

Exploring central and peripheral diversity in antibody evolution

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Abstract

The antigen-binding site, the paratope, of an antibody can be seen as being composed of a central core and a more peripheral area situated at its rim. Naturally these regions acquire their diversity using different mechanisms and they also have dissimilar roles, as they contribute differently to the binding interaction. Also, antigens of different size utilize these regions differently; while haptens mainly interact with the central core, larger antigens have additional interactions in more peripheral regions. Since haptens do not occupy the entire available paratope we hypothesized that hapten-specific antibodies, as they develop naturally or in the laboratory, have an imprint of the carrier protein they were once selected on. By using combinatorial library and phage display technologies on a hapten-specific antibody we were able to demonstrate that a peripheral carrier imprint indeed exists. We further show that such an imprint can act as a seed in the evolution of binders that recognize the carrier protein even in the absence of the hapten modification. The observed results provide a plausible mechanism for how haptenization of self-antigens can lead to the development of autoimmunity.

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1. Introduction

The human antibody repertoire provides a vast array of adaptable antigen-binding sites that allows for interaction with virtually any molecular target. The antigen-binding site, also known as the paratope, is largely composed of residues in the complementary determining region (CDR) loops, three each in the heavy and light chains. A beta-sheet framework in the form of an immunoglobulin fold brings the six CDRs together, enabling them to interact jointly with the antigen (Padlan, 1994). As the sequence and the length of these loops vary between antibodies, they form different surfaces with respect to shape and physiochemical properties allowing them to mediate different types of interactions (Collis et al., 2003; MacCallum et al., 1996; Vargas-Madrazo et al., 1995). Consequently, small molecules, haptens, tend to bind into cavities formed in the paratope, elongated molecules bind to groove-like surfaces, while large proteins generally bind to flat binding surfaces.

In the naïve antibody repertoire the diversity of paratopes is mainly focused to the central core, while diversity in the periph-

ery is much more restricted. At its early stage of development, diversity is mainly a result of the rearrangement of V, D and J genes that takes place in progenitors of B-lymphocytes. This relatively small number of genes is able to create vast diversity as the points of rearrangement are not fixed resulting in extensive sequence variability, in particular in the third hypervariable loop of the heavy chain. Following antigen recognition, the affinity of this primary repertoire is enhanced through somatic hypermutation in which mutations are introduced throughout the variable domain of the antibody. During this process of selection and expansion, the variants that have improved their binding the most will be positively selected. Interestingly, this mainly results in diversification of the peripheral parts of the paratope as opposed to the diversity pattern observed in the primary repertoire (Ramirez-Benitez and Almagro, 2001; Tomlinson et al., 1996), thus making the two diversity-creating processes complementary to each other.

Different roles have been ascribed these distinct parts of the paratope. The central core is considered the specificity-determining part of the binding site as it provides most of the free energy required to maintain the interaction. The region of surrounding residues on the other hand participates by modulating the interaction mainly by excluding water from the interaction surface. Thus, the primary (germ-line) repertoire harbours high

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variability in the part of the paratope that is likely to make up the hotspot of interaction. At this early stage, the binding sites are generally believed to be rather flexible to accommodate a variety of antigens. The subsequent affinity maturation process will result in fine-tuning of the peripheral parts of the binding site, a process that leads to a stabilized binding site with an enhanced antigen complementarity (Davis, 2004; Manivel et al., 2000; Wedemayer et al., 1997).

In this study we wanted to further explore the role that the different parts of the paratopes play in the evolution of antigen recognition. We have undertaken an *in vitro* study of the FITC-specific antibody fragment, FITC8, which serves as a model of a high affinity binder recognizing a hapten. Except for their importance in different bioanalytical applications, hapten-specific antibodies are encountered *in vivo* during conditions of hypersensitivity to low-molecular weight sensitizing agents, such as penicillin and formaldehyde (Bigazzi, 1997; Griem et al., 1998; Holsapple, 2002; Kimber and Dearman, 2002; Uetrecht, 2005). We postulated that although hapten-binders harbour a major specificity for the hapten in its hotspot of its binding site, other parts of the available interacting surface will most likely have an imprint of the protein to which the hapten inducing the immune response was originally attached. This is based on the fact that in many cases a small antigen will not occupy the entire antigen-binding surface of the antibody (Almagro, 2004). The hapten will, as stated above, insert itself into a cavity formed by the antigen-binding site and only interact with the central core, leaving room for contacts to be made between the carrier and the more peripherally located residues of the binding site. We therefore hypothesize that although such peripheral interactions contribute only marginally to the affinity of the interaction, they can have implications reaching beyond the recognition of the hapten alone. In particular they may be the starting point for developing immunity targeting the carrier protein.

