Protein Engineering, Design & Selection vol. 21 no. 5 pp. 343–351, 2008
doi:10.1093/protein/gzn013

In vitro DNA recombination by L-Shuffling during ribosome display affinity maturation of an anti-Fas antibody increases the population of improved variants

Matthieu Chodorge[1], Laurent Fourage[2,3], Gilles Ravot[2], Lutz Jermutus[1] and Ralph Minter[1,3]

[1]Cambridge Antibody Technology, Milstein Building, Granta Park, Cambridge CB21 6GH, UK and [2]Proteus S.A., 70 allee Graham Bell, Parc Georges Besse, 30000 Nimes, France

[3]To whom correspondence should be addressed.
E-mail: ralph.minter@cambridgeantibody.com (R.M.); lfourage@proteus.fr (L.F.)

The use of random mutagenesis in concert with protein display technologies to rapidly select high affinity antibody variants is an established methodology. In some cases, DNA recombination has been included in the strategy to enable selection of mutations which act cooperatively to improve antibody function. In this study, the impact of L-Shuffling DNA recombination on the eventual outcome of an *in vitro* affinity maturation has been experimentally determined. Parallel evolution strategies, with and without a recombination step, were carried out and both methods improved the affinity of an anti-Fas single chain variable fragment (scFv). The recombination step resulted in an increased population of affinity-improved variants. Moreover, the most improved variant, with a 22-fold affinity gain, emerged only from the recombination-based approach. An analysis of mutations preferentially selected in the recombined population demonstrated strong cooperative effects when tested in combination with other mutations but small, or even negative, effects on affinity when tested in isolation. These results underline the ability of combinatorial library approaches to explore very large regions of sequence space to find optimal solutions in antibody evolution studies.

Keywords: affinity maturation/directed evolution/Fas/*in vitro* recombination/ribosome display

Introduction

Since the discovery of hybridoma technology in the 1970s, therapeutic applications of monoclonal antibodies have expanded and the number of antibodies approved for clinical use has risen significantly. This is expected to continue as most major pharmaceutical companies are now developing one or more antibody drugs in the clinic (Pavlou and Belsey, 2005).

Apart from the isolation of lead candidates to relevant targets, another field of research for therapeutic antibodies is focused on the optimisation of their efficacy, pharmacokinetics and safety profile. Molecular or *in vitro* directed evolution is a proven approach for protein optimisation and has been successfully used on antibodies to improve their biologic potency (Thom *et al*., 2006) or generate very high affinity binders in the picomolar range that, for instance in the oncology field, could be used to modulate tumour retention and *in vivo* efficacy (Graff and Wittrup, 2003).

A directed evolution experiment follows a well-defined process. First, new DNA sequences are generated to create libraries of variants. This can be done from a single gene using non-rational or semi-rational methods such as random mutagenesis, randomisation of a complementarity determining region (CDR) loop in an antibody or saturation mutagenesis of previously identified ‘hotspot’ positions. Diversity could also be generated from several parent genes using a family shuffling recombination step (Crameri *et al*., 1998). Variant libraries are screened or selected using stringent conditions to identify improved variants in the desired property that can then be used as starting material for the next evolution round.

To select antibodies with improved affinity, display technologies, such as phage (Clackson *et al*., 1991), yeast (Boder and Wittrup, 1997) or ribosome display (Jermutus *et al*., 1998), are methods of choice as they are high throughput, provide a physical link between sequence information and protein function and have the ability to discriminate for antigen binding at the single molecule level. Ribosome display technology for single chain variable fragment (scFv) optimisation (Hanes and Plückthun, 1997) offers a large literature background with many successful experiments. It has one of the most powerful display ranges (typically 10^{12} molecules) and the advantage of being performed entirely *in vitro*.

As protein sequence space is far too vast to be explored extensively, one key requirement for a successful protein optimisation is the use of the most comprehensively diversified libraries. *In vitro* DNA recombination is a powerful tool to create such libraries (Stemmer, 1994; Zhao *et al*., 1998). In a family shuffling experiment, DNA recombination enables one to focus primarily on sequences coding for functional proteins and thus to reduce greatly the screening requirement (Stemmer, 1995). More specifically, an *in vitro* DNA recombination step enables the isolation of synergistic combinations of mutations and reduces the burden of deleterious mutations from an evolution lineage (Harayama, 1998). Advantages of *in vitro* DNA recombination are well described from computational analysis (Maheshri and Schaffer, 2003) but have also been demonstrated in numerous successful molecular evolution experiments to improve properties such as enzyme efficiency (Castle *et al*., 2004), enantioselectivity (Rui *et al*., 2005), thermostability (Giver *et al*., 1998) and substrate specificity (Sio *et al*., 2002).

Although the advantage of a recombination step is well established for molecular evolution of enzymes, where the screening is usually limited to 10^5–10^6 molecules, it is less straightforward for an antibody affinity maturation experiment. Indeed in such a case, the search in sequence space is already very efficient when using a powerful display

© 2008 The Author(s).
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

technology such as ribosome display and the need to apply a recombination step to generate the best evolution solution is less obvious. In other words, the high throughput nature of display technologies could balance the lack of recombination to nonetheless still identify the best possible variants.

Several antibody affinity maturation experiments integrating an *in vitro* DNA recombination step have already been conducted and have succeeded in identifying scFv with very high affinity for various antigens such as fluorescein (Jermutus *et al*., 2001) or a prion-derived peptide (Luginbühl *et al*., 2006) using ribosome (Zahnd *et al*., 2004) or yeast display (Boder *et al*., 2000). Despite impressive results, it is difficult from these studies to fully evaluate the impact of the DNA recombination on the optimisation process. Furthermore, affinity maturation without a recombination step has also been successful in identifying very high affinity antibodies against CEA (Graff *et al*., 2004), the tumour antigen c-erbB-2 (Schier *et al*., 1996) and digoxigenin (Daugherty *et al*., 2000).

Tumour necrosis factor (TNF) receptor superfamily members such as Fas (Apo-1/CD95) or TNF-related apoptosis inducing ligand (TRAIL) receptors 1 and 2 are promising drug targets for the development of new therapeutics in oncology (Fulda and Debatin, 2004; Wajant *et al*., 2005). Stimulation of these death receptors using agonistic antibodies, natural or engineered ligands (Pukac *et al*., 2005; Greaney *et al*., 2006) triggers the external apoptotic pathway that alone or in combination with cytotoxic molecules, or other cancer therapies, could lead to significant improvement in tumour remediation (Gong *et al*., 2006).

