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Comprehensive Functional Maps of the Antigen-binding Site of an Anti-ErbB2 Antibody Obtained with Shotgun Scanning Mutagenesis

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Shotgun scanning combinatorial mutagenesis was used to study the antigen-binding site of Fab2C4, a humanized monoclonal antibody fragment that binds to the extracellular domain of the human oncogene product ErbB2. Essentially all the residues in the Fab2C4 complementarity determining regions (CDRs) were alanine-scanned using phage-displayed libraries that preferentially allowed side-chains to vary as the wild-type or alanine. A separate homolog-scan was performed using libraries that allowed side-chains to vary only as the wild-type or a similar amino acid residue. Following binding selections to isolate functional clones, DNA sequencing was used to determine the wild-type/mutant ratios at each varied position, and these ratios were used to assess the contributions of each side-chain to antigen binding. The alanine-scan revealed that most of the side-chains that contribute to antigen binding are located in the heavy chain, and the Fab2C4 three-dimensional structure revealed that these residues fall into two groups. The first group consists of solvent-exposed residues which likely make energetically favorable contacts with the antigen and thus comprise the functional-binding epitope. The second group consists of buried residues with side-chains that pack against other CDR residues and apparently act as scaffolding to maintain the functional epitope in a binding-competent conformation. The homolog-scan involved subtle mutations, and as a result, only a subset of the side-chains that were intolerant to alanine substitutions were also intolerant to homologous substitutions. In particular, the 610 Å² functional epitope surface revealed by alanine-scanning shrunk to only 369 Å² when mapped with homologous substitutions, suggesting that this smaller subset of side-chains may be involved in more precise contacts with the antigen. The results validate shotgun scanning as a rapid and accurate method for determining the functional contributions of individual side-chains involved in protein–protein interactions.

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Introduction

Monoclonal antibodies have proven invaluable as reagents in biological chemistry, and more recently, as therapeutic agents.[1] The field of antibody engineering is concerned with technologies that can be used to

Table 1. Shotgun scanning codons

Wild-type[c]	Alanine-scan[a]				Homolog-scan[b]	
	Codon[d]	m1	m2	m3	Codon[d]	m4
A	GST	G			KCT	S
C	KST	A	G	S	TSC	S
D	GMT	A			GAM	E
E	GMA	A			GAM	D
F	KYT	A	S	V	TWC	Y
G*	GST	A			GST	A
H	SMT	A	D	P	MAC	N
I*	RYT	A	T	V	RTT	V
K	RMA	A	E	T	ARG	R
L	SYT	A	P	V	MTC	I
M	RYG	A	T	V	MTG	L
N*	RMC	A	D	T	RAC	D
P*	SCA	A			SCA	A
Q*	SMA	A	E	P	SAA	E
R	SST	A	G	P	ARG	K
S*	KCC	A			KCC	A
T	RCT	A			ASC	S
V	GYT	A			RTT	I
W	KSG	A	G	S	TKG	L
Y	KMT	A	D	S	TWC	F

For each scan, degenerate shotgun codons were designed to encode the wild-type amino acid and one or more substitutions. Asterisks (*) indicate wild-type amino acid residues for which both the alanine and homolog-scan codons encode a common substitution.
[a] The shotgun alanine-scan codon for each amino acid ideally encodes only the wild-type or alanine (m1), but the nature of the genetic code necessitates the occurrence of two other amino acid residues (m2 and m3) for some substitutions. In the case of wild-type alanine, the shotgun codon was designed to encode alanine and glycine.
[b] For the homolog-scan, binomial shotgun codons were designed to encode the wild-type and a similar amino acid (m4).
[c] Amino acid residues are represented by the single letter amino acid code.
[d] Equimolar DNA degeneracies in shotgun codons are represented by the IUB code (K = G/T, M = A/C, R = A/G, S = G/C, W = A/T, Y = C/T).

dissect and rationalize the requirements for antibody structure and function.[2] This knowledge can then be used to improve or alter particular antibody–antigen interactions, or even to engineer completely novel-binding specificities.