In order to test this hypothesis we created molecular libraries evolving different parts of the FITC8 binding site. Results from phage selections on the carrier protein that was originally used to obtain FITC8 indicated that there indeed exists a carrier imprint in the FITC8 paratope. The results further suggest that this peripherally located imprint can be evolved to recognize the carrier protein even in absence of the hapten. Thus, residues that have been considered as those fine-tuning the initial, central interaction may be the seed for the development of a new specificity. These results are discussed in particular with respect to their implications for the understanding of how autoimmunity may develop following modification of self-antigens by haptens.

2. Materials and methods

2.1. Construction of libraries and preparation of phage stocks

A modified version of the FITC8 gene (Persson et al., 2006) encoding an anti-FITC scFv, was used for library construction. Different parts of the binding site were evolved creating two different synthetic scFv (single chain antibody fragment) libraries,

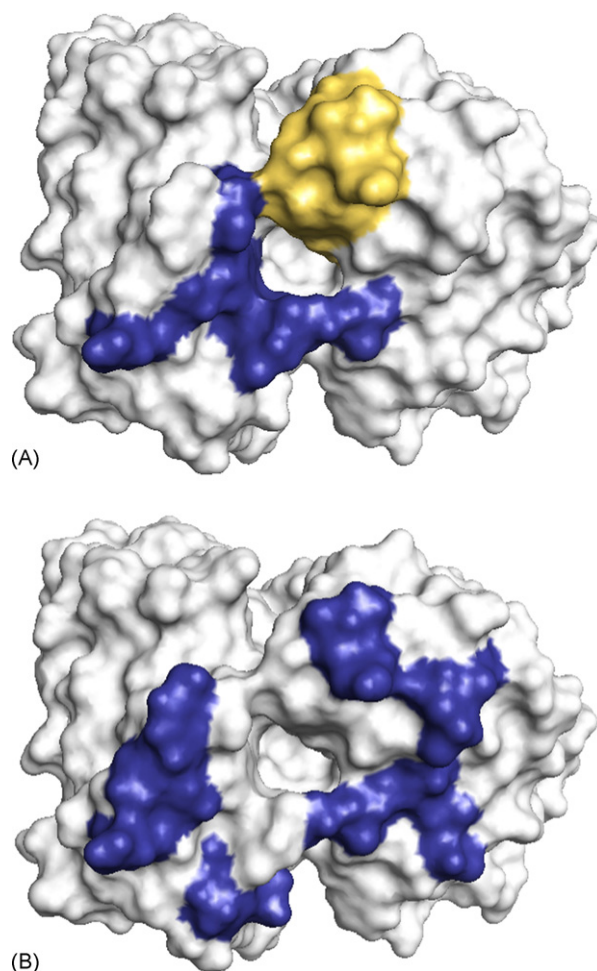


Fig. 1. Model structure of the library scaffold, FITC8, with residues targeted for modifications shown in blue for the two libraries: (A) the core library and (B) the rim library. Additionally, in the core library the full variability of the n-CoDeR library (Söderlind et al., 2000) was introduced in CDRL3 (yellow) and N32, Y62 and F67 were back-mutated to their respective germ-line sequence (not shown). In Table 1, the targeted positions together with the allowed variability are listed. Amino acids are abbreviated according to the standard one letter code. The model structure was obtained using the program MODELER (Söderlind et al., 2000) and the figure was generated using PyMol (<http://pymol.sourceforge.net/>).

the core and the rim libraries (Fig. 1, see Section 3 for a detailed description of the design of these libraries).

The construction of the core library has been described elsewhere (Persson et al., 2006). Similarly, the diversity of the rim library was introduced in a step-wise manner of six gene-fragments using degenerate primers (Fig. 2), designed in a way to include as many of the desired residues as possible without introducing too many unwanted residues. The PCR-amplified fragments were treated with *DpnI* (New England Biolabs, Beverly, MA, USA), assembled by overlap-extension PCR, cleaved with *SfiI/NotI* (New England Biolabs) and ligated into a modified pFab5c.His phagemid (Engberg et al., 1995). The ligated library was transformed into electrocompetent Top10F' *E. coli* cells giving a size of approximately 2×10^8 clones, of which over 99% contained the insert. Two-thirds of the clones were in frame and sequencing confirmed the intended diversity. Phage stocks were prepared using VCSM13 helper phage (Stratagene, La Jolla,