A fully human antibody scFv fragment targeting the human death-receptor Fas and able to trigger apoptosis in several cancer cell lines (anti-Fas scFv E09) has been identified from naïve antibody phage display (manuscript in preparation). Affinity maturation of the E09 antibody to improve Fas binding was considered as a possible strategy to enhance its apoptotic potency and broaden its potential therapeutic applications.

In this study, the affinity maturation of the anti-Fas antibody E09 has been used as a model system to analyse the impact of a DNA recombination step on a ribosome display optimisation experiment. We have used an efficient ligation-based method called L-Shuffling (Dupret *et al*., 2002; Ravot *et al*., 2006) to recombine DNA *in vitro*. Output libraries resulting from parallel selection processes but using different gene diversification strategies, namely with or without an L-Shuffling DNA recombination step, were compared for both the proportion and quantitative improvement of high affinity binders and also the diversity of evolution pathways. Affinity improvements were identified in both strategies but the highest affinity scFv were found in recombined outputs.

Materials and methods

Library construction

The initial mutant library of anti-Fas E09 scFv in the ribosome display format was generated by error prone PCR (ep-PCR) using the protocol described by Cadwell (Cadwell and Joyce, 1992). A 30 cycle PCR was performed using T7B and MycRestore (Groves *et al*., 2006) primers in presence of 7 mM of $MgCl_2$ and 0.3 mM of $MnCl_2$. The *Taq* DNA polymerase (ABgene) was added after the first denaturation step to reduce non-specific amplifications.

DNA from the output library 4 was amplified using a T7B/T6te PCR (Groves *et al*., 2006) to generate the material for the L-Shuffling *in vitro* recombination (Dupret *et al.*, 2002; Ravot *et al*., 2006). After purification using a Qiaquick column (Qiagen) and elution in H_2O, ~100 μg of DNA were randomly digested for 5 min by 0.05 units of DNase I (Promega) and purified on a P6 column (Bio-Rad) equilibrated in H_2O. About half of the digested fragments were added to a mix of thermostable L-Shuffling enzymes (Dupret *et al.*, 2002) in the ligation reaction buffer. The L-Shuffling reaction was performed in a MJ-Research PTC-225 thermocycler using the program 94°C, 5 min/(94°C, 1 min/60°C, 5 min) 50 times. Reaction products at ~1–1.2 kpb were gel-purified (Qiagen) and PCR amplified using SDCATC/MycRestore primers (Groves *et al*., 2006) in order to prepare a functional ribosome display construct.

Ribosome display selection

The different elements to obtain a ribosome display construct [T7 promoter and Shine-Dalgarno (SD) sequence in 5′ and geneIII tether in 3′] were added to the scFv genes using first a SDCATC/MycRestore PCR on the diversified libraries followed by a T7B/T6te PCR in the presence of the geneIII tether sequence.

Ribosome display selections for improved affinity were performed as previously described (Hanes *et al*., 2000). Ribosome–mRNA–scFv complexes were incubated for 2 h at 4°C with decreasing concentrations of Fc-tagged recombinant human Fas (R&D Systems) before a 1 h capture step with Protein G coated magnetic beads (Dynal Biotech). This was preferred to the streptavidin/biotin capture system to avoid random biotinylation that could lead to the loss of protein function and create steric constraints on the binding interface.

Four selection rounds at 50, 10, 2 and 0.5 nM of antigen were performed on the initial ep-PCR library. The final selections on the Round 4 output library or the recombined library were performed at 0.1 nM of Fas.

Output library analysis

Cloning Round 5 libraries were cloned into pCantab6 (McCafferty *et al*., 1994) using *Not*I and *Nco*I restriction sites and ligation products used to transform *E. coli* TG1 cells. A total of 528 colonies were picked for both mutated and recombined libraries into 96 well plates filled with 2TY medium supplemented with 2% glucose and 100 μg/ml ampicilin.

Initial screening Monsterblock culture plates (Greiner Bio-One) filled with 500 μl of 2TY plus 100 μg/ml ampicilin and 0.7 mM IPTG were inoculated from the master plates and incubated overnight at 30°C and 500 rpm shaking. Fifty microlitter culture supernatants, containing scFv variants, were transferred into high binding capacity plates (Costar 3925) and incubated for 2 h at room temperature to allow scFv coating.

As a first binding screen, affinity matured antibodies were identified after a 2 h incubation with 0.35 ng/ml of Fc-tagged recombinant human Fas (R&D Systems) in PBS plus 4% skimmed milk and detection using 0.5 μg/ml of

europium-labelled anti-human IgG (Perkin Elmer) according to the manufacturer's protocol. Relative binding was calculated by normalisation to the signal from wild-type scFv and the negative control signal as well as to the OD_{600} of *Escherichia coli* culture after expression in the Monsterblock to take into account any difference in cell growth. Graphs were plotted and analysed using the software Prism (GraphPad).

Confirmatory assay Selected scFvs from the initial screen were re-tested using the same binding assay principle. Coated scFvs on an ELISA plate were tested in quadruplicate using a 6-point titration of Fas antigen from 27 to 0.11 nM. Equilibrium binding titrations were analysed for each variant to determine the apparent Fas affinity using the curve fitting software Prism (GraphPad).

Sequencing Escherichia coli cultures of selected scFvs were used as templates for a PUCrev/FdSeq PCR and amplified DNA were sequenced using Big Dye Terminator reaction (Applied Biosystems) on an Applied Biosystems 3700 DNA Analyser.

ScFv directed mutagenesis, expression and purification

Oligonucleotide-directed mutagenesis was performed using the QuickChange II Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer instructions. After a sequence check, scFv antibodies were expressed in *E. coli* XL-1 Blue and purified as previously detailed (Bannister *et al.*, 2006). Briefly, expression was induced overnight at 30°C using 0.1 mM ITPG and scFv was purified from cell culture supernatants in a three steps process: cation exchange as capture, IMAC purification using the scFv C-terminal His-tag and a final gel filtration to buffer exchange into PBS. Protein concentrations were determined using a BCA assay (Sigma) and purity checked on SDS–PAGE.

