Phage Display and Selection of a Site-Directed Randomized Single-Chain Antibody Fv Fragment for Its Affinity Improvement†

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ABSTRACT: The affinity of an antibody Fv fragment was improved by semirational design involving site-directed randomization and phage display. On the basis of the predicted model of an anti-2-phenyloxazol-5-one (phOx) antibody Fv fragment, into which the ligand was inserted with the help of nuclear Overhauser enhancement (NOE) data, residues close to the hapten were identified. Seven of these residues in the third hypervariable regions of light and heavy chains were randomized in polymerase chain reactions (PCR) using degenerate oligonucleotides. Resulting clones were expressed as single-chain Fv (scFv) fragments on the surface of filamentous phage and selected for binding to phOx-conjugated bovine serum albumin. Selected Fv fragments were analyzed for hapten affinity by fluorescence quenching, and several mutants with improved affinities were identified. Phage selection on the basis of binding was very successful when phage scFv mutants differed in affinity by at least a factor of 6. Smaller differences did not result in predominant selection of the best binder. Combination of the two point mutations most crucial for improved hapten binding decreased the dissociation constant of the Fv for phOx 11–14-fold. Hapten binding of the improved Fv was analyzed in NOE experiments.

Structural information about antibody–antigen interactions helps to predict the effect of mutations on antigen affinity. The removal of favorable interactions like hydrogen bonds or ionic interactions as a consequence of a changed amino acid residue will usually result in loss of affinity. To increase antigen affinity by generation of new, favorable interactions or removal of unfavorable ones is in general more difficult, requiring the introduction of new side chains in the correct orientations. Successful prediction of mutational effects on antigen affinity depends on correct high-resolution structures, which are rarely available.

However, the general location of the antigen within the combining site of an antibody can be determined with limited effort: for example, by use of molecular modeling, sequence comparisons, mutagenesis experiments, and low-resolution X-ray or nuclear magnetic resonance (NMR)[1] analyses. We propose to take advantage of such information for a semirational design of antibodies to improve antigen affinity involving their site-directed randomization and expression on the surface of phage (Smith, 1985; Parmley & Smith, 1988). The surface display of antibody fragments on filamentous phage allowed the selection and rescue of specific antibody variable regions from libraries of different origins (e.g., Marks et al., 1991; Barbas et al., 1991; Hoogenboom et al., 1992). It was also successfully used for competitive selection to improve the affinity after exchange (Marks et al., 1992) of one of the two variable regions or randomization (Hawkins et al., 1992; Gram et al., 1992). However, random libraries need to be of considerable size to contain a clone with the desired properties. If, on the basis of structural information, randomizing is restricted to a few residues, the number of possible mutations can be adjusted so that the size of the library, which should cover all of them, is no longer a technical obstacle.

The approach of a restricted, site-directed randomization is demonstrated here for an antibody Fv fragment specific for the hapten 2-phenyloxazol-5-one (phOx). A structural model of the Fv was built according to the principles of the canonical antibody hypervariable loop structures (Chothia & Lesk, 1987; Chothia et al., 1989). The hapten phOx was docked into the model with the help of nuclear Overhauser enhancement data (McManus & Riechmann, 1991). On the basis of the model of the complex, we previously increased the antigen affinity of the Fv by design (Riechmann et al., 1992). The side chain of an aspartyl residue appears to be too close to the hydrophobic ligand phOx, and its mutation to alanine resulted in a 3-fold increase of hapten affinity.

Such a rational approach has obvious limitations, when no high-resolution structure is available. Uncertainties of side-chain orientations make the prediction of mutational effects more than difficult, and mutations for affinity improvements are rarely obvious. However, modeling combined with very little NOE data has proven to be sufficient to identify residues located close to the ligand. Therefore we widened our strategy to improve the affinity of the anti-phOx Fv by randomization of residues chosen simply on the basis of their neighborhood to the hapten, even if they do not cause obviously unfavorable contacts. Several libraries of phage displaying single-chain Fv fragments (scFv) randomized at such positions were constructed and selected for hapten affinity. Mutants selected from these libraries were analyzed for affinity by fluorescence quenching and for structural changes of hapten binding by NMR spectroscopy.

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[1] Abbreviations: CDR, complementarity-determining regions; g3p, fd-geneIII-protein; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; NMR, nuclear magnetic resonance; pelB, pectate lyase B; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; phOx-BSA, 2-phenyloxazol-5-one-conjugated bovine serum albumin; OxGABA, 4-amino-γ-butyryl-2-phenyloxazol-5-one; OxGly, 4-glycyl-2-phenyloxazoyl-5-one; scFv, single-chain Fv fragment; TOCSY, total correlated spectroscopy; VH, heavy-chain variable region; VL, light-chain variable region; 2D, two-dimensional. Mutants are denoted by a number for the residue involved, with a preceding letter for the wild-type amino acid and a succeeding letter for the mutant amino acid.

