hufton).

Abbreviations: CDR, complementarity determining region; VH, variable region of an antibody heavy chain; VL, variable region of an antibody light chain; FACS, fluorescent-activated cell sorting; LC, light chain; HC, heavy chain

generally been accomplished using phage display [9,10]; however, recent advances in yeast display technology have made this process much more efficient [11,12]. The successful application of yeast display to antibody affinity maturation can be largely attributed to the fact that repertoires can be selected by flow cytometric cell sorting. This selection process with untethered antigen allows for quantitative assessment of antigen binding and display level of each individual antibody-displaying yeast cell [13,14]. In contrast, selection for improved affinity can be less predictable using phage display, particularly with immobilised target, and can lead to selection for parameters other than improved affinity [15–18].

Previous studies using yeast display have described the affinity maturation of single chain molecules such as scFv antibody fragments [11,19] and scTCR molecules [20]. However, as yet this technology has not been used for the maturation of heterodimeric molecules anchored by one member of a pair of distinct polypeptides. We describe for the first time the display and affinity maturation of a heterodimeric Fab antibody specific for the tetravalent protein target streptavidin, using fluorescent-activated cell sorting (FACS) selection of yeast-displayed repertoires.

2. Materials and methods

2.1. Construction of yeast Fab display vector

The pYD1 yeast display vector (Invitrogen) was engineered for the display of antibody Fab fragments into vector pTQ3 (Fig. 1A). This modified vector allows the tandem expression of soluble light chains (LC) and heavy chains (HC) fused to the Aga2p yeast cell surface anchor protein. To make the vector compatible with our phage display and IgG expression systems, three ApaLI sites (1393, 3047, 4293) were removed from pYD1 by Quikchange site-directed mutagenesis (Stratagene) according to the manufacturer's instructions. In the AGA2 signal sequence a NheI cleavage site was introduced by site-directed mutagenesis. A polylinker was constructed by annealing complementary oligonucleotides and cloned as a NheI/PmeI fragment into the modified pYD1 vector. This polylinker introduced unique cloning sites (ApaLI, AscI, BamHI, PstI, EcoRI, PacI and PmeI) in the vector and a haemagglutinin (HA) epitope tag at the 3′ end of the AGA2 signal sequence. The ApaLI/AscI unique restriction sites were to facilitate cloning of LC fragments. The α1 mating factor transcriptional terminator (Matα) was amplified by PCR from the original pYD1 vector and cloned behind the HA tag as a BamHI/PstI fragment. A part of the expression cassette of pYD1 containing the GAL1 promoter, AGA2 signal sequence, AGA2 gene and glycine/serine linker was amplified by PCR using a reverse primer that incorporates the c-myc epitope tag and the SfiI/NotI restriction sites for HC cloning. This fragment was cloned behind the first Matα terminator sequence as a EcoRI/PacI fragment to give the final vector construct pTQ3.

2.2. Flow cytometric analysis of yeast cells

Induction of antibody expression and flow cytometric analysis was performed as described previously [21]. Briefly, yeast cells were grown to logarithmic phase and induced with galactose 2% (w/v) for 48 h at 20°C with shaking. After induction cells were labelled with anti-c-myc (25 μg/ml) or anti-HA (25 μg/ml) (Roche) for 1 h at room temperature. Subsequently cells were incubated on ice for 1 h with 1:40 rabbit anti-mouse fluorescein isothiocyanate (FITC) and 500 nM streptavidin-PE. Cells were washed once with 0.5 ml phosphate-buffered saline (PBS) between all labelling steps and were analysed on an Epics Altra flow cytometer (Beckman Coulter). For determination of dissociation rate constants cells were resuspended in 1 μM unlabelled streptavidin after the final wash and antigen binding was monitored over a 30 min period. The decrease in fluorescence over time was fitted to an exponential decay model from which off rate was determined as described [21].