The specificity and affinity of an antibody for its cognate antigen is determined by the sequence and structure of the variable fragment (Fv): a heterodimer consisting of the N-terminal domains of the heavy and light chains. Even within the Fv, antigen binding is primarily mediated by the complementarity determining regions (CDRs), six hypervariable loops (three each in the heavy and light chains) which together present a large contiguous surface for potential antigen binding. Aside from the CDRs, the Fv also contains more highly conserved framework segments which connect the CDRs and are mainly involved in supporting the CDR loop conformations,[3,4] although in some cases, framework residues also contact antigen.[5,6] As an important step to understanding how a particular antibody functions, it would be very useful to assess the contributions of each CDR side-chain to antigen binding, and in so doing, to produce a functional map of the antigen-binding site.

Site-directed mutagenesis is a powerful tool for mapping binding energetics at protein–protein interfaces.[7,8] In this process, individual DNA codons are systematically altered and the corresponding mutant proteins are expressed, purified, and assayed for activity relative to the wild-type. The effects of individual side-chain substitutions can then be assessed in terms of $\Delta\Delta G_{mut-wt}$, the difference in binding free energy between the mutant and wild-type protein. By analyzing panels of point mutants, a detailed map of the binding energetics can be obtained, but the process can be very laborious because individual mutant proteins must be made and analyzed separately. In particular, a comprehensive analysis of an antigen-binding site would ideally encompass all CDR residues, and this would require the analysis of dozens or even hundreds of point mutants.[9,10]

Recently, a general and rapid combinatorial mutagenesis strategy has been developed for exploring protein structure and function.[11] "Shotgun scanning" mutagenesis uses phage-displayed libraries of protein mutants constructed using degenerate codons with restricted diversity. For example, codons may be chosen to preferentially allow the wild-type (wt) or alanine in the case of a shotgun alanine-scan. The library pool is then subjected to binding selections to enrich for clones that retain affinity for a binding partner, and following selection, DNA sequencing is used to determine the ratio of wild-type/mutant (wt/mut) at each varied position. This ratio can be used to assess binding contributions of each side-chain with good correlation to those obtained with traditional site-directed mutagenesis. The method is very rapid because many side-chains are simultaneously scanned with a single library, and the analysis is based on DNA sequencing which circumvents the need for protein purification and biophysical analysis.

We used the shotgun scanning approach to study the antigen-binding site of a humanized monoclonal antibody (humAb2C4) that binds to the extracellular domain of the human receptor tyrosine kinase ErbB2 (ErbB2-ECD, K_d = 8.5 nM), and in so doing, inhibits tumor growth (C.W.A., unpublished results). The antigen-binding portion of humAb2C4 was displayed on M13 bacteriophage in an Fab format (Fab2C4), i.e. a heterodimer consisting of the light chain and the variable and first constant domains of the heavy chain. We conducted two different shotgun scans, with each scan covering essentially the complete sequences of all six CDRs. With a shotgun alanine-scan, we assessed the effects of removing all side-chain atoms past the β-carbon, fairly drastic mutations that can be used to infer the roles of individual side-chains in protein structure and function.[7] We also conducted a more subtle scan, termed a shotgun homolog-scan, in which we substituted

L1	24 K	25 A	26 S	27 Q	28 D	29 V	30 S	31 I	32 G	33 V	34 A					

L2	50 S	51 A	52 S	53 Y	54 R	55 **Y**	56 T

L3	89 Q	90 Q	91 **Y**	92 Y	93 I	94 Y	95 **P**	96 Y	97 T

H1	26 G*	27 F*	28 T	29 F*	30 T	31 <u>D</u>	32 **Y**	33 <u>T</u>	34 M	35 <u>D</u>

H2	50 <u>D</u>	51 V	52 <u>N</u>	52a <u>P</u>	53 <u>N</u>	54 <u>S</u>	55 <u>G</u>	56 G	57 S	58 <u>I</u>	59 Y	60 N	61 Q	62 R	63 F	64 **K**	65 G

H3	95 **N**	96 **L**	97 <u>G</u>	98 <u>P</u>	99 S	99a **F**	99b **Y**	100 **F**	101 <u>D</u>	102 Y

Figure 1. Sequences of the Fab2C4 CDRs. The sequence of each CDR is shown along with the position of each residue in the numbering scheme† of Kabat *et al.*[37] Residues shown to be important for ErbB2-ECD binding in either the shotgun alanine or homolog-scan are shown in bold or underlined, respectively ($F_{wt/mut} > 10$, see Tables 3 and 4). Asterisks (*) indicate residues that were not analyzed in the shotgun scans.