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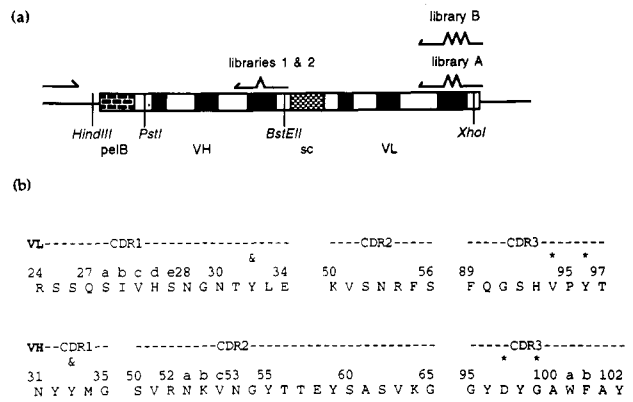

FIGURE 1: (a) Diagram of the template DNA used for the PCR amplification of the NQ11.7.22 scFv. CDRs in VH and VL (empty) are drawn in black. The locations of the PCR primers and restriction sites used for generation and cloning of the libraries are indicated (pelB = pectate lyase B leader DNA). (b) Amino acid sequence of the NQ11.7.22 complementarity-determining regions in light and heavy chain. Full details about protein and DNA sequence can be found elsewhere (Berek et al., 1985; McManus & Riechmann, 1991). CDR3-residues randomized in the libraries (asterisks) and the two Tyr residues (ampersands) in the CDR1s of VL and VH directly in contact with phOx are marked. Residues are numbered according to Kabat et al. (1991).

EXPERIMENTAL PROCEDURES

DNA Technology. Residues VH-99 and VH-97 of the NQ11.7.22 antibody Fv fragment (McManus & Riechmann, 1991) were randomized separately in a PCR amplification (Saiki et al., 1985) of the pelB leader–NQ11.7.22 heavy-chain variable region (VH) fragment (Figure 1) in M13 using the synthetic oligonucleotides LMB3 (Marks et al., 1991) and 99RAND (5′-GGA GAC GGT GAC CGT GGT CCC TTG GCC CCA GTA AGC AAA CCA GGC (C/G)NN GTA AGC ATA-3′) or 97RAND (5′-GAG GAG ACG GTG ACC GTG GTC CCT TGG CCC CAG TAA GCA AAC CAG GCC CCG TA(C/G) NNA TAG CCT C-′3) containing a *Bst*EII restriction site (underlined) for subcloning. For all libraries PCR was performed with 30 cycles of 92 °C (50 s), 32 °C (2 min), and 72 °C (90 s) using an oligonucleotide concentration of 0.5 pmol/μL each and Taq polymerase. PCR products were digested with *Hin*dIII (part of M13mp19/pUC19 polylinker) and *Bst*EII and cloned into the phagemid vector pHEN1 (Hoogenboom et al., 1991) containing a single-chain linker (sc)–NQ11.7.22 light-chain variable region (VL)–fd gene III fusion gene for expression of fd gene III protein (g3p)/scFv fusions. The sc linker consists of the peptide $(Gly_4Ser_3)_3$ (Houston et al., 1988). Two libraries, 1 (VH-99) and 2 (VH-97), were prepared by transformation of the *Escherichia coli* strain TG1. ScFv-displaying phage were prepared using VCS-M13 helper phage (Stratagene).

Residues VL-94 and -96 or VL-92, -93, and -97 were randomized by PCR amplification of the scFv gene (Figure 1) of NQ11.7.22 in pUC119 (McManus & Riechmann, 1991) using LMB3 and the synthetic oligonucleotides L3FOR1A3 (5′-CCG TTT GAT CTC GAG CTT GGT CCC CCC TCC GAA CGT (C/G)NN CGG (C/G)NN ATG TGA ACC TTG-3′) or L3FOR2B3 (5′-CCG TTT GAT CTC GAG CTT GGT CCC CCC TCC GAA (C/G)NN GTA CGG AAC (C/G)NN ACC TTG AAA GCA-3′) containing an *Xho*I site (underlined) for cloning. PCR products were cloned as *Pst*I (in the VH)/*Xho*I fragments into the fd vector fd-tet-DOG1 (Hoogenboom et al., 1991) for fusion with g3p. Ligated DNA was electroporated (Dower et al., 1988) into the *E. coli* strain MC1061 resulting in libraries A (VL-94 and -96) and B (VL-92, -93, and -97).

Table I: Affinities of the NQ11.7.22 Antibody Fv Fragment and Its Mutants for phOx

VH	VL	k_d[a] (nm), OxGABA	K_d[a] (nM), OxGly
WT[b]	WT	334 ± 46	366 ± 69
		Library 2	
D97A	WT	99 ± 21	134 ± 27
D97P	WT	291 ± 47	304 ± 48
D97S	WT	276 ± 65	245 ± 61
D97G	WT	271 ± 89	392 ± 144
		Library A	
WT	V94T, Y96F	163 ± 37	165 ± 57
WT	V94P, Y96F	86 ± 32	66 ± 3
WT	Y96F	53 ± 9	60 ± 9
WT	V94L, Y96F	135 ± 17	110 ± 38
WT	Y96L	>600	>600
		Library B	
WT	S92S, H93S, T97S	224 ± 40	243 ± 45
WT	S92S, H93R, T97S	126 ± 12	166 ± 32
		Combined Effects	
D97A	V94T, Y96F	50 ± 13	37 ± 11
D97A	V94P, Y96F	24 ± 4	31 ± 3
D97A	Y96F	30 ± 2	32 ± 5

[a] Dissociation constants were determined by fluorescence quenching of hapten-titrated heterodimeric Fv's at 25 °C in PBS, pH 7.0, as described (Foote & Milstein, 1991; Eisen, 1964). [b] WT = wild-type VH or VL.

PCR-aided colony screening (Güssow & Clackson, 1989) for inserts of the correct size was performed with the oligonucleotides LMB3 and pHEN-SEQ (Marks et al., 1991) in the case of phagemid libraries 1 and 2 or with fdSEQ1 and fdPCRBAK (Hoogenboom et al., 1991) in the case of fd libraries A and B.