Measurement of binding kinetics

The affinity and kinetic parameters of the scFv antibodies for the Fas receptor were determined by surface plasmon resonance using a BIAcore 2000 instrument (BIAcore). Protein G (Sigma) was immobilised on a CM5 sensor chip using the amine coupling method and 25 µl of Fc-tagged recombinant human Fas (R&D Systems) at 100 nM was first run to obtain around 300–400 RUs. For kinetic analysis, 50 µl of a serial dilution of scFv from 200 to 6.25 nM were injected at 30 µl/min following a 5 min dissociation. Experiments were performed in duplicate at 25°C in HBS-EP buffer (BIAcore) with 0.1% BSA. Curve fitting was done on the BIAevaluation 3.2 RC1 software (BIAcore) using a 1:1 Langmuir binding model. Kinetics of dissociation were analysed for a short time (90 s) to minimise the avidity effects on affinity calculation of potential scFv dimer.

Results

Random mutagenesis and ribosome display selections

Affinity maturation of the anti-Fas antibody E09 was performed using ribosome display after an initial ep-PCR random mutagenesis to create the sequence diversity. The average number of mutations was determined to be 4.5 base changes per scFv gene after sequencing 30 randomly selected variants (data not shown). After conversion to the ribosome display format, the mutant library was expressed on ribosomes and selected for improved Fas binding over four rounds with decreasing antigen concentrations from 50 to 0.5 nM (Fig. 1). The first selection was performed using a Fas antigen concentration four times higher than the E09 affinity for Fas (which is 13.1 nM) to ensure an efficient capture of the library. The selection output at Rounds 3 and 4 were recombined by L-Shuffling and also selected directly without recombination (Fig. 1).

L-shuffling in vitro DNA recombination

In vitro DNA recombinations of the output from Round 3 or 4 were performed using the L-Shuffling ligation based method (Dupret *et al.*, 2002; Ravot *et al.*, 2006) and recombination products were amplified in a final PCR to convert back into the ribosome display construct.

In order to verify the efficiency of the L-Shuffling method, a model experiment was performed whereby 10 variant *E. coli ponB* genes, each with a restriction site introduced at a different position, were recombined and the recombination efficiency was compared with a theoretical prediction of recombination events, as described previously (Moore *et al.*, 2001). This analysis showed that there was no significant difference between the observed and expected results, indicating that L-Shuffling is an efficient method for DNA recombination (Supplementary Material 1, Supplementary data are available at *PEDS* online).

Comparison of mutated and recombined library output

Initial screening Five hundred and twenty-eight variants from the recombined outputs SH3.2, SH4.1 and the non-recombined output EP5 were screened for Fas binding. Fluorescence signals were normalised by OD_{600} of the bacterial culture after overnight expression to take into account

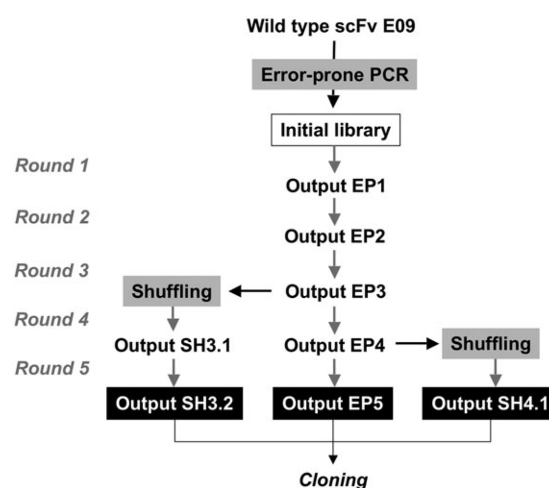

Fig. 1. Experimental strategy to analyse the effect of a recombination step at different stages in a ribosome display affinity maturation. After an initial random mutagenesis, the scFv gene library was selected on decreasing concentrations of antigen. Three different maturation schemes were then compared. One strategy (output SH3.2) introduced recombination after Round 3, another strategy (output SH4.1) introduced recombination after Round 4 and a third strategy (output EP5) did not include a recombination step. The three Round 5 output libraries were cloned and analysed for comparison.

any difference in cell growth. Screening results are shown in Fig. 2 and demonstrate that the recombined library SH4.1 contains a larger proportion of high affinity variants than the mutated library EP5. Recombined output SH4.1 contains 38% of variants with a binding signal five times above the wild-type compared with 31% of the non-recombined EP5 library. In addition, the area under the curve using a baseline of five times the wild-type signal, which measures the global improvement in the population and is a function of both the fraction of improved variants and the quantitative improvement of each variant, is around 30% larger in the recombined library than in the non-recombined library (5.6 and 7.2 for the library EP5 and SH4.1, respectively). Statistical analysis of the data showed that the differences between the two populations were significant ($P < 0.005$). It is also of interest to note that when the recombination step was attempted at an earlier stage (after Round 3), followed by two rounds of selection, the same statistical analysis showed that the difference in binding scores between 528 variants from the recombined pool and 528 variants from the non-recombined (EP5) pool was not significant ($P = 0.35$).

Confirmatory assay The highest affinity variants, identified after the initial screening, were cherry-picked and analysed for Fas binding using a confirmatory assay. A surrogate affinity binding screen, which used a 6-point titration of Fas to calculate K_D for each variant and showed a good correlation ($R^2 = 0.7$) with BIAcore measurements (Fig. 3A), was used to calculate affinities for the 44 variants from each strategy. Seven out of 44 variants from the recombined output SH4.1 were able to achieve at least a 20-fold improvement over the wild-type affinity in this assay compared with only 3/44 in the non-recombined output EP5 (Fig. 3B). Furthermore, a 1 to 1 comparison of the 10 best variants identified after the confirmatory assay (Fig. 3C) suggests that higher affinity antibodies were found in the recombined library.

Affinity determination by BIAcore Affinity was determined by BIAcore for the most improved variant SH4.1_H10 as 600 pM $\pm$ 10 and corresponded to a 22-fold affinity improvement compared with the wild type (13.1 nM). Full sequences and BIAcore data for the wild-type and affinity matured variants are shown in Supplementary Material 2, Supplementary data are available at *PEDS* online.