Table 2. Fab2C4 shotgun scanning libraries

		Mutated regions			Diversity	
Library	CDRs	Residues	Shotgun codons	Mutagenic oligonucleotides	Theoretical	Actual
HAa	H1, H2, H3	T28, T30, D31, Y32, T33, D50, V51, N52, N53, S54, I58, N60, Q61, N95, L96, P98, S99	Alanine	H1-A1, H2-A1, H3-A1	3.3×10^7	1.5×10^{10}
HAb	H1, H2, H3	D35, P52a, G55, G56, S57, Y59, R62, F63, K64, G65, G97, F99a, Y99b, F100, D101, Y102	Alanine	H1-A2, H2-A2, H3-A2	1.7×10^7	2.4×10^{10}
LAa	L1, L2, L3	Q27, D28, S30, I31, G32, S50, S52, Y53, Y55, Y91, Y92, I93, Y94, Y96	Alanine	L1-A1, L2-A1, L3-A1	8.3×10^7	1.4×10^{10}
LAb	L1, L2, L3	K24, A25, S26, V29, V33, A34, A51, R54, T56, Q89, Q90, P95, T97	Alanine	L1-A2, L2-A2, L3-A2	1.6×10^4	2.5×10^{10}
HHa	H1, H3	T28, T30, D31, Y32, T33, M34, D35, N95, L96, G97, P98, S99, F99a, F100, Y99b, D101, Y102	Homolog	H1-H, H3-H	1.3×10^5	2.4×10^{10}
HHb	H2	D50, V51, N52, P52a, N53, S54, G55, G56, S57, I58, Y59, N60, Q61, R62, F63, K64, G65	Homolog	H2-H	1.3×10^5	2.2×10^{10}
LH	L1, L2, L3	K24, A25, S26, Q27, D28, V29, S30, I31, G32, V33, A34, S50, A51, S52, Y53, R54, Y55, T56, Q89, Q90, Y91, Y92, I93, Y94, P95, Y96, T97	Homolog	L1-H, L2-H, L3-H	1.3×10^8	2.4×10^{10}

Libraries were designed to replace the codons for the indicated residues with either alanine-scan or homolog-scan shotgun codons (Table 1). Libraries were constructed using the indicated mutagenic oligonucleotides (see Materials and Methods), and in each case, the theoretical diversity (the number of amino acid combinations encoded by the mutagenic oligonucleotides) was exceeded at least 100-fold by the actual diversity of the constructed library.

each wild-type residue with a similar amino acid, to gain insight into which positions require precise side-chain geometries and chemistry. When the mutagenesis results were mapped onto the three-dimensional crystal structure of Fab2C4, each scan provided a comprehensive view of how the CDR side-chains contribute to the formation of a functional antigen-binding site. The two views are distinct yet complementary: together, they provide a clearer understanding of antibody structure and function than would be possible with either scan alone.

Results

Shotgun alanine-scan of Fab2C4

For the shotgun alanine-scan, we replaced wt codons with degenerate codons that ideally encoded the wt amino acid or alanine (m1 in Table 1), although the nature of the genetic code

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† Antibody residues are designated by a letter in lower case italics denoting the heavy or light chain (*h* or *l*, respectively), followed by the amino acid in the one-letter code, followed by the position in the chain. For example, *h*D101 denotes an aspartic acid residue at position 101 in the heavy chain.