2.3. Construction of diversified repertoires by error-prone PCR

The anti-streptavidin Fab fragment F2 was randomly mutated us-

ing error-prone PCR as described previously [22]. Briefly, the Fab was amplified from pTQ3 vector using primers 5′-ACG ACG TTC CAG ACT ACG C-3′ and 5′-TTC AGA TCC TCT TCT GAG ATG AG-3′. After purification of the PCR products they were digested with ApaLI and AscI for the LC, SfiI and NotI for the HC and ApaLI and NotI for the cloning of the whole Fab fragment. The digested products were gel purified by Wizard PCR and cloned separately into similarly treated empty pTQ3 vector or vector containing either wild-type LC or wild-type HC. For the construction of the Fab repertoire the phage expression cassette was replaced by the yeast expression cassette from pTQ3 as an AscI/SfiI fragment. Plasmid DNA was prepared from these libraries using a Qiagen plasmid purification kit and was subsequently transformed into the yeast strain EBY100 as described previously [23].

2.4. Library selection

The mutant F2 repertoires were grown overnight and antibody expression was induced for 48 h as described above. The repertoire was labelled with anti-HA (25 μg/ml) for 1 h at room temperature followed by a secondary incubation with rabbit anti-mouse Ig-FITC (1:40) and 6 nM streptavidin-PE (Dako). Cells were washed once with 0.5 ml PBS following all incubation steps, after the final wash cells were kept on ice to prevent antigen dissociation. Samples were sorted on a Epics Altra flow cytometer with a sorting rate of approximately 2000 cells/s.

2.5. Protein analysis

Monoclonal yeast cultures were grown and induced as described above. After induction yeast cells were microfuged for 10 min at 13 000 rpm, washed with 1 ml milli-Q water and resuspended in 250 μl sodium dodecyl sulphate (SDS) sample buffer (10 mM Tris–HCl pH 6.8, 2% SDS, 15% glycerol). Glass beads (Sigma) were added to just below the meniscus and cells were vortexed four times for 1 min on ice. Finally, cell debris was removed by centrifugation at 13 000 rpm and samples were boiled for 10 min.

Protein samples were analysed by SDS–polyacrylamide gel electrophoresis through 12.5% Tris/glycine gels as described [3].

2.6. Surface plasmon resonance analysis

Kinetic measurements were preformed on a Biacore 3000™ (Biacore, AB). Approximately 500 RU streptavidin was immobilised on a CM5 sensor chip by standard amine coupling. Association and dissociation rate constants were determined by injection of a concentration range of each soluble Fab protein at a constant flow rate of 30 μl/min to avoid rebinding effects. All injections were performed in duplicate. Sensorgrams were analysed using BIAevaluation software 3.1™.

3. Results

3.1. Construction and evaluation of a yeast Fab display vector

A single yeast display vector was constructed comprising two expression cassettes driven by identical inducible GAL1 promoters (Fig. 1A). We reasoned that this format would lead to the production of stoichiometric amounts of separate LC and HC polypeptides and thus optimise the yield of functional Fab antibodies. The HC was fused at its N-terminus to the Aga2p yeast cell surface anchor protein, whereas the LC was produced as a soluble fragment. This format is also the most frequently used for Fab display on phage by the p3 coat protein [3]. The AGA2 signal sequence was appended to both LC and HC to drive processing of each polypeptide through the yeast secretion apparatus, ultimately leading to an assembled heterodimeric protein on the yeast cell surface. The addition of different epitope tags to the N-terminus of the LC and the C-terminus of the HC allows simultaneous and independent monitoring of display of each polypeptide chain and permits normalised selection [11].