Heavy-chain variable regions were subcloned as *Hin*dIII/*Bst*EII fragments into a pUC119-based expression vector for the expression of heterodimeric NQ11.7.22 Fv (McManus & Riechmann, 1991). Light-chain variable regions were subcloned as *Sac*I/*Xho*I fragments. Mutants in Table I, which were not isolated from phage libraries, were engineered by site-directed mutagenesis in M13.

Phage Selection. From the libraries about 2×10^{10} phage particles were prepared for each single experiment. Phage were resuspended in phosphate-buffered saline (PBS), pH 7.0, containing 3% BSA for blocking of nonspecific binding. Selection for binding to 2-phenyloxazol-5-one-conjugated BSA (phOx-BSA) was carried out for libraries 1 and 2 in Immunotubes (Nunc, 3.5-mL volume) and for libraries A and B in microtiter-well plates (Falcon, 140-μL volume). Prior to selection, tubes or wells had been incubated with phOx-BSA in PBS at a concentration of 1 mg/mL overnight at room temperature and blocked for 1–2 h with 3% PBS at 37 °C. Binding of phage was carried out for 2–4 h, followed by extensive washing (at least 10 volumes) with first PBS and then PBS containing 0.1% Tween-20. Phage were eluted from wells with 0.1 M triethylamine for 1 min and immediately afterward neutralized with 1 M Tris, pH 7.4. From tubes, phage were eluted with 1.5 mM 4-glycyl-2-phenyloxazol-5-one (OxGly) in PBS. Eluted phage were used for infection of the *E. coli* strain JM109 to prepare phage (25-mL cultures grown at 37 °C overnight) for the next round of selection.

Fv Expression. Fv protein was basically expressed and purified as described before (McManus & Riechmann, 1991) except that induction was performed at 30 rather than 37 °C.

Spectroscopic Techniques. The dissociation constants for 4-amino-γ-butyryl-2-phenyloxazol-5-one (OxGABA) and

OxGly were determined as described (Foote & Milstein, 1991). NMR experiments were performed on a Bruker AMX-500 spectrometer as described (McManus & Riechmann, 1991) and analyzed with FELIX software (D. Hare). Spectra were recorded at 313 K with about 0.8 mM Fv and 4.8 mM OxGly in 99% deuterium oxide containing 10 mM sodium phosphate, pH 6.2, and 200 mM NaCl.

RESULTS

Hapten Binding Site. The antigen binding site of the anti-phOx antibody Fv fragment originating from the hybridoma NQ11.7.22 (Berek et al., 1985) was investigated previously by molecular modeling, site-directed mutagenesis, and NMR spectroscopy (McManus & Riechmann, 1991). We found that the binding site for the hapten phOx was formed mainly by the complementarity-determining regions (CDRs) 1 and 3 in both heavy and light chain. Direct proton interactions in the form of NOEs were identified from the side chains of VH-Y33 (VH-CDR1) and VL-Y32 (VL-CDR1) to the benzyl ring of the hapten (McManus & Riechmann, 1991). On the basis of this information, when the phOx hapten was inserted into the model, within VH-CDR3 residues VH-G99 and VH-D97 were closest to the hapten. For a glycine α-proton (identified in experiments with the ^{13}C-labeled Fv; Riechmann et al., 1991), probably belonging to VH-G99, a strong NOE to phOx was seen (L.R., unpublished results). For residue VH-97 an NOE interaction to the hapten was only observed when the wild-type aspartic acid was mutated to alanine (VH-D97A) (Riechmann et al., 1992). This mutant had a 3-fold increased affinity for the phOx derivatives OxGly and OxGABA when compared to wild-type Fv.

Randomizing Residues in VH-CDR3. To analyze the involvement of residues VH-97 and -99 in hapten binding further, we randomized them separately and expressed the resulting clones as scFv/g3p fusion proteins on the surface of phage. Two libraries, each with about 10^4 members, randomized at position VH-99 (library 1) or position VH-97 (library 2) were constructed using degenerate oligonucleotides as forward primers in a PCR amplification of the wild-type VH. The randomized scFv genes were cloned into a phagemid vector, which made it necessary to use helper phage for the preparation of scFv-displaying phage. As a consequence of the endogenous helper phage gene III, scFv was not fused to each g3p copy (on average three; Gray et al., 1981; Glaser-Wuttke et al., 1989), thereby minimizing avidity effects through multivalent hapten binding of phage scFv. The libraries were random and essentially without ligation background according to 12 sequenced clones.

Both libraries were separately selected on phOx-BSA (14:1 phOx/BSA ratio) coated tubes and eluted with free hapten. From library 1 all clones rescued and sequenced after the first (9 clones) and second round (12 clones) had a Gly at position VH-99. The result was not unexpected, as this Gly was presumably crucial for phOx binding. The side chains of other amino acids in this position, which would be surface-exposed (Chothia & Lesk, 1987), might impede phOx binding through steric hindrance and/or might cause the VH-CDR3 hairpin loop to fold in a way unsuitable for phOx binding. For example the mutant most similar to the wild type, VH-G99A, engineered by site-directed mutagenesis, had no binding activity at all.

From library 2, in which residue VH-97 was randomized, different scFv clones were selected. After the first round of selection on phOx-BSA, clones with Ser (3), Thr (2), Ala, Glu, Pro, Gln, and Val (1 each) were sequenced. After a second round of selection, clones had Pro (8), Ser, Thr, Ala, and Phe (1 each) at position VH-97, and after a third round, Pro (10), Ala (1), and Ser (1) were found. We determined the phOx affinities of the three VH-97 mutants rescued after three rounds of selection, indicating that Ala in this position resulted in the best hapten affinity (Table I). This result was in agreement with the previously designed affinity improvement at position VH-97 (Riechmann et al., 1992).