Table 3. Fab2C4 light chain shotgun scan

Residue[a]	Antigen selection				Display selection				$F_{wt/mut}$			
	Wt/m1	Wt/m2	Wt/m3	Wt/m4	Wt/m1	Wt/m2	Wt/m3	Wt/m4	m1	m2	m3	m4
K24	0.89	4.2	0.96	0.88	0.42	0.79	0.52	1.0	2.1	5.3	1.8	0.86
A25	3.7			2.8	2.0			1.6	1.8			1.8
S26*	3.5			2.8	2.9			1.5	1.2*			1.9*
Q27*	0.67	1.5	2.5	0.51	0.88	1.2	0.94	0.73	0.76	1.3*	2.7	0.70*
D28	1.1			1.8	0.99			1.9	1.1			1.0
V29	6.1			3.5	2.5			2.0	2.4			1.8
S30*	1.8			1.1	1.5			0.87	1.1*			1.3*
I31*	0.91	2.8	0.57	0.64	1.7	2.7	0.56	0.55	0.53	3.7	1.0*	1.2*
G32*	3.3			4.8	2.9			3.9	1.1*			1.2*
V33	16			3.1	3.3			2.8	4.8			1.1
A34	16			5.5	3.6			2.5	4.6			2.2
S50*	1.0			0.78	1.3			0.87	0.77*			0.89*
A51	1.7			1.6	0.90			0.85	1.9			1.8
S52*	1.3			1.2	1.5			1.7	0.85*			0.70*
Y53	1.9	97	4.4	1.4	1.6	3.5	1.2	1.3	1.2	**28**	3.7	1.1
R54	3.2	4.1	1.9	3.0	1.7	3.7	1.0	2.4	1.8	1.1	1.9	1.3
Y55	32	80	53	4.8	1.4	2.3	0.89	0.95	**23**	**35**	**60**	5.1
T56	0.49			0.88	0.89			0.76	0.55			1.2
Q89*	8.8	10	70	3.6	0.77	2.4	3.4	1.9	11	4.2*	21	1.8*
Q90*	2.4	1.1	>36	0.67	0.88	1.9	2.3	0.71	2.7	0.58*	**>16**	0.94*
Y91	>166	>166	166	0.94	1.8	3.5	0.97	1.2	**>92**	**>47**	138	0.76*
Y92	1.2	3.7	1.1	0.88	1.3	2.1	0.84	0.6	0.96	1.8	0.76	1.5
I93*	1.7	1.6	0.81	0.69	1.7	1.5	0.64	0.53	1.0	1.1	1.3*	1.3*
Y94	6.7	30	5.5	1.3	1.9	3.0	1.7	0.63	3.6	**10**	3.2	2.0
P95*	13			9.7	1.1			1.74	**12***			5.6*
Y96	0.99	>66	2.1	0.36	2.1	18	2.2	0.91	0.48	>3.7	0.95	0.40
T97	0.56			0.28	0.89			0.35	0.62			0.80

For each of the listed light chain residues, the effect of each mutation (Table 1) was assessed using data from either the alanine-scan libraries (m1, m2, and m3) or the homolog-scan libraries (m4) described in Table 2. The wt/mut ratios were determined from the sequences of binding clones isolated after selection for binding to either the ErbB2-ECD (antigen selection) or an anti-tag antibody (display selection). The function ratio ($F_{wt/mut}$) for each mutation was derived by dividing the antigen selection wt/mut ratio by the display selection wt/mut ratio. $F_{wt/mut}$ provides a quantitative estimate of the effect of each mutation on the binding affinity of Fab2C4 for ErbB2-ECD. Deleterious effects are indicated by $F_{wt/mut}$ values greater than 1.0, and mutations that have large deleterious effects ($F_{wt/mut} > 10$) are shown in bold text. In cases where a particular mutation was not observed amongst the antigen selection sequences, only a lower limit could be defined for the wt/mut ratio and the $F_{wt/mut}$ (indicated by a greater than sign). Asterisks (*) indicate residues for which the alanine and homolog-scan codons encoded a common substitution.