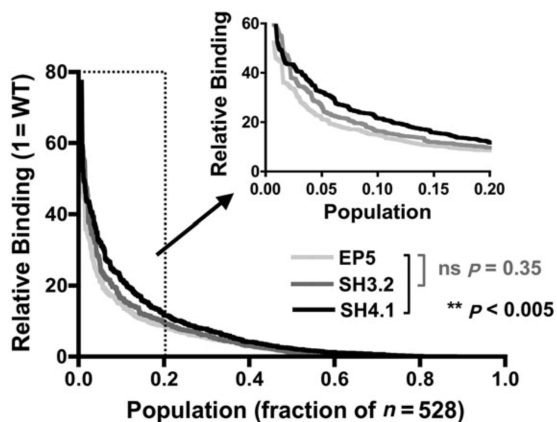

Fig. 2. Results of affinity screen for output libraries at Round 5. Recombined library SH4.1 is shown in black and the control (non-recombined) library EP5 in grey. Relative binding signals compared with the wild-type variant are plotted by decreasing order as a function of the population expressed in fraction of the number of screened variants (528 for each strategy). The inset is a zoomed view of the 20% highest affinity variants. For statistical analysis, the unpaired Student's *t*-test was applied.

Sequence analysis The 44 highest affinity variants in the recombined SH4.1 and the mutated EP5 libraries were sequenced to analyse the evolution process at the amino acid level. Five V_L mutations were defined as mutational hotspots (Thom *et al*., 2006) in both the recombined and non-recombined populations (Table I). Tyrosine in position V_L50 is frequently mutated in over 80% of variants examined and the four remaining positions are mutated in 25–50% of variants. Interestingly, all highly selected mutations are in the light chain and, except V_L70, all are located into CDR loops, as observed previously (Thom *et al*., 2006).

A comparison between the pool of sequences coding for the highest affinity variants in the two outputs shows that five positions are preferentially mutated in the recombined SH4.1 rather than in the non-recombined EP5 library (Table II). These positions show a higher mutation rate than the background mutation level in the unselected library and at least a 2-fold higher mutation rate in the SH4.1 pool than in the EP5 pool. More particularly, mutations at positions V_H26 and V_H110 were found three and four times, respectively, in the pool of the 44 recombined variants but could not be detected in the non-recombined pool of variants.

Additivity/cooperativity effect of recombination-selected mutations

To analyse the relative contribution to affinity of the five mutations preferentially found in the recombined library population, site-directed mutagenesis followed by analysis of the energy changes was performed (Yang *et al*., 2003). Putative beneficial, recombination-selected mutations were introduced into the wild-type sequence (referred to as wild-type mutant) but also removed from the backbone of a recombined variant (referred to as revertant).

Amino acid sequences, compared with the wild-type scFv, of the four recombined variants used as template for the reversion mutagenesis are illustrated in Fig. 4A. These variants were chosen because they derive from the recombined strategy and contain the mutations preferentially selected in the recombined pool, as listed in the recombined pool, as listed in Table II.

Kinetic measurements were performed for each of the three variants (wild-type mutant, recombined variant and revertant) for all five selected mutations to analyse their effects on both the wild-type and the improved sequence context (Table III). There is a large difference in affinity between the four recombined variants used for this study. Variant SH4.1_H10 has the largest improvement in affinity (22-fold) compared with the wild-type scFv, whereas variants SH4.1_D08 and SH4.1_H09 are slightly improved to a $\sim$4 nM affinity. Variant SH4.1_A07 has a comparable affinity to the wild type due to a 3-fold improvement in off-rate combined with a 3-fold decrease in on-rate from 13.1×10^4 to 3.5×10^4 M^{-1} s^{-1}.

Binding energy analysis was performed for the five mutations to precisely quantify their cooperative or additive effects on Fas binding (Yang *et al*., 2003; Bernat *et al*., 2004). For each of the five targeted mutations, Fig. 5 reports