Because the phOx affinities of wild-type *scFv* and *heterodimeric* Fv were identical (L.R., unpublished results), we assumed that the affinities of scFv mutants and their heterodimeric counterparts were also virtually identical, especially as the NMR analysis (see below) showed very similar intramolecular NOEs for all mutants analyzed. Assuming further that soluble and surface-displayed scFv were identical with respect to hapten binding, selection of library 2 must have been based on factors other than the competition for binding sites alone, since in most cases the best binding mutant (VH-D97A) was not found. However, all mutants rescued after three rounds had phOx affinities different by no more than a factor of 3, and all bound hapten slightly better than the wild type.

Randomizing Residues VL-94 and -96. The phage display system was next used to analyze the effects of randomizations within CDR3 of the NQ11.7.22 light chain. VL-CDR3 formed part of the hapten binding pocket in the Fv model, but no NOEs to phOx were observed for residues within this CDR. The side chains of residues VL-V94 and VL-Y96 pointed toward the hapten in our model (Figure 2a). The hydroxyl group of VL–Y96 could according to the model be in direct contact with the hapten. The side chain of VL-V94 was about 7–8 Å distant from the hapten. We chose these two residues (VL-94 and -96) for randomization in a scFv phage display library, for which an fd vector was used. In contrast to libraries 1 and 2 (for which a phagemid/helper phage system was used) here all g3p copies of the phage were fused to scFv, as no helper phage was necessary for the preparation of phage.

Residues VL-94 and VL-96 of the scFv were randomized together in PCR amplification of the wild-type scFv using a single degenerate oligonucleotide. The resulting library A contained about 8.5×10^5 clones with an insert of expected size and an additional 1.5×10^5 members containing the cloning vector as ligation background, according to PCR-aided colony screening. This library size guaranteed easily that all possible mutations were indeed present (see statistical analysis in the Discussion).

Initially library A was selected for binding to phOx-conjugated BSA in wells derivatized with a phOx-BSA preparation containing on average 14 phOx groups/BSA molecule. This ratio is likely to enable the phage to bind multivalently through the multiple scFv copies, creating an avidity effect. The selection was carried out for six rounds. In a second experiment, wells used for selection were derivatized with a phOx-BSA preparation containing on average 1.9 phOx groups/BSA molecule. This ratio should diminish multivalent binding. Selection in this case was carried out for five rounds. The results are summarized in Table II.

In both experiments the number of phage bound to the well reached a plateau in the second round of selection (10^6–10^7 eluted phage), indicating an enrichment for phOx binding phage after the first round. The ligation background was virtually eliminated in this first round, and no non-scFv phage outgrew scFv phage later. The higher number of eluted phage in the first round of the experiments performed with the high-ratio phOx-BSA as opposed to the one with the low-ratio