Name	Sequence	Location
a-5'	5'-GGCCGATTCATTAAATGCAGC	Vector specific primer
a-3'	5'-ACTAAAGGTGAATCCAGAGG	Codons H30-H24
b-5'	5'-CCTCTGGATTACACCTTTAGTRRCHATTGGATGAGCTGGGTCCGCCAG ^a	Codons H24-H39
b-3'	5'-CACGGAGTCTGCGAAGTATGTGWACYACCGYYACCKYTAATACCTGAGACCCACTCCAG ^a	Codons H63-H45
c-5'	5'-ACATACTTCGCAGACTCCGTG	Codons H57-H63
c-3'	5'-ACCTCCGCACAGTAATACACAGC	Codons H95-H88
d-5'	5'-GCTGTGTATTACTGTGCGGGAGGTGATRGCRGTGGCTGGTCTTCTGGGGC ^a	Codons H88-H104
d-3'	5'-GAGCTGCTGATACCACTGTACAKYATAAYGYGBCGATGTTGGAGCTGCTCCAGT ^a	Codons L39-L24
e-5'	5'-CACTGGTATCAGCAGCTCCAGGAACGGCCCCAACTCCTCATCTATVGTAAACAACAMCGGCCCTCAGGGGTCCCT ^a	Codons L34-L59
e-3'	5'-GAACACTCGACCACTCAGGSYKYATCCCAAGCCGCACAGTA ^a	Codons L98-L87
f-5'	5'-CTGAGTGGTCGAGTGTTCGG	Codons L95-L99
f-3'	5'-GCCTTTAGCGTCAGACTGTAG	Vector specific primer

^aB = C, G or T; H = A, C or T; K = G or T; M = A or C; R = A or G; Y = C or T, V = A, C or G, W = A or T.

Fig. 2. Oligonucleotide primers used for the construction of the rim library (for primers used in the construction of the core library see Persson et al., 2006). Six fragments (a, b, c, d, e and f) were generated by PCR and subsequently assembled into complete scFv genes by overlap-extension PCR. The primers anneal to one or more nucleotides in the given codons.

CA, USA) and phages were concentrated using conventional polyethylene glycol/NaCl precipitation.

To allow for comparison, a phage library based on the n-CoDeR principle (Söderlind et al., 2000) (kindly provided by BioInvent International AB, Lund, Sweden), containing an entirely different source of diversity, was used. Due to the presence of an amber codon in this library, the *E. coli* suppressor strain HB101F' was used for phagemide rescue.

2.2. Phage selections

The libraries were subjected to three rounds of phage display selection on gradually decreasing amounts of streptavidin-coated paramagnetic beads (DynaL A/S, Oslo, Norway). In the first round 50 µl of beads were used, in the second and third rounds 10 µl were used. Prior to selection, the beads were washed twice in PBS. Phage stocks containing 10¹⁰ to 10¹¹ particles were added in selection buffer (1% BSA, 0.05% (v/v) Tween 20 in PBS). The mixture was incubated for 1.5 h on rotation at room temperature. After five washes in selection buffer, followed by two washes in PBS, the bound phages were eluted with trypsin (Invitrogen, Pasley, UK), which cleaves the trypsin-site located between the displayed scFv and protein III. The enzymatic activity was inhibited by the addition of aprotinin (Roche Diagnostic Corporation, IN, USA). Eluted phage were propagated in *E. coli* (Top10F' or HB101F') and used for further rounds of selection.

2.3. Phage ELISA and sequencing of selected clones

To assess the outcome of the selections, the binding specificities of the phage-displayed scFv were analyzed by ELISA (enzyme-linked immunosorbent assay). Phage stocks of individual clones were made on randomly picked clones from the second and third rounds of selection as previously described (Cicortas Gunnarsson et al., 2004). The clones were tested for binding to streptavidin (Sigma–Aldrich Inc., St. Louis, USA), NeutrAvidin (Pierce Biotechnology, Rockford, IL, USA), which is a modified variant of avidin, and BSA. Antigen-bound phages were detected using a peroxidase-conjugated anti-M13 monoclonal antibody (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The colorimetric reaction was developed using *o*-phenylenediamine and recorded at 490 nm.

To determine whether the streptavidin-specific clones bound to the vicinity of the biotin-binding site of streptavidin, a second phage ELISA was performed in which differently sized molecules coupled to biotin (FITC (fluorescein isothiocyanate) (Sigma–Aldrich), a 12-mer peptide (GVTSAPDTRPAP) and a 63-mer peptide (Ala₃-(PAHGVTAPDTRPAPGSTAP)₃) (kindly provided by Prof. Elias Krambovitis, IMBB, Heraklion, Greece)) were allowed to block this site. If specific for the vicinity of the biotin-binding site, the binding of the scFv should decrease as the size of the inhibiting molecule increases.