This new display system was validated using three Fab antibodies isolated from a large Fab repertoire [3], specific for
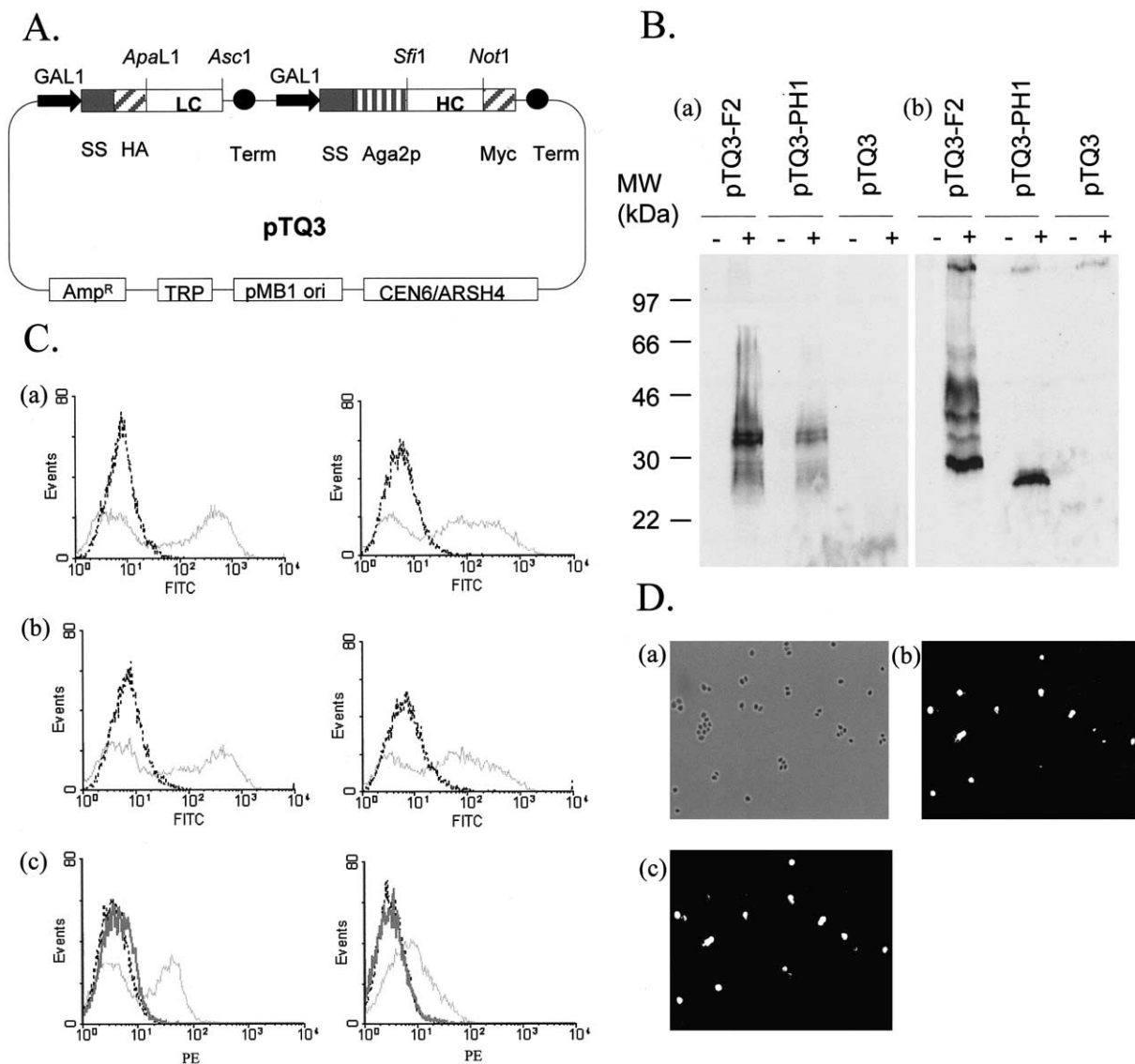
Fig. 1. Functional evaluation of Fab display on yeast cells. A: Two identical GAL1 promoters allow the expression of LC- and HC-Aga2p fusion. Display of LC and HC can be independently monitored using the appended epitope tags HA (LC) and c-*myc* (HC). Aga2p refers to the Aga2p subunit of the a-agglutinin yeast cell surface anchor protein; SS refers to the Aga2p signal sequence. B: Expression of Fab antibody fragments F2 and PH1 in *Saccharomyces cerevisiae* before (−) and after (+) 48 h induction, detection of (a) HC-Aga2p fusion product and (b) LC fragments. C: Yeast cells transformed with pTQ3-F2 (left panels) and pTQ3-PH1 (right panels) were left untreated (dotted line) or induced for 48 h at 20°C (light grey line). HC display (a), LC display (b) and antigen binding (c) were analysed using flow cytometry. Specific binding was demonstrated by competition with unlabelled antigen (dark grey). D: Two-colour immunofluorescent detection of assembled Fab antibodies on the yeast cell surface; (a) phase contrast, (b) detection of HC, (c) detection of LC.