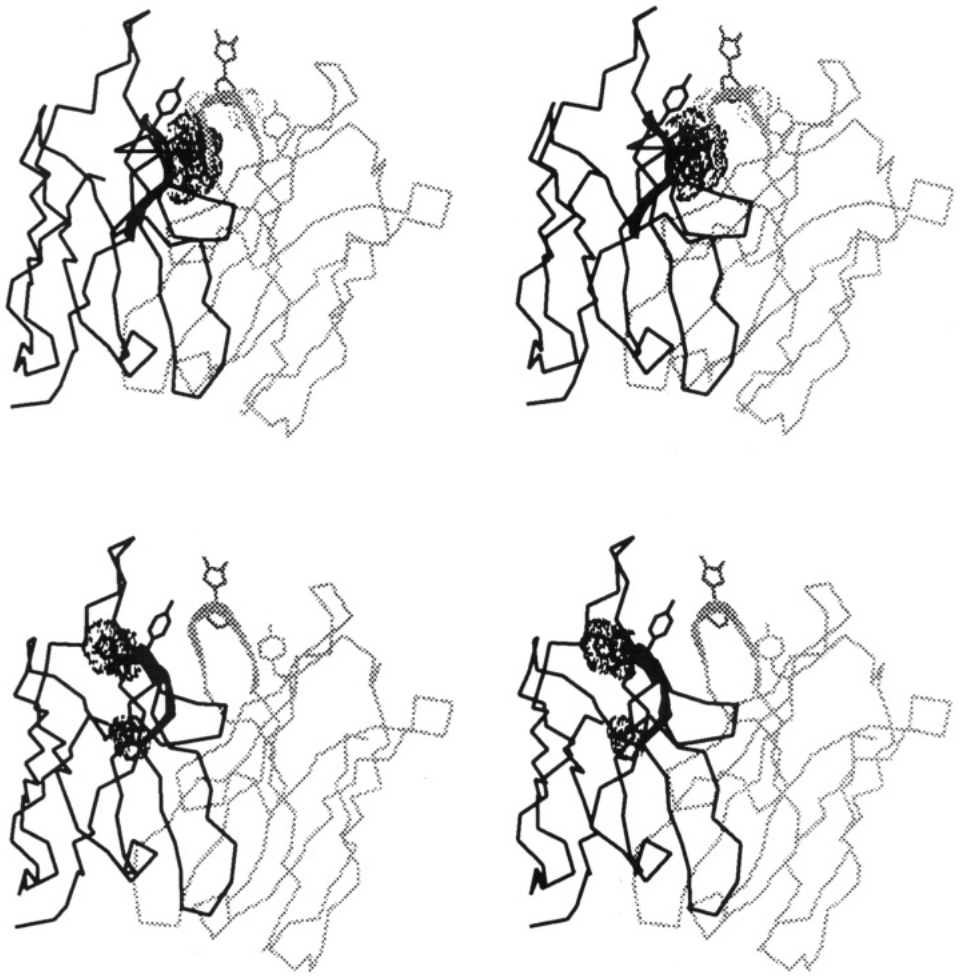

FIGURE 2: Model of the NQ11.7.22-Fv/phOx complex. (Top) The α-carbon trace of the VL is shown in black; that of the VH is in gray. The side chains of residues VL-94 and -96 and VH-97 and -99 are attached with a van der Waals surface. The hapten phOx and the side chains of VL-Y32 and VH-Y33 are also shown in detail. The trace of the CDR3 loops in VH and VL is highlighted with a ribbon. (Bottom) The same model as shown at the top except here the side chains of VL-92, -93, and -97 are attached with a surface cloud.

Table II: Selection of VL-CDR3 Library A (Residues VL-94 and -96 Randomized)

selection after round	eluted[a]	with insert[b]	sequences[c]
	(A) Selection on High-Ratio phOx-BSA (14:1)		
1	1.5×10^5	14/14	Y,Y; S,F; L,L
2	1.0×10^7	nd[d]	nd
3	3.4×10^6	6/6	S,F; T,M; P,H; H,Y; P,F; A,F
4	1.1×10^7	5/5	T,F; T,F; T,F; R,Y; G,F
5	4.7×10^6	5/6	T,F; T,F; T,F; T,F; P,F
6	1.5×10^7	5/6	T,F; T,F; T,F; T,F; A,F
	(B) Selection on Low-Ratio phOx-BSA (1.9:1)		
1	2.5×10^3	6/6	T,S; Y,R; L,F; A,H
2	2.5×10^6	4/6	T,R; A,R; T,F; T,L
3	6.6×10^6	5/6	P,F; I,L; N,F
4	5.2×10^6	6/6	P,F; P,F; T,F
5	5.0×10^6	6/6	P,F; P,F; S,F; I,L

[a] Number of eluted phage was estimated by titration of infective events. [b] Number of randomly picked clones with insert of size expected for intact scFv compared with number of clones analyzed by either PCR-aided colony screening or sequencing. [c] Sequences of randomly picked colonies; 94,96 = V,Y in WT. [d] nd, not determined.

phOx-BSA might have been caused by avidity effects, which enabled low-affinity scFv phage to bind more strongly.

With the high-ratio phOx-BSA after four rounds of selection the most frequently found scFv contained the sequence VL-V94T,Y96F, whereas with the low-ratio phOx-BSA the predominant sequence was VL-V94P,Y96F (Table II). The selection result was unambiguous with respect to position VL-96, for which Phe was clearly preferred over any other residue. Only two of 22 randomly picked clones after four and five rounds did not have a Phe but instead a Leu or Tyr (wild-type residue) at this position. Less clear was the result for residue 94.

For the VL double mutants (VL-V94T,Y96F and VL-V94P,Y96F), soluble heterodimeric Fv fragment was produced to measure the affinity for phOx (Table I). The VL-V94T,-Y96F Fv had an affinity 2-fold increased for two phOx derivatives when compared to wild type. The VL-V94P,-Y96F Fv had a 4–5-fold increased affinity. Thus, with use of the low-ratio phOx-BSA the library was predominantly selected for a mutant with a higher affinity than in the case of the high-ratio phOx-BSA. Seemingly the competition for phOx binding sites was more effective when the number of sites was smaller and/or if they were spatially more distant.

Possible reasons for the failure to select for the best binder on the matrix with the higher ligand concentration were discussed and analyzed in detail by Hawkins et al. (1992). In short, selection might not have been effective enough to overcome differential growth properties of the various phage scFv mutants. Phage scFv could have bound to the hapten groups more often multivalently via their several g3p-scFv copies in the case of the high-ratio versus the low-ratio phOx-BSA. Then, despite the relatively high off rate of the Fv's with respect to soluble hapten ($k_{off} = 34\ s^{-1}$ for the wild type, $k_{off} = 16\ s^{-1}$ for the VL-94T96F Fv assuming $k_{on} = 10^8\ M^{-1}\ s^{-1}$; McManus & Riechmann, 1991), multivalent attachment

could have decreased the off rate so much that phages scFv once bound would have rarely dissociated during selection and binding was more diffusion-controlled than competition-controlled. As a consequence more abundant phage would have been favored over phage with higher affinity. Hawkins et al. (1992) solved this problem by using monovalent, soluble antigen at limiting concentration for selection. Here the low-ratio phOx-BSA was able to diminish these effects. Another solution to reduce multivalent binding is theoretically the use of the phagemid system, which, however, also did not solve the problem completely (see library 2).

Selection for suboptimum binders could also be due to differential on and off rates. Thus, scFv with weaker affinity could have lower off rates than scFv with stronger affinity, if their respective on rates differed significantly. Then high-ratio phOx-BSA and multivalent binding would favor selection on the basis of off rate rather than affinity.