To estimate the binding affinity for soluble fluorescein, clones of the core and rim library were further analyzed in a competitive phage ELISA. A fixed concentration of phage, diluted to yield signals in the linear range, were pre-incubated with serial dilutions (0.1–10,000 ng/ml) of fluorescein for 2 h at 37 °C. The mixtures were transferred to streptavidin-coated plates or to plates coated with biotinylated FITC (bound to the plate via streptavidin). After 30 min of incubation, the detection was performed as described above and IC₅₀ values, defined as the concentration of soluble hapten that blocked 50% of phage binding to the immobilized antigen, were determined.

Phagemid vectors encoding the streptavidin-binding scFv were purified and subsequently sent for automated DNA-sequencing (MWG Biotech, Ebersberg, Germany).

3. Results

3.1. Construction of combinatorial libraries

The hapten (FITC)-specific scFv, FITC8, that was used as scaffold for library construction had originally been obtained from the n-CoDeR library (Söderlind et al., 2000). This library had been constructed by shuffling human CDR sequences from mature B-cells into a single scaffold. Thus, the variability found in this library was a result of both V–D–J rearrangement as well as somatic hypermutation. By doing phage selections on paramagnetic beads carrying a streptavidin–biotin–FITC complex the FITC8 scFv was identified (Söderlind et al., 2000). This clone recognizes the hapten-carrier complex as well as the soluble hapten with high affinity (Persson et al., 2006). According to ELISA and BIAcore analyses it is, however, not able to establish a measurable binding to the carrier protein, streptavidin, itself (data not shown). Nevertheless, it is reasonable to believe that

Table 1
Library design

	Core library		Rim library	
	Target positions ^a	Designed modifications	Target positions	Designed modifications
H1	N32 (31)	S	N32 (31)	DGNS
			Y33 (32)	HNY
	W34 (33) S40 (35)	FHLYW HNRS		
H2	G55 (50)	GRYW		
	S57 (52)	NSTY	S57 (52)	NKRS
	N59 (53)	FINSTY	N59 (53)	DGNS
			G61 (55)	GS
	Y62 (56)	S	Y62 (56)	NYST
	Y66 (58) F67 (59)	FHLY Y		
H3	G107 (95)	GS		
	D108 (96)	DGNS		
	G109 (97)	GS	G109 (97)	GS
	S113 (98)	GS	S113 (98)	GS
L1			G31 (28)	AEG
			A32 (29)	DGNS
			G33 (30)	DGNS
			D35 (32)	ADNT
	H40 (34)	HNY		
L2			G56 (50)	GRS
			N66 (53)	KN
L3	X106–X117 (89–97)	n-CoDeR diversity ^b		
			D109 (93) S110 (94)	DEKN AGST

^a Residues are numbered according to the IMGT standard (Lefranc et al., 2003) and the corresponding Kabat nomenclature (Kabat et al., 1991) is given in parenthesis.

^b The diversity found in the n-CoDeR library (Söderlind et al., 2000) was introduced in CDRL3 of the core library.

FITC8, like other hapten-binders, harbours, in addition to the major specificity for the hapten in the hotspot of its binding site, an imprint of the carrier protein in its paratope periphery.

In order to explore the role of such an imprint and to investigate how different parts of a paratope can contribute to the evolution of antigen recognition, two synthetic libraries were created based on the FITC8 sequence. The diversity of the libraries was focused either to residues believed to be centrally resided in the combining site, the core library (designated the cavity library in previous work), or to residues that were more likely to make up the periphery of the paratope, the rim library (Fig. 1 and Table 1). The core library was originally designed to create a library with broad specificity against different haptens (Persson et al., 2006). Briefly, the model structure of FITC8 together with known antibody–antigen interaction patterns (Honegger and Plückthun, 2001; MacCallum et al., 1996) were used to identify those residues that were most likely to interact with the hapten, which resulted in modification of mainly centrally located residues. In addition, because of the original purpose of generating a library with an increased hapten-binding ability, some residues in the periphery of the binding site were also mutated. More specifically, N32, Y62 and F67 were back-mutated to their respective germ-line sequences to decrease the carrier-dependence of the library members. Due to a possible conformational change of the CDRH2 resulting from the length

variation that was introduced in this loop, S57 and N59 were also targeted for modification. In the present study, the diversity in the rim library was designed using the same tools but, in contrast to the core library, mutations were introduced into residues that are mainly contacted by large antigens, resulting in variation of 16 surface-exposed and peripherally located residues, giving a theoretical library size of 1.1×10^8 variants. For stability reasons the substitutions introduced into the two libraries were primarily restricted to what is found in the germ-line genes that encode the CDR loops of the n-CoDeR library (Söderlind et al., 2000) from which FITC8 originates. In the core library, residues commonly found in hapten-binders were also considered. To summarize, this diversification strategy gave rise to two libraries with diversity largely complementary to each other (Fig. 1).