streptavidin (clone pTQ3-F2), the breast epithelial cancer marker mucin-1 (clone pTQ3-PH1) [24] and cytotoxic T-lymphocyte-associated antigen-4 (data not shown). Whole cell extracts were made of normal and induced yeast cells containing the pTQ3-F2 or pTQ3-PH1 antibody expression constructs. Western blotting showed a HC product of the expected molecular weight of 33 kDa (Fig. 1B). The doublet band probably corresponds to Aga2p-HC protein with and without a signal sequence. LC product was also detected at the expected molecular weight of approximately 25 kDa.

To evaluate if yeast cells can functionally assemble heterodimeric Fab antibodies on their surface we induced antibody expression followed by testing for Fab display and antigen binding in yeast whole cell enzyme-linked immunosorbent assay (data not shown), flow cytometry (Fig. 1C) and immuno-

fluorescence microscopy (Fig. 1D). Yeast cells were labelled for the detection of HC, LC and antigen binding. In all cases LC and HC could be detected on the yeast cell surface and the Fab was functionally accessible for binding to antigen. Antigen binding was specific with no cross-reactivity with nonrelevant antigens (data not shown). Specificity could be further confirmed by competition with unlabelled antigen (Fig. 1C). Simultaneous labelling of HC and LC using two-colour immunofluorescent microscopy demonstrated the pairing of both chains on the surface of individual yeast cells. Analysis of a monoclonal culture expressing target-specific Fab antibody fragments showed that approximately 60% of the total cell population displayed functional Fab with antigen binding activity (Fig. 1C), this is consistent with previous studies using scFv fragments [21].

3.2. Model enrichment experiments using FACS

We performed single pass enrichment experiments using a mixture of yeast cells displaying an anti-streptavidin Fab (pTQ3-F2) antibody and control cells not displaying an antibody. Positive and negative yeast cells carried expression plasmids with different selectable markers for tryptophan and leucine respectively. This allowed simple calculation of enrichment factors by titration on selective plates before and after selection. Enrichment factors of up to 212-fold were obtained using FACS depending on the initial purity of the specific Fab fragment. Enrichment factors of the same order of magnitude have been reported using model selection experiments with antibodies in the scFv format [11].

Similar experiments were performed to determine if we could preferentially enrich a high affinity clone from an excess of lower affinity clones with the same specificity. Mixtures of pTQ3-F2 (34 nM) and pTQ3-A12 (500 nM) carrying different auxotrophic markers were labelled at five antigen concentrations spanning the affinities of the two antibodies (Table 1). The cell mixture was simultaneously labelled for antigen binding and Fab display, and cells were sorted for both parameters in high purity mode with a sort window of approximately 1%. Enrichment factors were determined by titration on selective plates and the optimum enrichment factor of 38-fold was found at an antigen concentration of 50 nM. This empirical value showed good agreement with the theoretical optimum labelling concentration of 62 nM determined using the mathematical model of Boder and Wittrup [13].

3.3. Affinity maturation of streptavidin-specific Fab fragment

We chose to affinity-mature a streptavidin-specific Fab antibody fragment F2. Antibody V genes were diversified using error-prone PCR [22] introducing mutations into the LC only, HC only or into the whole Fab gene, resulting in three repertoires with 5×10^6, 1.7×10^6 and 1×10^6 individual yeast clones respectively. The mutation frequency at the amino acid level was determined as 3% for the LC and 1.3% for the HC.