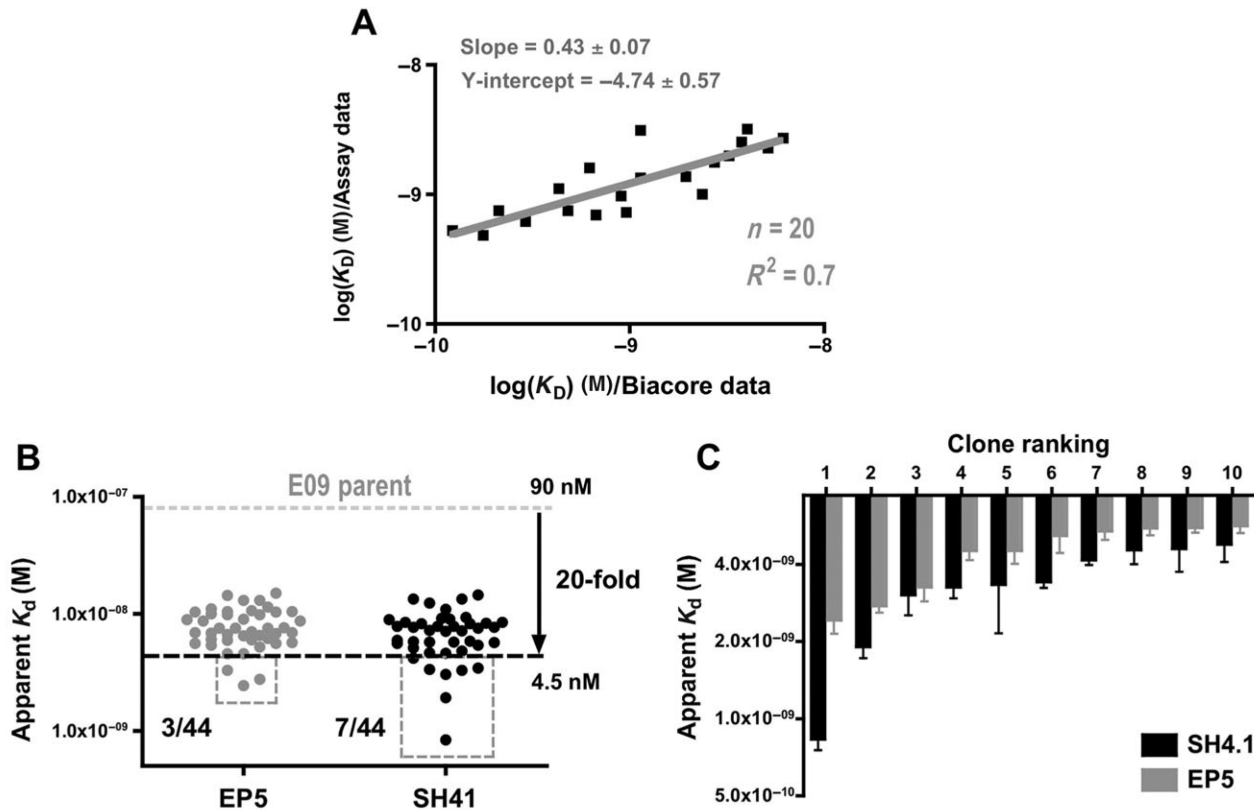

Fig. 3. Comparison of the highest affinity variants from both the recombined library SH4.1 (in black) and the non-recombined library EP5 (in grey) for Fas antigen binding. (**A**) A correlation plot of affinity measurements for 20 variants tested by both BIAcore and the 6-point surrogate binding assay. (**B**) Binding confirmatory assay of the 44 highest affinity variants identified after the first screen. Each point represents one clone and is sorted for apparent affinity determined using a 6-point titration of antigen concentrations in the standard binding assay. The dashed grey box represents the proportion of variants in each population showing an increase in affinity of >20-fold compared with the wild type. (**C**) Direct comparison of the surrogate affinity values for the 10 highest affinity variants in the two libraries identified after the confirmatory assay. Error bars are calculated from four independent binding experiments.

the change in free energy of binding for the wild-type mutant, the recombined variant and the revertant scFv but also the theoretical sum of the values for the wild-type mutant and revertant.

Mutations V_H26_G>D and V_L42_K>E are found to be additive as changes of free energy for the corresponding recombined variant are comparable to the sum of the wild-type mutant and the revertant. However, the three mutations V_H50_S>G, V_H110_T>A and V_H79_G>D show a positive cooperative effect with the other mutations found in recombined variants SH4.1_H09, SH4.1_H10 and SH4.1_D08, respectively. Cooperativity energies are calculated as -0.3, -1.5 and $-0.4\ \text{kcal mol}^{-1}$ for mutations V_H50_S>G, V_H110_T>A and V_H79_G>D in their corresponding recombined variants, respectively.

The most striking result of the free energy analysis is the effect of the mutation T>A in position VH110. This mutation has a significant negative effect on Fas binding when introduced into the wild-type scFv but a strong positive effect, notably due to cooperative interactions, when combined with other mutations to yield the highest affinity anti-Fas antibody identified in our study.

Discussion

DNA recombination technologies based on recombination of homologous genes, selected from natural diversity or created from a single gene (Chodorge *et al.*, 2005), have already shown their great potential for the optimisation of proteins

Table I. Hotspot positions in both recombined and non-recombined libraries

Position	Dominant mutation[a]	Number in best variant pool ($n = 44$)	
		EP5	SH4.1
V_L _ 27a	F>S	15	15
V_L _ 50	Y>S	39	36
V_L _ 51	*N*>D	21	19
V_L _ 70	S>T	13	9
V_L _ 95a	K>E	16	13

[a]Amino acid replacement found in >50% of variants.

Table II. Preferentially mutated positions in the recombined library SH4.1

Position	Dominant mutation[a]	Number in best variant pool ($n = 44$)	
		EP5	SH4.1
V_H _ 26	G>D	0	3
V_H _ 50	S>G	3	7
V_H _ 110	T>A	0	4
V_L _ 42	K>E	2	6
V_L _ 79	L>Q	1	6

[a]Amino acid replacement found in >50% of variants.