3.2. Core-type diversity is helped by a peripheral imprint

Extensive studies of the library carrying diversity in the core of the binding site have previously demonstrated that this mode of diversification allowed for selection of not only high affinity variants specific for fluorescein but also binders specific for entirely different haptens not recognized by the original binding site (Persson et al., 2006). In the current investigation we wanted to further explore how antibodies can evolve their specificities,

	CDRH1				CDRH2							CDRH3				CDRL1					CDRL2		CDRL3											Additional substitutions
IMGT	32	33	34	40	55	57	59	61	62	66	67	107	108	109	113	31	32	33	35	40	56	66	105	106	107	108	109	110	112	113	114	115	116	
Kabat	31	32	33	35	50	52	53	55	56	58	59	95	96	97	98	28	29	30	32	34	50	53	89	90	91	92	93	94	95	95a	95b	95c	96	97
wt	N	Y	W	S	G	S	N	G	Y	Y	F	G	D	G	S	G	A	G	D	H	G	N	A	A	W	D	D	S	L	S	G	R	V	
SC-1	S	-	-	R	-	N	Y	-	S	F	Y	-	G	S	-	-	-	-	-	Y	-	-	Q	S	Y	-	S	-	-	-	-	T	V	-
SC-2	S	-	-	R	-	N	F	-	S	L	Y	-	G	S	-	-	-	-	-	N	-	-	Q	T	-	-	T	-	-	-	-	-	-	-
SC-3	S	-	-	R	-	N	I	-	S	H	Y	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	N	-	-	-	-	-	-
SC-4	S	-	-	R	-	N	T	-	S	H	Y	-	G	S	-	-	-	-	-	N	-	-	-	-	-	-	-	N	-	-	-	-	-	-
SC-5	S	-	-	N	-	-	I	-	S	H	Y	-	S	-	G	-	-	-	-	-	N	-	-	Q	S	Y	-	S	-	-	-	H	W	-
SC-6	S	-	-	R	-	N	S	-	S	-	Y	-	G	-	-	-	-	-	-	N	-	-	-	V	-	-	-	-	-	N	-	-	-	-
SC-7	S	-	-	R	-	N	I	-	S	L	Y	-	N	-	G	-	-	-	-	-	-	-	-	T	-	-	G	-	-	N	-	-	-	-
SC-11	S	-	-	R	-	N	-	D*	-	S	L	Y	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	N	-	-	-	-
SC-14	S	-	-	R	-	N	T	-	S	L	Y	-	G	S	G	-	-	-	-	N	-	-	Q	T	-	-	T	-	-	-	-	-	-	-
SC-15	S	-	-	R	-	N	T	-	S	H	Y	-	G	S	-	-	-	-	-	Y	-	-	-	T	-	-	-	-	-	N	-	-	-	-
SC-16	S	-	-	R	-	N	-	-	S	F	Y	-	G	S	-	-	-	-	-	N	-	-	-	T	-	-	H	-	-	-	-	-	-	-
SC-17	S	-	-	R	-	N	S	-	S	H	Y	-	G	S	G	-	-	-	-	-	N	-	-	Q	S	F	-	S	-	-	-	S	T	-
SR-1	S	N	-	-	-	N	-	S	-	D*	-	-	-	-	-	-	S	-	T	-	S	K	-	-	-	-	-	-	-	G*	-	-	-	-
SR-2	-	-	-	-	-	N	-	-	-	D*	-	-	-	-	-	-	G	N	-	-	-	K	-	-	-	-	N	G	-	-	-	-	-	-
SR-3	-	N	-	-	-	N	-	S	-	D*	-	-	-	-	-	A	S	N	-	-	-	K	-	-	-	-	-	T	-	-	-	-	-	-

Fig. 3. Sequences of selected scFv. Streptavidin-specific clones were sequenced from the core library (SC) and the rim library (SR). Dashes indicate positions that were fixed as wild-type, whereas dots indicate positions that were diversified but where the wild-type residue was selected. Positions marked with asterisks indicate residues not intended by design.

specifically by studying the impact of the untouched part of the binding site, i.e. the peripheral parts. The core library was therefore displayed on filamentous phage and selected on streptavidin, the carrier protein originally used to obtain FITC8 (Söderlind et al., 2000). This resulted in identification of a number of different sequences (Fig. 3, for full-length sequences see GenBank accession nos. EF089236–EF089247). Phage ELISA analysis showed that streptavidin binding of 2/3 of these were negatively affected by more than 30% in the presence of the fluorescein-biotin ligand in the binding site (Fig. 4A). In no case was binding enhanced by the presence of biotinylated fluorescein. This was so despite the fact that they had evolved from a scFv that originally recognized fluorescein. An increase in the size of the biotinylated molecule further led to a decrease in signal of all nine clones (Fig. 4A). This suggests that the evolution process, which was, due to design, mainly focused to the central part of the paratope, managed to evolve the binding site in a direction that promoted recognition of streptavidin in the immediate vicinity of its biotin-binding site.