Residue VL-94. Because of the ambiguous selection results with library A concerning the amino acid at position VL-94, we consulted the structural model. The wild-type VL-V94 side chain was 7–8 Å distant from the hapten in our Fv model and probably not in direct contact with the hapten. Therefore, its effects on hapten affinity were likely to be indirect via interaction with, for example, the side chain of residue VL-96. We were curious whether Thr and Pro at position VL-94 were indeed better for binding than the wild-type Val. Consequently, we engineered the single mutant VL-Y96F, which had the wild-type Val at position VL-94. Its affinity was slightly better than that of VL-V94P,Y96F Fv (Table I). However, an increase in side-chain size beyond that of Val had a negative effect on the affinity. Thus the engineered VL-V94,Y96F Fv had an affinity lower than both the VL-V94L,Y96F and the VL-V94P,Y96F mutant Fv's (Table I).

Randomizing Residues VL-92, -93, and -97. In library A, residues for randomization (VL-94 and -96) were chosen on the basis of their side-chain orientation toward the hapten. Another library, B, was generated, in which residues within VL-CDR3 were randomized whose side chains were expected to point away from the hapten (Figure 2b). Thus any affinity improvement observed for clones from this library should be caused by a rearrangement of the peptide backbone, resulting in improved contacts of the backbone or the side chains of unmutated residues.

The degenerate oligonucleotide L3FOR2B3 used for the generation of library B was designed to randomize residues 92 (S in WT), 93 (H in WT), and 97 (T in WT) of the light chain (Figure 2b). Library B was constructed and selected (Table III) using the same conditions as for library A. It contained about 9×10^5 clones with an insert of expected size and an additional 1.25×10^5 clones containing the cloning vector as ligation background.

Selection of library B on high-ratio phOx-BSA was successful insofar as almost all clones analyzed contained scFv inserts after all of the five rounds of selection. In contrast, selection on low-ratio phOx-BSA was more problematic, because in two attempts non-scFv phage began to outcompete scFv phage after three rounds of selection. This result illustrated that adverse combinations of phage growth properties, ligand affinity, and ligand concentration could cause the selection process to collapse after several rounds of selection in favor of probably well-growing but nonspecific phage.

However, altogether 39 clones with scFv inserts were rescued and sequenced after selection of library B. Selection was most unambiguous with respect to residue VL-92, for which 59% of the clones had a Ser (Thr was the next most frequent with 25.6%). For residue VL-93, Ser (23%), Thr (15.3%), His, Arg, and Ile (each 12.8%) were most frequently found. In the case of residue VL-97, most clones had either an Arg (25.6%) or a Ser (23%). For none of the positions randomized was a preference as unambiguous as in the case of library 1 (VH-G99) and library A (VL-Y96F) observed, suggesting that no hugely improved mutated Fv was present in library B.

Table III: Selection of VL-CDR3 Library B (Residues VL-92, -93, and -97 Randomized)

selection after round	eluted	with insert	sequences[b]
	(A) Selection on High-Ratio phOx-BSA (14:1)		
1	3.8×10^5	14/14	S,Q,S; T,H,S; T,S,R; H,R,R; T,I,S; T,S,T
2	1.6×10^6	6/6	S,S,G; A,T,T; V,N,T; T,T,A; S,L,R; S,R,L
3	4.8×10^6	6/6	T,V,S; S,I,Q; N,T,T; N,H,G; S,T,S; S,T,A
4	1.1×10^7	6/6	T,I,R; S,H,T; S,H,K; S,R,H; S,E,R; S,S,S
5	1.0×10^7	5/6	S,S,R; S,S,R; S,S,R; V,N,S
	(B) First Attempt: Selection on Low Ratio phOx-BSA (1.9:1)		
1	1.6×10^5	9/10	nd
2	8.4×10^5	9/10	nd
3	4.0×10^6	1/10	nd
	(C) Second Attempt: Selection on Low Ratio phOx-BSA (1.9:1)		
1	1.4×10^5	10/10	S,E,T; S,S,H; S,R,L; T,N,Q
2	5.3×10^5	5/10	S,H,R; T,S,R; T,T,S; S,I,A
3	2.1×10^7	3/10	S,I,K; S,R,S; S,I,A

[a] For explanations see Table II. [b] 92,93,97 = S,H,T in WT.

The result showed that affinity improvement could also be obtained via randomization of residues whose side chains pointed away from the ligand and most likely did not directly participate in binding. However, the affinity improvement was less than that obtained with the VL-Y96F mutation.

Three VL mutants were chosen for expression as heterodimeric Fv's in combination with the wild-type VH to analyze their hapten affinity. From these the VL-S92S,H93S,-T97R Fv was expressed but not secreted from *E. coli* as judged from Western blot analysis. Possibly the side chain of residue VL-T97R was unsuitable for VH/VL association in a heterodimeric Fv but might be tolerated in the g3p-fused scFv. The heterodimeric versions of the other two mutants, the VL-S92S,H93S,T97S Fv and the VL-S92S,H93R,T97S Fv, were secreted and could be purified on phOx–Sepharose. Both had an affinity for phOx moderately higher than the wild type (Table I).

Combining Mutations. To combine the affinity-improving effects of independently selected mutants, these effects also had to be independent from each other. The two mutations most effective for affinity improvement were part of VH-CDR3 (VH-97) and VL-CDR3 (VL-96), therefore located on opposite sites of the hapten binding site and spatially relatively distant (Figure 2). When the mutated heavy chain VH-D97A (resulting in a 3-fold increase on its own) was combined with any one of the three VL mutants, VL-V94T,-Y96F, VL-V94P,Y96F, or VL-Y96F, a further 2–3-fold increase in affinity compared to Fv containing wild-type heavy chain was observed (Table I). This indicated that the geometry of the hapten in the combining site was not crucially altered by the mutations, as improvements of contacts in VL-CDR3 were independent of the heavy-chain mutation VH-D97A and vice versa. Similar results after combination of affinity-improving mutations in light and heavy chain were observed for the Fv of the anti-lysozyme antibody D1.3 (Hawkins, Russell, Baier, and Winter, in preparation).