Three rounds of equilibrium selections were performed using FACS. An optimum antigen concentration of 6 nM was calculated, using a mathematical model [13], to be required for a predicted five-fold improvement in affinity. A reaction volume was chosen to maintain a 10-fold molar excess of labelled ligand over displayed Fab assuming approximately 10^5 copies of Fab per yeast cell. This was important to min-
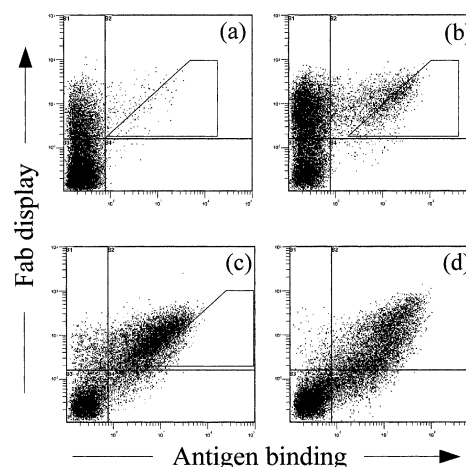

Fig. 2. Polyclonal flow cytometric analysis. Dotplots of antigen binding and Fab display are shown for the unselected library (a) and polyclonal outputs of selection rounds 1, 2 and 3 (b–d) The sorting gate used in each library selection is indicated.

imise selection on the basis of avidity since the selections were performed with the multimeric antigen streptavidin. During each selection round the stringency was increased by gating a smaller percentage of the highest antigen binding population (sequentially gate 5%, 1% and 0.5%). The selection campaign was monitored by performing flow cytometric analysis of the polyclonal outputs of each selection round (Fig. 2). It can be seen that the number of cells in the gated population increased during selection, indicating that selection was proceeding towards higher affinity antibodies.

Sequence analysis of the selected clones from the selection campaigns of all three repertoires had gone to completion with a limited number of clones dominating the third round of selection (Table 2). The sequence of selected variants had reduced to two different clones from the LC repertoire (H34R/Y96H and S2P/D85V), two different clones from the HC repertoire (H53R and H53R/S62A) and six different clones from the Fab repertoire. These selected clones were initially screened for a possible improvement in off rate using flow cytometric analysis of Fabs displayed on yeast cells [11]. The decrease in fluorescence over time was fitted to an exponential decay model from which the dissociation rate constants were calculated. These range from 3.5×10^{-4} s^{-1} to 0.5×10^{-4} s^{-1} compared to 2.2×10^{-4} s^{-1} for the starting

Table 1
Affinity discrimination of two yeast-displayed Fab antibodies of different affinities

Antigen (nM)[a]	Titre (−Trp)[b]	Titre (−Trp/−Leu) Fab-A12[c]	Titre (−Trp)−(−Trp/−Leu) Fab-F2[d]	Percentage Fab-F2[e]	Enrichment[f]
Input	3.7×10^7	3.7×10^7	4.7×10^5	1.3	
Output					
500	9×10^3	8.9×10^3	100	1.1	0.9
100	1.3×10^3	7.9×10^2	5.6×10^2	43	33
50	2.4×10^3	1.2×10^3	1.2×10^3	50	38
25	1.2×10^3	9.1×10^2	3.2×10^2	26	20
10	1.53×10^3	1.49×10^3	45	3	2.3