  [a] Residues are denoted by the single letter amino acid code and are numbered according to the scheme of Kabat *et al.*[37]

necessitated two other amino acid substitutions for some residues (m2 and m3 in Table 1). In positions where alanine was the wt, we used a degenerate codon that encoded alanine or glycine. The six CDRs of Fab2C4 encompass a total of 64 residues (Figure 1). We constructed two libraries (HAa and HAb) that together covered 33 of the 37 heavy chain CDR residues and two libraries (LAa and LAb) that together covered all 27 light chain CDR residues (Figure 1 and Table 2). Each library contained $>10^{10}$ unique members, and thus in each case, the theoretical diversity for combinatorial mutagenesis at the scanned positions was exceeded by at least 100-fold (Table 2).

Phage pools from each library were subjected to two different selections. The first selection (display selection) isolated variants capable of binding to a monoclonal antibody specific for the epitope tag fused to the N terminus of the Fab2C4 light chain. The second selection (antigen selection) isolated variants capable of binding to ErbB2-ECD. Close to 100 binding clones were sequenced from each

selection; the sequences were aligned, and at each mutated position, the occurrences of wt or each designed substitution were tabulated (see Materials and Methods for details). For each selection, these data were used to calculate the wt/mut ratio for each mutation at each position (Tables 3 and 4).

Because the wt/mut ratio is the statistical preference for the wt relative to the mutant, it correlates with the effect of each mutation on the selected trait (i.e. binding to the anti-tag antibody or ErbB2-ECD). Ratios greater than or less than 1 indicate deleterious or beneficial mutations, respectively.

The anti-tag antibody selected for phage variants that displayed assembled Fab2C4 fragments containing both the heavy and light chains. This is because the heavy chain was fused directly to a bacteriophage coat protein while the epitope tag was fused to the light chain N terminus. Thus, the anti-tag antibody only binds to phage particles that contain a light chain associated with the

Table 4. Fab2C4 heavy chain shotgun scan

	Wt/mut ratios								$F_{wt/mut}$			
	Antigen selection				Display selection							
Residue[a]	Wt/m1	Wt/m2	Wt/m3	Wt/m4	Wt/m1	Wt/m2	Wt/m3	Wt/m4	m1	m2	m3	m4
T28	4.5			0.94	0.7			0.47	6.4			2.0
T30	0.33			0.27	0.7			0.39	0.47			0.69
D31	170			29	1.4			1.1	**120**			**26**
Y32	>161	>161	>161	17	2.0	3.1	1.1	0.85	**>81**	**>52**	**>150**	**20**
T33	20			8.9	0.94			0.38	**21**			**23**
M34	ND[b]	ND	ND	2.2	ND	ND	ND	0.88	ND	ND	ND	2.5
D35	2.8			14	0.14			0.90	**20**			**15**
D50	170			>91	0.24			0.41	**710**			**>220**
V51	10			1.3	1.1			1.8	9.4			0.73
N52*	>168	168	84	>91	0.41	0.34	0.80	0.83	**>410**	**490***	**110**	**>110***
P52a*	72			14	6.1			0.62	**12***			**23***
N53*	>166	166	>166	>91	1.4	0.97	2.6	0.57	**>120**	**170***	**>64**	**>160***
S54*	84			>91	0.33			1.1	**260***			**>83***
G55*	14			90	0.40			2.9	**34***			**31***
G56*	0.60			0.36	5.0			2.6	0.12*			0.14*
S57*	7.0			0.47	4.4			0.86	1.6*			0.55*
I58*	45	45	4.5	2.1	0.86	0.95	0.51	0.61	**53**	**47**	8.8*	3.4*
Y59	33	>59	9.8	0.78	8.7	10.4	1.8	0.58	3.8	>5.7	5.4	1.3
N60*	4.8	4.4	120	3.0	1.2	0.91	15	1.8	4.0	4.8*	8.0	1.7*
Q61*	2.6	0.98	1.1	0.69	0.53	0.42	2.0	0.71	4.8	2.3*	0.55	0.97*
R62	4.3	>44	4.0	1.3	1.2	15	0.24	1.2	3.6	2.9	17	1.0
F63	26	26	4.6	3.2	6.6	2.2	8.8	4.0	4.4	**12**	0.52	0.81
K64	54	54	6.0	0.57	4.9	7.7	2.7	0.67	**12**	7.0	2.2	0.85
G65*	5.8			9.1	2.50			3.9	2.3*			2.4*
N95*	>170	21	>170	21	1.8	2.0	2.1	3.1	**>98**	**11***	**84**	6.9*
L96	23	>45	0.35	1.5	0.11	0.33	0.19	1.2	**210**	**>140**	1.8	1.3
G97*	>78			89	3.3			2.1	**>24***			**42***
P98*	>178			29	1.9			0.44	**>94***			**65***
S99*	2.8			7.0	0.55			1.6	5.0*			4.4*
F99a	>75	>75	>75	10	2.4	5.4	1.3	1.1	**>31**	**14**	**58**	9.1
Y99b	>74	74	74	1.7	0.8	4.1	1.7	0.49	**>93**	**18**	**44**	3.5
F100	77	>77	77	17	2.6	5.9	1.5	5.1	**30**	**13**	**51**	3.3
D101	9.1			>87	1.1			2.5	8.3			**>35**
Y102	8.3	7.5	3.2	2.8	2.3	1.9	2.1	0.92	3.6	3.9	1.5	3.0