Improved Hapten Binding. Combination of the light-chain mutants VL-Y96F and VL-V94P,Y96F with the heavy-chain mutant VH-D97A resulted in Fv's with the highest affinities

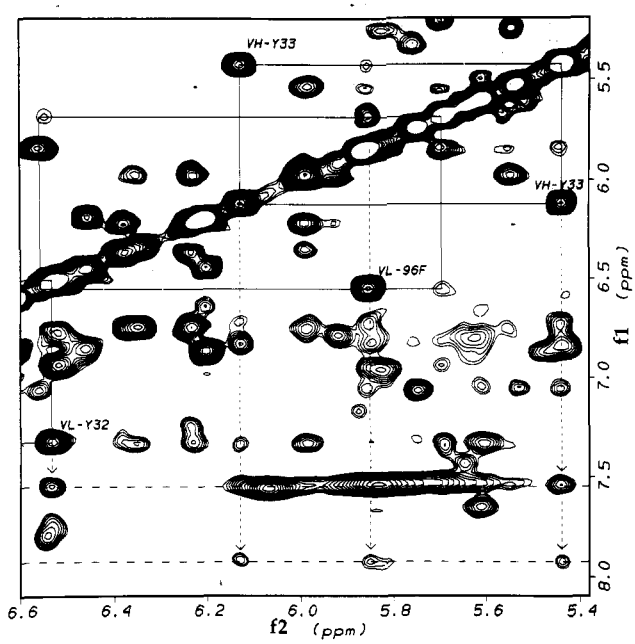

FIGURE 3: Two-dimensional ^{1}H NOESY (mixing time τ_m = 100 ms) recorded with the VH-97A,VL-94P,96F mutant Fv of the NQ11.7.22 antibody in the presence of a 6-fold excess of OxGly. Spin system of side chains involved in phOx binding (solid lines), their NOEs (broken lines with arrows) to phOx, and the frequencies of the benzylic protons of the free hapten (broken lines; *ortho* at 7.9 ppm, *meta*/*para* at 7.55 ppm) are indicated.

for the phOx derivatives obtained in our experiments yet: 24–32 nM. Two point mutations (VL-96 and VH-97) in combination increased the affinity 11–14-fold compared to wild type. As the choice of residues for randomization was based on the predicted orientation of their side chains toward the ligand, we were interested whether the achieved affinity improvement could indeed have been connected with an interaction of these side chains with the hapten. This would also increase faith in our model of the Fv/hapten complex. Hapten binding of the affinity-improved VH-D97A,VL-V94P,Y96F Fv was analyzed directly by two-dimensional (2D) ^{1}H NMR spectroscopy.

In NOESY experiments (Jeener et al., 1979; Figure 3) transfer NOE cross peaks (Clore & Gronenborn, 1982, 1983) between protons of the bound hapten OxGly and the Fv were observed for the free hapten similar to those seen in the case of the wild-type Fv (McManus & Riechmann, 1991). The NOEs from both the VH-Y33 ring protons and the α-proton of presumably VH-G99 to *ortho* and *meta*/*para* protons of phOx and from the VL-Y32 ring to the degenerate *para*/*meta* protons of phOx (Titman et al., 1979) remained. Of the two NOEs between the *ortho* and the *meta*/*para* protons of phOx and the α-proton of Ala97 determined for the mutant Fv VH-D97A (combined with the wild-type VL; Riechmann et al., 1992) the NOE involving the *ortho* proton of phOx was not seen for the affinity-improved VH-D97A; VL-V94P,Y96F Fv (not shown).

The residue most crucial for the affinity improvement achieved in this study was VL-96. The NMR signals of the VL-Y96F ring protons were identified in 2D ^{1}H TOCSY experiments (Braunschweiler & Ernst, 1983) by comparison with the wild-type Fv. With the help of remote peaks in 2D ^{1}H double quantum experiments (Rance et al, 1989) these were further characterized with respect to their positions on the benzyl ring. One weak NOE from the *meta* protons of the VL-Y96F side chain to the *ortho*-benzyl protons of phOx was observed (Figure 3). However, possible NOEs from VL-Y96F to the other phOx protons could be obscured by the strong exchange peaks of free and bound phOx.

On the whole these results confirmed our model, according to which the VL-96 side chain was expected to be in direct contact with the ligand. The changes in the transfer-NOE pattern would be in agreement with a slightly different positioning of phOx in the VH-D97A,VL-V94P,Y96F Fv compared to the VH-D97A Fv. The hapten could have moved slightly away from Ala VH-97 toward the VL-Y96F side chain, which lacked the hydroxyl oxygen of the wild-type VL-Y96 side chain.

In summary, given a suitable amino acid for the three residues (VH-97 and -99 and VL-96), which were randomized and also expected to directly participate in hapten binding, interactions with phOx were seen in form of transfer NOE cross peaks. Only for residue VH-99 was an NOE already observed for the wild-type amino acid. For VH-97 and VL-96, NOEs were only seen after mutation to a new amino acid (VH-D97A and VL-Y96F). Both mutations caused an increase in hapten affinity (3- and 6-fold, respectively). However, the lack of hypothetical NOEs for the wild-type residues at these positions (VH-D97 and VL-Y96) could be due to the fact that only the fast-exchanging oxygen-bound protons of their side-chain carboxyl (although presumably deprotonated) and hydroxyl groups might have been in NOE distance to the ligand but were not observable by NMR spectroscopy under physiological conditions. Thus it cannot be excluded that the newly observed transfer NOEs were incidental and that other conformational changes in the binding site were the cause for the affinity improvement. However, the principally very similar NOE pattern (McManus & Riechmann, 1991; Riechmann et al., 1992) between aromatic side-chain protons in the binding site would argue against major structural differences between the mutants. The principal location and interactions of phOx in the mutant Fv seemed indeed to remain unchanged.