[a]Antigen concentration used for labelling yeast cells prior to FACS.
[b]Titre on −Trp selective plates.
[c]Titre on −Trp/−Leu selective plates representative of the number of yeast colonies containing antibody construct pTQ3-A12.
[d]Titre on −Trp plates minus titre on −Trp/−Leu selective plates representative of the number of yeast colonies containing antibody construct pTQ3-F2.
[e]The percentage of yeast cells containing the higher affinity antibody pTQ3-F2.
[f]The ratio of positive to negative yeast cells before and after selection.
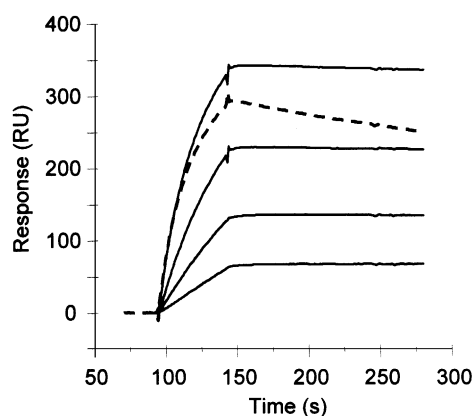
Fig. 3. Affinity determination using Biacore™. Association and dissociation rate constant analysis of clone R2H10. Continuous lines, injection of purified R2H10 Fab at (from top to bottom) 462 nM, 232 nM, 116 nM and 58 nM. Broken line, injection of purified wild-type F2 Fab at 500 nM.

wild-type antibody which corresponds to up to a 4.4-fold improvement in off rate (Table 2).

A more accurate affinity screening was performed with soluble Fab fragments. For this the majority of the selected variants were re-cloned into an *Escherichia coli* expression vector [3]. Affinity analysis of purified soluble Fab fragments obtained using Biacore™ and immobilised streptavidin showed that the dissociation rate constants were significantly faster than the dissociation rate constants obtained with flow cytometry. Although the absolute dissociation rate values were different for the two techniques the relative ranking of clones remains the same. The overall improvement in affinity varied from 1.6- to 10.7-fold compared to the wild-type F2, with clone R2H10 (Fig. 3) and R3B1 giving the greatest improvements in affinity to 3.2 nM and 5.5 nM respectively (Table 2).

We also investigated if consensus mutations in the LC could be combined with those identified in the HC to have a synergistic effect. The best LC mutants (R2H10 and R3B1) were combined with a dominant HC (R3G4), resulting in two new clones called Hyb1 and Hyb2 (Table 2). Affinity screening of

the soluble Fab fragments revealed two-fold improved on rates for both clones; however, this was accompanied by a faster off rate. Although there was modulation of the off and on rates no additive effect could be seen.

4. Discussion

Lead antibodies from primary selections of naive phage antibody libraries generally contain antibodies in either the scFv or Fab format. Although both formats are widely accepted there may be advantages in adopting the Fab antibody format for antibody development. The first potential advantage lies in reformatting to whole IgG, which is generally required prior to clinical development [25–28]. Although both scFv and Fab antibodies have been successfully reformatted into whole IgG, the conversion of Fab fragments may be more predictable due to the more natural format in comparison to the artificially restrained scFv format. Secondly there is a stronger association between the two chains of a Fab fragment compared to the pairing of the variable regions of antibody heavy and light chains (VH and VL) in a scFv fragment. As this stable pairing is essential for effective antigen binding there is more scope for simple improvement in the associations of VL and VH domains at the expense of real affinity improvements via optimisation of the antigen binding site. One report on the maturation of scFv by yeast display reported that four out of 10 selected mutations were located at the VH–VL interface suggesting that improved stability and/or orientation of the VH–VL domain pairing may represent important initial changes required prior to changes at the antigen binding site [19]. Such mutations may not be of any relevance for the affinity of an antibody after conversion to whole IgG and may simply be related to an optimisation of the scFv format. When using Fabs such artefacts may well be avoided. Furthermore, the tendency of scFv antibodies to dimerise through possible acquired mutations in the V gene or flexible linker can complicate affinity selection using phage display [15,17] and may be expected to be the same for yeast display.