To evaluate whether the recognition of this epitope is an intrinsic property of the core library or can be explained by the antigenic nature of streptavidin itself, selections on this antigen were performed using a library belonging to the same family of libraries, n-CoDeR, from which FITC8 was originally established. The diversity distribution of the n-CoDeR library is bigger than the combined diversified area of the core and rim library (Persson et al., 2006) and in contrast to these two libraries the n-CoDeR library is naïve with respect to streptavidin as its origin is not dependent on the selection process that involved this biotin-binding protein. A set of streptavidin-specific and highly diverse scFv was selected using the n-CoDeR library, none of which were similar to the FITC8 sequence (GenBank, accession nos. EF089251–EF089257). In contrast to the clones selected from the core library, these clones could in most cases recognize the antigen when a small hapten (fluorescein) was sitting in the biotin-binding site (Fig. 4D). The recognition of streptavidin was in only one of eight cases disturbed by the presence of this biotinylated molecule. As expected, the larger the molecule that occupied the binding site of streptavidin, the more the interaction was disturbed and the 63-mer peptide

regularly prevented the binding of the scFv to their target protein. These blocking experiments demonstrate that antibodies selected from a large naïve library bind epitopes less closely related to the biotin-binding site of streptavidin compared to antibody fragments selected from the core library. Thus, the wild-type clone FITC8, and as a result the variants derived from the core library, have a built-in propensity in the periphery of its paratope that allows for interaction with the hapten modification site of the carrier. This was so despite the fact that the peripheral located amino acids N32, Y62 and F67 had been mutated in the core library to decrease carrier-dependence (Persson et al., 2006).

3.3. Peripheral diversification retains hot-spot interaction but enhances binding to the carrier

Having established the existence of a peripheral imprint for streptavidin in FITC8 we next explored whether this imprint could be evolved to enhance the binding to the carrier without losing the specificity for the hapten target. The rim library, designed to preferentially target residues in the periphery of the combining site, i.e. remote from the hot-spot interaction of the hapten, was selected on streptavidin. This resulted in the identification of three different clones that specifically were able to bind streptavidin (Fig. 3, GenBank, accession nos. EF089248–EF089250). Binding was enhanced by the presence of biotinylated FITC but completely inhibited by all other biotinylated ligands (Fig. 4B). Recognition of soluble fluorescein was also retained although the affinity for this ligand had dropped by 1000-fold compared to the wild-type scFv (as determined by inhibition ELISA, data not shown). Thus diversification of peripheral parts of the binding site had evolved a specificity that was able to recognize the carrier itself but, in contrast to the core library, still retained a capacity to interact with the hapten. The interaction of two of these clones was also affected by the nature of the carrier as biotinylated fluorescein immobilized onto NeutrAvidin was less well recognized as compared to antigen immobilized onto streptavidin (Fig. 4C). Since the interaction of these clones was very sensitive to other ligands sitting in the biotin-binding site of streptavidin, we propose that this mode of evolution, just as the core library, directs