DISCUSSION

The NMR analysis of the interactions between hapten and the affinity-improved Fv illustrated how our aim to increase the affinity of an antibody Fv fragment by optimizing the nature of side chains oriented toward the hapten was achieved. The anti–phOx Fv NQ11.7.22 was randomized at positions identified as being close to the bound ligand by model building and NOE experiments. Antibody fragments with improved phOx affinities were selected from the resulting libraries of randomized and phage surface-displayed scFv.

Which conclusions can be drawn from the results with regard to the use of the phage display system for the semirational design of antibody combining sites to improve their antigen affinity? For two of the seven randomized residues, a very strong selection for one particular amino acid was observed. The wild-type Gly was the only amino acid selected for at position VH-99 and presumably no other amino acid in this position gave hapten binding. For position VL-96 a Phe resulted in the best phOx affinity. The VL-Y96F Fv had a 6-fold higher affinity than the next best Fv, the wild-type VL-Y96-Fv. This difference in affinity was sufficient to select the optimal amino acid using the phage display technique.

More ambiguous results were obtained for the other residues randomized. We previously determined by site-directed mutagenesis that in position VH-97 Ala was better for phOx binding than the wild-type Asp (Riechmann et al., 1992). Ala resulted in an about 2–3-fold higher affinity than several other amino acids, among them Pro (Table I). However, phage

selection favored the VH-D97P scFv over the VH-D97A scFv despite its lower affinity. Similarly, for position VL-94 the phage display experiments selected predominantly for Thr or Pro, although the wild-type Val had at least the same if not a slightly higher affinity. Therefore in cases of similar affinities (within a factor of 3) scFv phage were selected not solely on the basis of affinity but also on the basis of probably phage growth-related properties. The selection for Pro residues in the turn region of the hypervariable loops (VH-97 in VH-CDR3 and VL-94 in VL-CDR3) could for example be connected with improved folding characteristics of the scFv–g3p fusion proteins. Pro residues play an important role in folding initiation via formation of turns within nascent helices of peptides (e.g., Chandrasekhar et al., 1991, and references therein).

Despite this last complication, affinity-improved antibody fragments could be selected and rescued after site-directed randomization of residues crucial for the formation of the combining site and selection of page-displayed scFv libraries. However, when small differences in affinity (2–3-fold) were not sufficient to ultimately select for the best binding mutant, a more rational approach based on the structure of the combining site was more successful (VH-D97A, VL-V94). Therefore the fine-tuning of affinity might in the future still depend on structural information about the ligand binding site.

A semirational approach via site-directed randomization of antibody variable regions for their affinity improvement was successful in case of the NQ11.7.22 Fv, but how practical can one expect it to be in general? If randomization is restricted, as it was here, to very few residues, all possible mutants or combination of them can be covered with medium-size libraries and none (i.e., the one with the crucial mutation for the affinity improvement) should be missed by chance. Thus when two residues are randomized using the codons NN(G/C) or NN(A/C) and assuming an unbiased nucleotide incorporation, the probability of a library with only 10^4 members *not* to contain one particular of $32^2 = 1024$ possible combinations is 4.5×10^{-5} according to Poisson's law ($P = e^{-N/n}$; P = probability of zero to fine one particular combination, N = library size, n = possible combinations; Sokal & Rohlf, 1981). If three residues are randomized, there are $32^3 = 32\,768$ possible combinations and a library of 3×10^5 gives only a 1% probability to *miss* one particular clone.

As is is not difficult to generate libraries of such size, an oligonucleotide-mediated site-directed randomization should prove a meticulous and successful route to improve affinities. Even a stepwise randomization of all CDR residues (or at least all CDR3 residues) within an antibody is feasible, in cases where no structural information at all about the antigen-binding site is available. If some details about the binding site are known, it may be more economical to develop a strategy for such experiments. One possible route was successfully demonstrated here by randomization of residues whose side chains point toward the antigen or the cavity of the presumed binding site. It seems likely that any structural changes introduced this way are of a more local nature than if whole CDRs or residues whose side chains point toward the interior of the protein domains would have been randomized. As a consequence, conservation of the general architecture of the Fv/antigen complex should be expected and was observed in the case of the improved NQ11.7.22 Fv. However, we were able to increase the hapten affinity, although to a lesser degree, by randomization of residues whose side chains were not directly involved in antigen binding. Affinity improvement was in this case probably achieved indirectly via slight backbone rearrangements.

Also critical for the success of the experiments is the selection procedure. Thus we would expect that selection of libraries containing clones with higher affinities (K_d < 100 nM) is more likely to be successful, when little and/or univalent ligand is presented during selection. Although not analyzed systematically in this study, in the case of higher affinities the use of a phagemid system involving the use of helper phage seems preferrable to the use of fd-DOG1-type vectors, with which avidity effects due to the multiple Fv copies must be expected. In contrast, libraries containing only medium- and low-affinity clones might be more successfully and reproducibly selected with higher ligand concentrations and using fd-based vectors enabling multivalent binding.

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