We have shown that Fab antibodies can be displayed on the

Table 2
Characterisation of affinity-improved Fab fragments

Clone	Library	Mutation		FACS[c]	Biacore[c]			
		Variable LC[a,b]	Variable HC[a,b]	k_d (10^{-4} s^{-1})	k_d (10^{-3} s^{-1})	k_a (10^4 M^{-1}s)	K_D (nM)	Improvement
wt F2	–	wt	wt	2.2 ± 1.0	1.52 ± 0.15	4.51 ± 0.01	34	–
R2H10	LC	**H34R, Y96H**	none	0.5 ± 0.1	0.18 ± 0.01	5.69 ± 0.02	3.2	10.7
R3A9	LC	S2P, D85V	none	1.3 ± 0.1	1.53 ± 0.57	7.84 ± 0.08	19.5	1.7
R3H4	HC	none	**H53R**	1.9 ± 0.7	N.D.	N.D.	N.D.	N.D.
R3D2	HC	none	**H53R**, S62A	1.6 ± 0.6	N.D.	N.D.	N.D.	N.D.
R2D3	Fab	**H34R**	1 silent mut.	2.1 ± 0.3	1.04 ± 0.10	5.76 ± 0.08	18.1	1.9
R3H1	Fab	**Y96F**	A23V, **S65R**	1.0 ± 0.4	0.28 ± 0.04	3.25 ± 1.14	8.7	3.9
R3G4	Fab	S2P, D85V	**H53R**, A84T	3.5 ± 1.1	2.37 ± 0.25	10.9 ± 1.13	21.7	1.6
R3B1	Fab	**Y96F**	P40L	0.9 ± 0.2	0.22 ± 0.05	4.00 ± 1.30	5.5	6.3
R3E1	Fab	S2P, D85V	K14E	2.1 ± 1.2	1.03 ± 0.10	7.64 ± 0.95	13.5	2.5
R3H3	Fab	Q79R	Q3R	2.0 ± 1.0	1.04 ± 0.04	11.3 ± 2.64	9.2	3.7
Hyb1	Constructed hybrid	**H34R, Y96H**	**H53R**, A84T	N.D.	0.49 ± 0.03	13.7 ± 0.13	3.6	9.4
Hyb2	Constructed hybrid	**Y96F**	**H53R**, A84T	N.D.	0.34 ± 0.24	7.71 ± 0.04	4.4	7.6

wt: wild-type; N.D.: not determined.
[a]Antibody residue numbering according to Kabat et al. [32].
[b]Mutations in bold are in the CDR loops.
[c]Reported values are the means with standard deviation of three independent experiments.

yeast cell surface and that these antibodies are functionally accessible to large protein antigens. Our yeast display vector was designed so that the polarity of the two chains was compatible with our phage display Fab libraries for initial lead identification [3] (Ladner et al., manuscript in preparation) and also with vectors used for whole IgG reformatting and production. This compatibility in format and design allows for the 'batch transfer' or the 'combinatorial transfer' of selected Fab fragments between phage, yeast and mammalian expression systems. The 'batch transfer' of selected pools of Fab fragments is distinguished from 'combinatorial transfer' in that it retains selected LC and HC combinations. This may be the preferred cloning strategy when the pool of Fabs is so large that the separate transfer of LC and HC risks losing optimum antigen-positive LC/HC combinations. The 'combinatorial transfer' on the other hand can be used for chain shuffling procedures in order to create new LC and HC combinations.