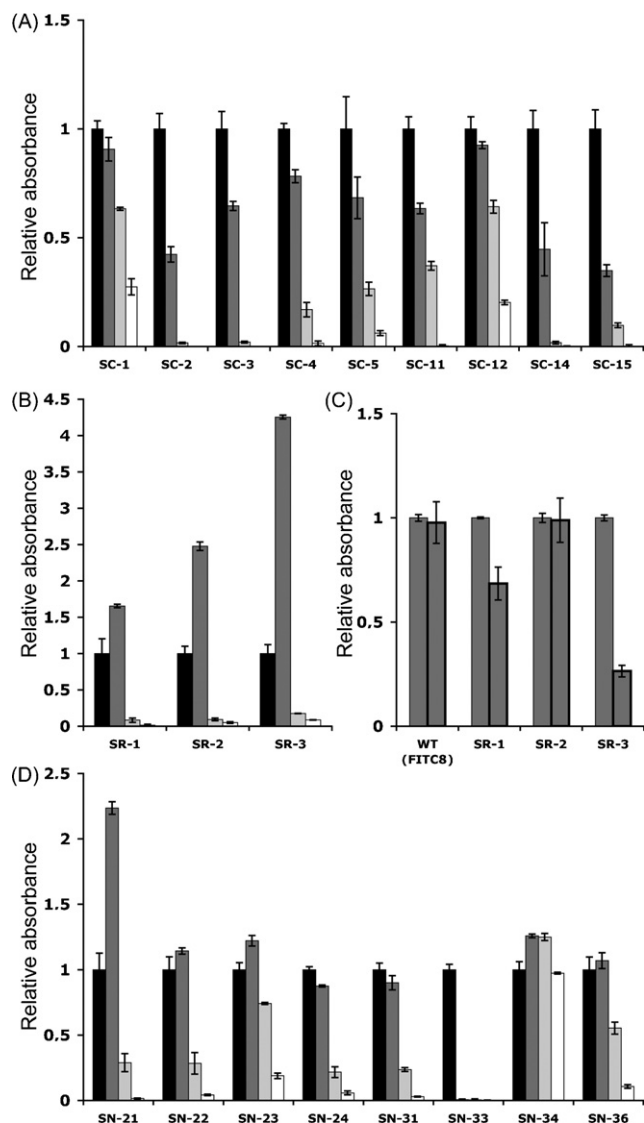


Fig. 4. Binding characteristics of phage-displayed scFv as determined by phage ELISA. Analyses were performed on clones selected from the core library (A), the rim library (B) and the n-CoDeR library (D) on streptavidin (black) and on streptavidin complemented with differently sized biotinylated molecules; FITC (dark grey), a 12-mer peptide (light grey) or a 63-mer peptide (white). The rim library clones were also analyzed on FITC–biotin coupled to NeutrAvidin (C, bars with thick outline). In (A), (B) and (D) the y-axis shows the relative signal intensity compared to that obtained for streptavidin. In (C), the y-values are instead normalized based on the absorbance for the FITC–biotin–streptavidin complex. None of the clones showed any reactivity to BSA, which was used as a negative control to confirm specificity. Numbers are average of triplicates.

the selection to preferentially recognize the area surrounding the biotin-binding site of streptavidin. The fact that clones selected from the two libraries show a high degree of sequence similarity further indicates that they bind to the same epitope (Fig. 3).

Taken together these results imply that the carrier imprint that is found in FITC8, and very likely in many other hapten-specific paratopes allow for interactions with surfaces in the immediate vicinity of the hapten modification. Evolution of such a clone may not only lead to a better hapten-binder but also to a carrier-specific binder.

4. Discussion

The aim of this study was to explore the different regions of the antibody binding site and their different roles in antigen recognition. As model antibody we chose a hapten-specific antibody. Because of the small size of haptens, they will most likely only occupy a portion, usually the central part, of the available antigen-binding site. This allows for additional interactions with the protein, the carrier, to which the hapten is attached. As haptens and proteins in parts interact with different regions of the paratope (Almagro, 2004; MacCallum et al., 1996), with large molecular weight proteins contacting several more residues in the peripheral part, such dual interaction is likely. The carrier interactions may not be sufficient to establish a stable interaction with the antibody in the absence of the hapten modification but it can often be identified by an increased affinity for the hapten-carrier complex as opposed to the soluble hapten (Charlton et al., 2001; Moghaddam et al., 2001). We hypothesized that this built-in tendency to recognize not only the hapten but also the carrier may have implications for the ability of the antibody to evolve into other specificities, i.e. to spread its epitope recognition and in the end recognize the carrier even in the absence of the hapten modification.

Indeed, our results demonstrate that an antibody can evolve from being a hapten-specific binder into one of dual specificity, recognizing the soluble hapten as well as the carrier protein. Firstly, we have shown that the hapten-binding antibody fragment, FITC8, has a molecular imprint of streptavidin, the protein carrier used in the selections to obtain this clone (Söderlind et al., 2000). FITC8 binds fluorescein with high affinity, most likely by interacting with the central parts of the paratope. Binding of this clone to streptavidin could not be detected by neither ELISA nor BIAcore analyses. However, by introducing mutations in the central, hapten-binding part of the paratope, highly specific streptavidin binders could be obtained. These binders, which completely lost their fluorescein binding ability, were shown to bind to an epitope in a close vicinity to the biotin-binding site, i.e. closely related to the hapten (FITC) modification site. Importantly, as shown by binders selected from a naive library, this was not an intrinsic property of streptavidin but rather a feature of the FITC8 combining site. Thus, the peripheral parts of the FITC8 binding site were shown to be complementary with the area surrounding the biotin-binding site of streptavidin. Secondly, we demonstrated that this peripheral imprint could be evolved to increase the recognition of the carrier protein while still retaining the binding to the hapten. By focusing the diversity to the peripheral part of the FITC8 paratope, an antibody of dual specificity was created with the ability to bind both fluorescein and streptavidin, separately as well as in complex. Specifically, these clones were shown to recognize an epitope in close vicinity of the biotin-binding site of streptavidin.