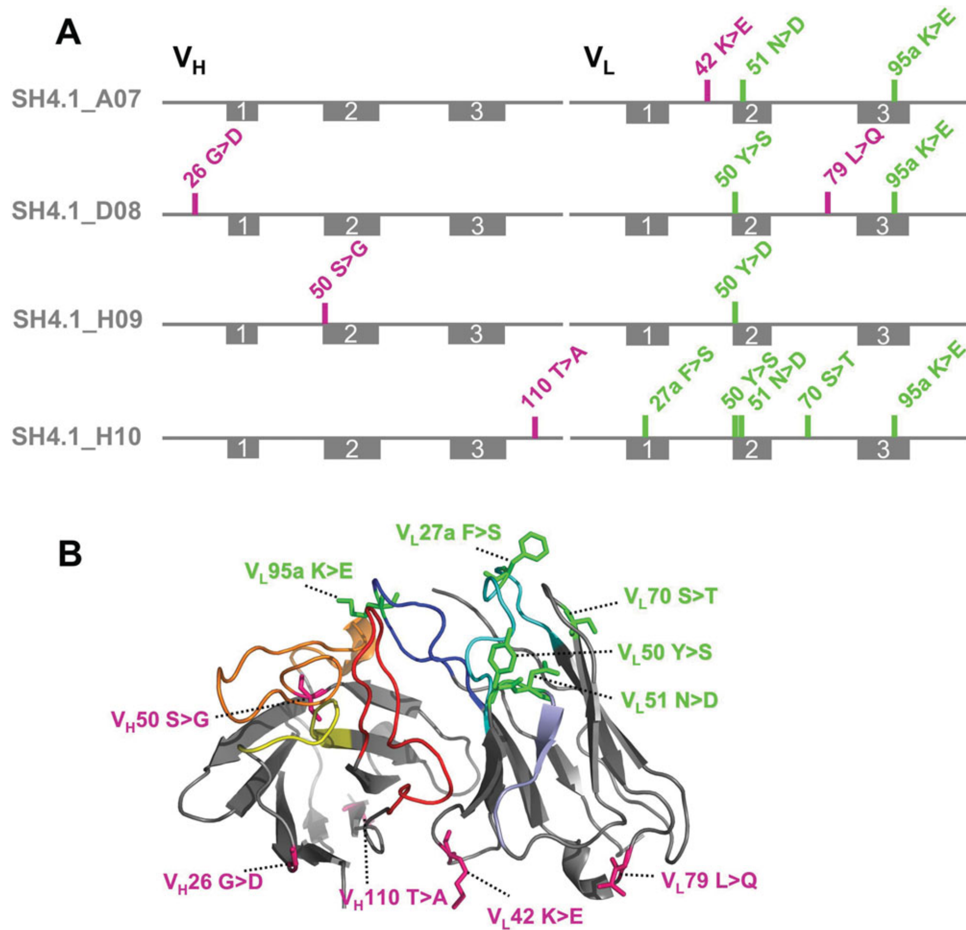

Fig. 4. Locations of the mutations present in the four recombined scFvs used for the additivity/cooperativity experiments. (**A**) Mutations are numbered according to the Kabat numbering scheme and are shown in green for hotspot mutations (those found in both recombined SH4.1 and non-recombined EP5 outputs) and pink for those more specifically selected in the recombined library. CDRs in the V_H region (left) and the V_L region (right) are indicated by grey boxes numbered 1–3. Additional mutations, omitted for clarity were present in SH4.1_A07 (V_L18T>A, V_L27S>G), SH4.1_D08 (V_H42G>E, V_H52dN>S, V_L3V>A, V_L13E>G, V_L69T>P, V_L103K>R), SH4.1_H09 (V_H17T>I, V_H74S>P, V_L26V>D) and SH4.1_H10 (V_H45L>P, V_H52aY>H, V_L16*R*>K, V_L24S>P). (**B**) Mutations selected during the affinity maturation process are represented on a wild-type E09 scFv model structure, with heavy chain on the left and light chain on the right. Heavy chain CDR 1, 2, 3 and light chain CDR 1, 2, 3 loops are shown in yellow, orange, red, turquoise, light blue and dark blue, respectively. Hotspot mutations are shown with side chains in green and map predominantly to the CDRs. Mutations preferentially selected in the recombined library are shown with side chains in pink and are mostly distal to the antigen binding site. Structure visualisation was performed using PYMOL software (DeLano Scientific LLC).

including antibodies. Among these technologies, the ligation based recombination process L-Shuffling (Dupret *et al.*, 2002) has been applied for the molecular evolution of industrial proteins such as alkaline proteases (Fourage *et al.*, 2005). The aim of this study was to measure the contribution of an L-Shuffling DNA recombination step to the affinity maturation of an antibody *in vitro*.

The maximal gain in affinity obtained in this study (22-fold) is somewhat lower than that previously reported in similar affinity maturation experiments (Jermutus *et al.*, 2001; Thom *et al.*, 2006). Although the 22-fold gain in affinity in the study was low, this is not believed to be due to the choice of the L-Shuffling method over other more established DNA recombination techniques. The recombination efficiency of L-Shuffling was shown to be close to the expected theoretical recombination rate in a model experiment (Supplementary Material 1, Supplementary data are available at *PEDS* online) and it is more likely that the screening of relatively few variants (528 in total) from each output limited the chances of finding variants with greater affinity gains. Also, ribosome display selections were performed at equilibrium where ligand concentrations directly determine the range of affinity improvement. The lowest Fas concentration employed was 100 pM and therefore possibilities to obtain low picomolar binders may have been limited. Furthermore, selection strategies such as a kinetic screen to discriminate for low dissociation rate could have led to the identification of higher affinity antibodies (Boder and Wittrup, 1998).

Although the overall improvements in antibody affinity were relatively modest, it was still possible to discern a significant difference between the recombined and non-recombined populations. Not only was the highest affinity variant isolated from the recombined approach, but the recombined population as a whole contained a larger number of high affinity variants. When the affinity was determined for the top 44 hits from each population, it was observed that 7/44 variants from the recombined and 3/44 from the non-recombined library, respectively, achieved a 20-fold improvement in affinity. This is particularly striking since only one

Table III. Kinetic and affinity parameters of recombined and engineered E09 variants for Fas antigen by surface plasmon resonance

Name	Variant type	k_{on} (M^{-1} s^{-1}) (10^4)	k_{off} (s^{-1}) (10^{-4})	K_A (M^{-1}) (10^7)	K_d (M) (10^{-9})	DG° (kcal mol^{-1})[a]	$DDG^{\circ b}_{(mut-WT)}$ (kcal mol^{-1})
Wild type	NA	13.1	17.1	7.7	13.1	−10.7	0.0
V_H26_G>D	Wild-type mutant	7.7	7.1	10.8	9.2	−11.0	−0.2
V_H50_S>G	Wild-type mutant	4.5	4.7	9.6	10.4	−10.9	−0.1
V_H110_T>A	Wild-type mutant	6.0	17.0	3.5	28.3	−10.3	0.5
V_L42_K>E	Wild-type mutant	6.8	16.1	4.2	23.7	−10.4	0.4
V_L79_G>D	Wild-type mutant	8.7	16.4	5.3	18.9	−10.5	0.2
SH4.1_ D08/V_H26_D>G	Revertant	6.0	3.9	15.4	6.5	−11.2	−0.4
SH4.1_ H09/V_H50_G>S	Revertant	4.9	3.9	12.6	8.0	−11.0	−0.3
SH4.1_ H10/V_H110_A>T	Revertant	8.7	2.5	34.8	2.9	−11.6	−0.9
SH4.1_ A07/V_L42_E>K	Revertant	6.4	5.4	11.9	8.4	−11.0	−0.3
SH4.1_ D08/V_L79_D>G	Revertant	6.7	4.1	16.3	6.1	−11.2	−0.4
SH4.1_ D08	Recombined variant	7.3	3.2	22.8	4.4	−11.4	−0.6
SH4.1_ H09	Recombined variant	13.0	5.0	26.0	3.8	−11.5	−0.7
SH4.1_ H10	Recombined variant	33.2	1.9	174.7	0.6	−12.6	−1.9
SH4.1_ A07	Recombined variant	3.5	5.1	6.9	14.6	−10.7	0.1

[a]DG° is calculated from K_A using the relationship DG° = −RT ln(K_A). *T* is the temperature in Calvin and *R* the gas constant is equal to 1.987 cal K^{-1} mol^{-1}.
[b]Changes in free energy were determined from previously calculated free energies using the wild type as reference.

round of selection was performed post-recombination and since a limited screening strategy was employed.

It is worth noting that in order to gain the maximum benefit from the recombination step, the point at which it is introduced must be chosen judiciously because at early stages in the selection process, the library diversity is high and there are a majority of neutral mutations for which recombination is unlikely to lead to great benefit. In this study, when the recombination step was performed too early in the selection process, it did indeed show that there was not a significant affinity gain for the recombined pool in relation to the non-recombined pool (Fig. 2). It is also possible, although not investigated here, that introducing recombination too late in the process, when diversity is low, could reduce the possibility for finding beneficial combinations. Further, optimisation experiments, where the optimal timing of the recombination step is determined empirically, may shed some light on a general rule for recombination timing.