Our study demonstrates that quantitative selection of yeast-displayed repertoires using FACS is an efficient method for affinity maturation of Fab antibody fragments. The affinity of a model antibody specific for the challenging multivalent target streptavidin was improved 10.7 fold to 3.2 nM, applying one cycle of mutagenesis in combination with FACS selection. We noted that all selected variants have improved affinity and showed an average of one to two mutations (at the amino acid level) per variable region. Clones with the highest improvement in affinity had mutations LC-H34R (CDR1) and LC-Y96H (CDR3) or LC-Y96F (CDR3). In all cases the mutations are conservative substitutions, either substituting like positive charges as in LC-H34R (CDR1) or exchanging aromatic groups like LC-Y96H or LC-Y96F (CDR3). Other clones with smaller affinity improvements due to improved association rate carried mutations LC-S2P (FR1) and LC-D85V (FR3) alone or in combination with HC-H53R (CDR2). The mutation of Y96 to two different amino acids suggests the importance of this residue in antigen binding and furthermore the substitution of other aromatic amino acids suggests particular structural requirements at this site. Each of these substitutions requires a single base change. The off rate of selected clones was determined both as yeast-displayed Fab by FACS and as soluble Fab by BIAcore™. The ranking of the clones was consistent between the two assays with the three clones R2H10, R3B1 and R3H1 being ranked in identical order of increasing off rate in both assays. However, we did note a consistent difference between the absolute off rate values of the two assays in that the FACS-based assay gave a slower off rate for wild-type F2 and the variants obtained after selection. This difference was most likely due to avid binding of the multivalent streptavidin molecule to multivalent Fab displayed on the yeast cell surface. As this relative difference between the two assays was consistent between clones we conclude that the normalised FACS selection was successful and we did not select clones on the basis of higher display. Since many clinically relevant target antigens are produced as multivalent molecules (e.g. Fc fusions) it is critical that any affinity maturation strategy is not compromised by the use of multivalent targets. We have shown that this is not a significant concern using yeast display and that by using normalised FACS selection of relatively small Fab repertoires specific for a tetravalent antigen we have recovered a panel of antibodies all with improved monovalent binding affinity.

The combination of the dominant CDR mutations LC-H34R (CDR1), LC-Y96H/F (CDR3) and HC-H53R (CDR2) was not retrieved from the Fab library. A possible explanation for this is that the clone was not present in the library since the sequence space sampled in this repertoire is smaller than that of the separate HC and LC libraries. Another explanation is that the mutations in LC and HC were not synergistic, resulting in a lower intrinsic affinity or stability. To test this, variants were constructed from the dominant LC and HC. The variants Hyb1 and Hyb2 gave improved affinity relative to wild-type; however, there appeared to be no synergistic effect. As such it seems that the former is the likely explanation for the absence of the combinatorial clones Hyb1 and Hyb2 from the starting Fab repertoire.

The mutation rate in our starting libraries was relatively high (1.4% for LC and 0.7% for HC at the nucleotide level) and the percentage of antigen binding clones was between 2 and 10% depending on the library. This percentage of active clones was in agreement with Georgiou and coworkers [22] who reported that a library with a mutation frequency of 0.5% nucleotide mutations gave 6.7% active clones. Although our libraries only sparsely sample a very large theoretical sequence space, we were able to select mutants with a significantly higher affinity after only one cycle of mutagenesis and selection. Greater affinity improvements can be obtained by iterating the process of mutagenesis and selection. This has been most effectively demonstrated by Wittrup and colleagues who using four cycles of DNA shuffling and selection of yeast-displayed scFv repertoires were able to get over a 1000-fold improvement in affinity to 48 fM for an anti-fluorescein scFv [19]. Our data suggest that this will also be possible for Fab antibodies.

The rapid and efficient generation of high affinity antibodies is an important step in the development of antibodies for immunotherapy of many disease indications including cancer [4,29] and microbial pathogenicity [30,31]. The affinity maturation process should be integrated not only with technologies for initial lead identification (such as from phage display repertoires) but also with technologies to build pharmaceutical leads (such as reformatting to whole IgG). Our yeast display system is fully compatible with both components of the antibody development pipeline. We have shown that antibodies in the Fab format can be affinity-matured without difficulty using quantitative FACS affinity selection of relatively small libraries of 10^6–10^7 yeast-displayed variants. Furthermore, our proof that Fab antibodies compromising two distinct polypeptides can be displayed on yeast cells invokes library construction strategies based on combinatorial yeast mating of separate LC and HC repertoires (Hufton, Blaise et al., manuscript in preparation). Such combinatorial yeast mating technology will find applications in both antibody affinity maturation and in the generation of large non-immune repertoires for initial lead identification.

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