Many antibodies are known to harbour a rather flexible binding site that through conformational changes, so called conformational isomerism, can adapt its binding site to fit quite different antigens (Foote and Milstein, 1994; James et al., 2003). The fact that the *in vitro* evolution process resulted in binders recognizing both streptavidin and fluorescein in a complex as

well as separately, however, suggests that the binding site was capable of adapting its paratope to a second antigen not primarily through conformational isomerism. Rather, a single antibody isomer capable of using different parts of the paratope to recognize different antigens was likely created through this process. The central parts of the paratope of these dual-specific clones bind the hapten (fluorescein), while residues located more in the periphery interact with the carrier protein (streptavidin). As mentioned above, the central residues are normally seen as the specificity-determining region of the paratope, while those found in the periphery are evolved during somatic hypermutation to complement and fine-tune the central, initial, interaction. However, here we demonstrate that the peripheral region can be evolved to have specificity in itself, strong enough to interact with an antigen without the need of the central part of the binding site. The way of making use of different parts of the paratope to bind to different antigens was recently demonstrated by Sethi et al. (2006). This multi-specificity mechanism, which has been termed “differential ligand positioning”, has together with conformational isomerism been suggested as a way of the immune defense to effectively increase the available antibody repertoire. Although there is no evidence that these types of multi-specificity mechanisms exist *in vivo*, there is no convincing reason to believe that they should not.

The fact that hapten-binding antibodies can evolve to recognize the carrier protein independently on the presence of the hapten obviously raises questions of tolerance and self-reactivity. Many molecules, including metal ions and low-molecular weight reactive compounds that are present in the environment or that are formed through metabolic processes, are known to bind to proteins and to structurally modify them. Even though haptens by themselves are unable to mount an immune response, such modification may make the low-molecular weight compound immunogenic, allowing an immune response against it to develop. Indeed, in many cases a clear connection between haptenization and autoimmunity has been established but the actual mechanism responsible for the induction is still largely lacking (Bigazzi, 1997; Griem et al., 1998; Holsapple, 2002; Pichler, 2003; Rao and Richardson, 1999). The results obtained in this study provide an explanation for how this process can occur. We propose that hapten-binders in general, found *in vitro* as well as *in vivo*, just as FITC8, have a peripheral imprint for the area surrounding the hapten-modification of the carrier. In the initial phase of an immune response the diversity of the antibody repertoire is a result of the V–D–J recombination event and consequently mainly focused to the central, hapten-binding, part of the paratope. This fact, in addition to the probable deletion of self-recognizing B-cells, suggests that the binding to the carrier at this point is minor. In order for B-lymphocytes to develop into antibody-producing cells it is essential that they receive appropriate stimuli from T-lymphocytes. Indeed, T-cells recognizing haptenated peptides have been found (Griem et al., 1998). Also, haptens have been shown to affect the level and the pattern of antigen processing, allowing the display of cryptic peptides (Griem et al., 1996, 1998; Jang et al., 1991; Kang et al., 2006). After appropriate T-cell signaling some of the initially selected B-cells undergo somatic hypermutation, in which the diversity

spreads outward to include more peripheral parts of the paratope (Tomlinson et al., 1996). During the subsequent cycles of affinity maturation the periphery of the paratope serves as a scaffold that could be evolved so as to perfect the binding to the original carrier even in the absence of the hapten modification, thereby providing a mechanism to by-pass immunological tolerance.

Although, a direct study of auto-reactive antibodies as they develop in humans has not been the purpose of the current study, literature data are available that hints that a peripheral imprint in hapten-specific immune responses may be involved in the development of auto-reactive antibodies. For example in the autoimmune disease primary biliary cirrhosis (PBC), the major auto-antigen recognized by both B- and T-cells is pyruvate dehydrogenase (PDC), more specifically a domain of this enzyme containing the hapten lipoic acid. It has been suggested that modifications of the lipoyl lysine residue by reactive metabolites could be the initiating event of PBC (Long et al., 2001). Sera from these patients contain different populations of antibodies that bind free lipoic acid, lipoylated PDC or non-lipoylated PDC (Bruggraber et al., 2003). Interestingly, except for differing in specificity, these antibodies populations also differ in isotype composition. The observed change in IgM–IgG ratio of these populations suggests that antibodies targeting the hapten modification are produced in the initial phase of the immune response, while the carrier (PDC)-dependent populations are created later. It thus seems likely that our hypothesis could be used to explain the onset of conditions such as PBC. Sequencing of the different antibody populations together with detailed epitope mapping of the same would provide valuable information whether this truly is the case.

5. Conclusions

In conclusion, we have shown that a new specificity can evolve from a previous one by using a subtle imprint in what was originally not the specificity-determining core of the paratope. We hypothesize that this process may contribute to the development of humoral autoimmune specificity and that it should be considered a potential mechanism behind the development of such specificities.

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