As well as the positive impact of the recombination step on the overall affinity of the population, a detailed free energy analysis of mutations preferentially identified in recombined sequences showed that some of these mutations were acting co-operatively. The clearest example of this was the mutation at position V_H110 which had a significant negative effect on Fas binding when introduced into the wild-type scFv but a strong positive effect, likely due to co-operative interactions, when combined with other mutations to yield the highest affinity Fas antibody identified in this study. The V_H110 mutation was not observed in the sequences derived from the non-recombined selection outputs, suggesting that the recombination step enabled the exploration of novel, beneficial combinations of mutations. However, it should be noted that the purpose of this study was to assess the impact

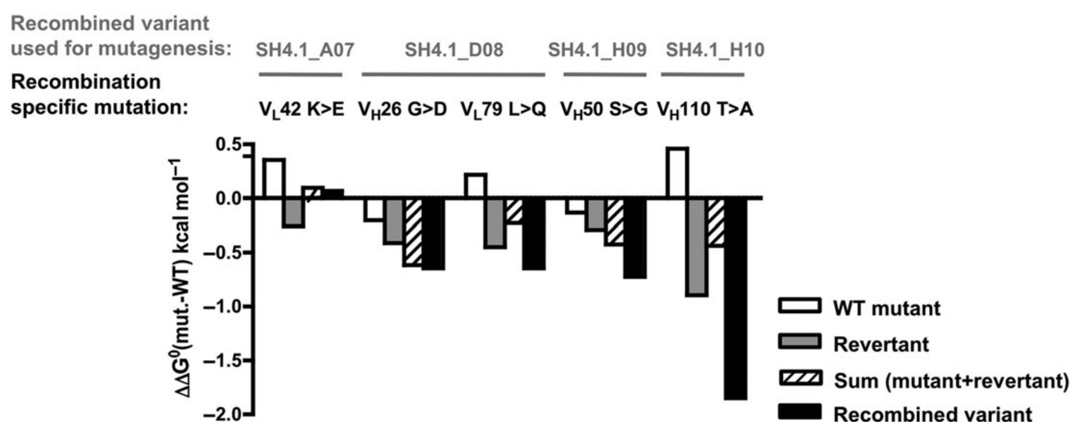

Fig. 5. Additivity/cooperativity effect on Fas binding of the mutations more specifically selected in the recombined output library. Free energies of binding compared with the wild-type scFv [$\Delta\Delta G^{\circ}_{(mut.-WT)}$] for the wild-type mutant (in white), the revertant (in grey) or the recombined variants (in black) are reported for each mutated position (shown on top). Theoretical additive values of the wild type and the revertant are shown in dashed black. The recombined variants from which the mutants were derived are shown at the very top of the figure.

of the recombination step on the final affinity of variants rather than to compare recombination against error-prone mutagenesis. As such, it is acknowledged that an additional error-prone mutagenesis step prior to the fifth round of selection could have introduced an analogous mutation at position V_H110 and such a variant could also have been selected on the basis of co-operativity.

When looking at the structural location of the beneficial mutations, the five hotspots which were present in both the recombined and non-recombined populations clearly map to the antigen binding site, either in CDRs or vernier positions (Fig. 4B). In contrast, the mutations found preferentially in recombined scFvs were predominantly located in the scFv framework and were distal to the antigen binding site. This suggested that such mutations were having more subtle, conformational effects on the overall structure of the scFv rather than directly affecting the contact interface. This is in agreement with previous recombination-based antibody evolution studies, which have all cited second-sphere or third-sphere mutations as being important in determining the final affinity (Boder *et al*., 2000; Jermutus *et al*., 2001; Luginbühl *et al*., 2006). Indeed, a detailed structural analysis of one such affinity-matured antibody concluded that very high-binding affinity is the cumulative result of many small structural alterations (Midelfort *et al*., 2004). When a sufficient number of such mutations are combined, the cooperative effect on antibody affinity can be significant, as seen with the V_H110 change in combination with nine other mutations in the highest affinity variant SH4.1_H10.

In conclusion, this study demonstrated that the inclusion of a recombination step in an *in vitro* antibody affinity maturation allowed the selection of novel combinations of mutations and therefore the exploration of more sequence space, compared with a parallel strategy which omitted recombination. Furthermore, free energy analysis showed that the recombined mutations were able to act cooperatively to improve affinity through subtle conformational changes. Importantly, the interesting finding that a mutation at position V_H110 had a negative impact on antigen binding in the context of the wild-type sequence and yet had a positive, co-operative effect when combined with other mutations is a strong argument for the combinatorial, library-based approach to protein evolution. Such a beneficial mutation would not have been discovered by methods which first test the contribution of mutations in the wild-type context before recombining only those with a positive effect (Wu *et al*., 1998; Rajpal *et al*., 2005). The new generation of protein evolution tools, such as the computational methods EvoSight (Chodorge *et al*., 2005) or ProSAR (Fox *et al*., 2007), have highlighted the importance of not limiting the amino acid diversity just to beneficial mutations which are present in variants with increased function. The inclusion of a recombination step, as described here, inherently allows a wider diversity of mutations to be included and selected for, in order to find the optimal protein solutions.

Acknowledgements

We thank Christophe Ullmann for helpful discussions on technical issues, the Cambridge Antibody Technology DNA chemistry and High-Throughput Expression teams for their contribution to DNA sequencing and scFv antibody purifications, respectively.

References

Bannister,D., Wilson,A., Prowse,L., Walsh,M., Holgate,R., Jermutus,L. and Wilkinson,T. (2006) *Biotechnol. Bioengineer.*, **94**, 931–937.

Bernat,B., Sun,M., Dwyer,M., Feldkamp,M. and Kossiakoff,A.A. (2004) *Biochemistry*, **43**, 6076–6084.

Boder,E.T. and Wittrup,K.D. (1997) *Nat. Biotech.*, **15**, 553–557.

Boder,E.T. and Wittrup,K.D. (1998) *Biotechnol. Prog.*, **14**, 55–62.

Boder,E.T., Midelfort,K.S. and Wittrup,K.D. (2000) *Proc. Natl Acad. Sci. USA*, **97**, 10701–10705.

Cadwell,R.C. and Joyce,G.F. (1992) *PCR Meth. Appl.*, **1**, 28–33.

Castle,L.A. *et al*. (2004) *Science*, **304**, 1151–1154.

Chodorge,M., Fourage,L., Ullmann,C., Duvivier,V., Masson,J.-M. and Lefevre,F. (2005) *Adv. Synth. Catal.*, **347**, 1022–1026.