For each of the listed heavy chain residues, the effect of each mutation (Table 1) was assessed using data from either the alanine-scan libraries (m1, m2, and m3) or the homolog-scan libraries (m4) described in Table 2. The wt/mut ratios were determined from the sequences of binding clones isolated after selection for binding to either the ErbB2-ECD (antigen selection) or an anti-tag antibody (display selection). The function ratio ($F_{wt/mut}$) for each mutation was derived by dividing the antigen selection wt/mut ratio by the display selection wt/mut ratio. $F_{wt/mut}$ provides a quantitative estimate of the effect of each mutation on the binding affinity of Fab2C4 for ErbB2-ECD. Deleterious effects are indicated by $F_{wt/mut}$ values greater than 1.0, and mutations that have large deleterious effects ($F_{wt/mut} > 10$) are shown in bold text. In cases where a particular mutation was not observed amongst the antigen selection sequences, only a lower limit could be defined for the wt/mut ratio and the $F_{wt/mut}$ (indicated by a greater than sign). Asterisks (*) indicate residues for which the alanine and homolog-scan codons encoded a common substitution.
[a] Residues are denoted by the single letter amino acid code and are numbered according to the scheme of Kabat *et al.*[37]
[b] ND indicates that these values were not determined, because we forgot to include this residue in the alanine-scan libraries.

phage-displayed heavy chain. Most of the wt/mut ratios for the display selection were close to 1.0, indicating that the mutations did not significantly affect Fab2C4 display levels (Tables 3 and 4). However, several mutations exhibited wt/mut ratios significantly greater than 1.0 (e.g. hP52aA, hY59A, hF63A), suggesting that these mutations reduced display. Conversely, for a few mutations, wt/mut ratios significantly less than 1.0 suggest that these mutations may actually increase display (e.g. hD35A, hL96A).

In the selection for binding to ErbB2-ECD, mutations could effect the selection either by altering the level of Fab2C4 display (as in the display selection), or alternatively, by directly or indirectly altering the side-chains that make binding contacts with the antigen. In this selection, alanine substitutions at three light chain positions (Table 3) and 21 heavy chain positions (Table 4) exhibited wt/mut ratios greater than 10.

To obtain a quantitative estimate of each mutation's effect on ErbB2-ECD binding affinity, we divided the wt/mut ratio from the antigen selection by the wt/mut ratio from the display selection. This operation corrected for effects on Fab2C4 display and provided a number which we termed the function ratio ($F_{wt/mut}$). As we have shown previously, the $F_{wt/mut}$ value for each mutation is approximately equal to the corresponding ratio of equilibrium binding constants ($K_{a,wt}/K_{a,mut}$),[11] and thus, it provides a good estimate of the effect of each mutation on the

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