Clackson,T., Hoogenboom,H.R., Griffiths,A.D. and Winter,G. (1991) *Nature*, **352**, 624–628.

Crameri,A., Raillard,S.A., Bermudez,E. and Stemmer,W.P. (1998) *Nature*, **391**, 288–291.

Daugherty,P.S., Chen,G., Iverson,B.L. and Georgiou,G. (2000) *Proc. Natl Acad. Sci. USA*, **97**, 2029–2034.

Dupret,D., Masson,J.-M. and Lefèvre,F. (2002) European Patent 1104457, US Patent 6951719.

Fourage,L., Lefèvre,F., Wieland,S., Weber,A., Beckers,A., Maurer,K.H. and Kottwitz,B. (2005) Patent WO 2005118793.

Fox,R.J. *et al*. (2007) *Nat. Biotechnol.*, **25**, 338–344.

Fulda,S. and Debatin,M. (2004) *Curr. Opin. Pharmacol.*, **4**, 327–332.

Giver,L., Gershenson,A., Freskgard,P.O. and Arnold,F.H. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 12809–12813.

Gong,J., Yang,D., Kohanim,S., Humphreys,R., Broemeling,L. and Kurzrock,R. (2006) *Mol. Cancer Ther.*, **5**, 2991–3000.

Graff,C.P. and Wittrup,K.D. (2003) *Cancer Res.*, **63**, 1288–1296.

Graff,C.P., Chester,K., Begent,R. and Wittrup,K.D. (2004) *Prot. Eng. Des. Sel.*, **17**, 293–304.

Greaney,P. *et al*. (2006) *Leukemia Res.*, **30**, 416–426.

Groves,M., Lane,S., Douthwaite,J., Lowne,D., Rees,G.D., Edwards,B. and Jackson,R.H. (2006) *J. Immunol. Methods*, **313**, 129–139.

Hanes,J. and Plückthun,A. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 4937–4942.

Hanes,J., Jermutus,L. and Plückthun,A. (2000) *Methods Enzymol.*, **328**, 404–430.

Harayama,S. (1998) *Trends Biotechnol.*, **16**, 76–82.

Jermutus,L., Ryabova,L.A. and Plückthun,A. (1998) *Curr. Opin. Biotechnol.*, **9**, 534–548.

Jermutus,L., Honegger,A., Schwesinger,F., Hanes,J. and Plückthun,A. (2001) *Proc. Natl Acad. Sci. USA*, **98**, 75–80.

Luginbühl,B., Kanyo,Z., Jones,R.M., Fletterick,R.J., Prusiner,S.B., Cohen,F.E., Williamson,R.A., Burton,D.R. and Plückthun,A. (2006) *J. Mol. Biol.*, **363**, 75–97.

Maheshri,N. and Schaffer,D.V. (2003) *Proc. Natl Acad. Sci. USA*, **100**, 3071–3076.

McCafferty,J., Fitzgerald,K.J., Earnshaw,J., Chiswell,D.J., Link,J., Smith,R. and Kenten,J. (1994) *Appl. Biochem. Biotechnol.*, **47**, 157–171.

Midelfort,K.S., Hernandez,H.H., Lippow,S.M., Tidor,B., Drennan,C.L. and Wittrup,K.D. (2004) *J. Mol. Biol.*, **343**, 685–701.

Moore,G.L., Maranas,C.D., Lutz,S. and Benkovic,S.J. (2001) *Proc. Natl Acad. Sci. USA*, **98**, 3226–3231.

Pavlou,A.K. and Belsey,M.J. (2005) *Eur. J. Pharma. Biopharma.*, **59**, 389–396.

Pukac,L. *et al*. (2005) *Br. J. Cancer*, **92**, 1430–1441.

Rajpal,A., Beyaz,N., Haber,L., Cappuccilli,G., Yee,H., Bhatt,R.R., Takeuchi,T., Lerner,R.A. and Crea,R. (2005) *Proc. Natl Acad. Sci. USA*, **102**, 8466–8471.

Ravot,G., Masson,J.-M. and Lefèvre,F. (2006) In Oren,A. and Rainey,F. (eds.), *Methods in Microbiology: Application of Extremophiles*. Elsevier, London, pp. 765–794.

Rui,L., Cao,L., Chen,W., Reardon,K.F. and Wood,T.K. (2005) *Appl. Environ. Microbiol.*, **71**, 3995–4003.

Schier,R., McCall,A., Adams,G.P., Marshall,K.W., Merritt,H., Yim,M., Crawford,R.S., Weiner,L.M., Marks,C. and Marks,J.D. (1996) *J. Biol. Chem.*, **263**, 551–567.

Sio,C.F., Riemens,A.M., Van der Laan,J-M., Verhaert,R.M. and Quax,W.J. (2002) *Eur. J. Biochem.*, **269**, 4495–4504.

Stemmer,W.P. (1994) *Nature*, **370**, 389–391.

Stemmer,W.P. (1995) *Biotechnology*, **13**, 549–553.

Thom,G., Cockroft,A.C., Buchanan,A.G., Joberty Candotti,C., Cohen,S.E., Lowne,D., Monk,P., Shorrock-Hart,C.P., Jermutus,L. and Minter,R.R. (2006) *Proc. Natl Acad. Sci. USA*, **103**, 7619–7624.

Wajant,H., Gerspach,J. and Pfizenmaier,K. (2005) *Cytokine Growth Fact. Rev.*, **16**, 55–76.
Wu,H., Beuerlein,G., Nie,Y., Smith,H., Lee,B.A., Hensler,M., Huse,W.D. and Watkins,J.D. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 6037–6042.
Yang,J., Swaminathan,C.P., Huang,Y., Guan,R., Cho,S., Kieke,M.C., Kranz,D.M., Mariuzza,R.A. and Sundberg,E.J. (2003) *J. Biol. Chem.*, **278**, 50412–50421.
Zahnd,C., Spinelli,S., Luginbühl,B., Amstutz,P., Cambillau,C. and Plückthun,A. (2004) *J. Biol. Chem.*, **279**, 18870–18877.
Zhao,H., Giver,L., Shao,Z., Affholter,J.A. and Arnold,F.H. (1998) *Nat. Biotech.*, **16**, 258–261.

Received November 7, 2007; revised March 5, 2008; accepted March 7, 2008

Edited by Hugues Bedouelle