Generation and Analysis of Random Point Mutations in an Antibody CDR2 Sequence: Many Mutated Antibodies Lose Their Ability to Bind Antigen

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Summary

We have investigated the impact of mutations on the binding functions of the phosphocholine (PC)-specific T15 antibody in the absence of antigen selection pressure. The H chain complementarity determining region 2 (CDR2) sequence of T15 antibody was saturated with point mutations by in vitro random mutagenesis. From the mutant library, 289 clones were screened by direct DNA sequencing. The point mutations generated by this method were randomly distributed throughout the CDR2 region and included all kinds of substitutions. 46 unique mutant antibodies, containing one to four point mutations each, were expressed in SP2/0 myeloma cells. Functional analysis on these antibodies has provided insights into several aspects of somatic mutation. (*a*) The majority (26/46) of mutant antibodies either lost (20/46) or had reduced (6/46) ability to bind PC-protein conjugates or R36a, a PC-expressing strain of *Streptococcus pneumoniae*. In contrast, none of the mutant antibodies displayed increased binding for these PC antigens. Taken together with calculations of destructive mutations elsewhere in the V region, the data suggest that somatic mutation may cause extensive wastage among B cells during clonal expansion after antigen stimulation. (*b*) The frequency of binding-loss mutants increased sharply when a second mutation was introduced into the CDR2 sequence; it appears that, in some cases, two or more mutations are needed to destroy binding. (*c*) The mutant antibodies were tested for their reactivity to 11 non-PC antigens as well as to three PC analogues. None of the mutants gained new reactivity or changed their ability to discriminate structural analogues, supporting the notion that the major role of somatic mutation is to increase or decrease affinity rather than to create new specificities. (*d*) Mutations in at least five different positions in CDR2 were deleterious, suggesting that these residues may be essential for antigen binding. Three of these positions are novel in that they had not been identified to be important for binding PC by previous crystallographic analysis. (*e*) Introduction of mutations into two highly conserved residues in CDR2 did not alter the overall conformation of the V region as judged by antiidiotypic analysis, and, in some cases, did not affect the antigen binding function. The results thus indicate that even nonconservative substitutions of invariant residues need not be deleterious, suggesting that their conservation may be due to reasons other than maintaining antibody structure or specificity.

In the course of an antigen-specific immune response, somatic mutations accumulate in antibody V region genes and their immediate flanking sequences at an extraordinarily high rate ($\sim 10^{-3}$/bp/generation) (1–4). Subsequent selection by the antigen of B cells expressing higher affinity antibodies frequently results in "affinity maturation" of the antibody pool (5). It is generally assumed that mutations occur randomly over the length of the V region (6), although recent studies have suggested that there may be mutational hot spots (3, 7, 8). The mechanism of somatic hypermutation is unknown, and its impact on the structure and function of antibodies is not well understood. Studies have been hampered by the lack of in vitro experimental systems. Antibodies recovered during an immune response have been subjected to selective pressures and thus may not provide an unbiased representation of the entire range of somatic mutational products. For example, the fraction of mutations leading to a non–antigen-binding antibody or to a structurally nonfunctional antibody is not known because antibodies having these mutations are seldom recoverable. Efforts have been made to recover such mutants either by antiidiotypic stimulation of B cells previously primed with antigen (9) or by molecular

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screening for expression of a canonical gene combination frequently used by antiarsonate antibodies (10). Several mutants with drastically reduced or undetectable binding for the immunizing antigen were isolated (9, 10), indicating that such deleterious mutations do occur in vivo. Another useful approach to obtain loss mutants has been to screen for spontaneous variants from cultured cell lines (11–14). These approaches, although providing important information about the effects of somatic mutation on antibody specificity, suffer from the inherent problem of low efficiency in the detection and recovery of such mutants (9, 15).

It is not clear why mutations accumulate in some V region genes less frequently than in others even when the B cells expressing them are induced by the same antigen (3, 16–18). One interpretation is that mutations in the CDRs of some genes might lead to antigen-binding loss mutants more frequently than in others; alternatively, different mutational rates may be exerted on different genes.

An experimental approach to generate random point mutations in V region sequences was used to address these questions. This approach utilizes automated DNA synthesis and allows rapid generation of a point mutation library that contains all the possible base substitutions in a defined target sequence. The heavy chain CDR2 sequence of the T15 antiphosphocholine (anti-PC)[1] antibody was chosen as the target because the high-resolution crystal structure of a closely related anti-PC antibody McPC603 (19–21) is available, and a model of the T15 antibody was established based on the McPC603 structure (13). Thus, it is possible to analyze the significance of particular mutations at the structural level. Furthermore, unmutated T15 antibodies exhibit optimal binding to *Streptococcus pneumoniae*, which expresses a carbohydrate-linked form of PC, and mutations in T15 usually result in decreased binding (21). Even in the response to PC-protein, mutated T15 antibodies have displayed only moderately increased hapten binding (less than twofold) (6). In contrast, non-T15 anti-PC antibodies display a high degree of somatic mutation concentrated in CDR regions (especially heavy chain CDR2), and mutations have usually caused an increase in affinity (6, 22–24). Thus, it was of considerable interest to test if T15 antibodies are particularly susceptible to somatic changes that result in loss of function.

Mutations covering every nucleotide position were randomly introduced into the V_H CDR2. The resultant mutants were analyzed for their antigen binding specificity. The results reveal several important features of the randomly mutated antibodies as well as the functional importance of individual residues.

Materials and Methods

Plasmids and Vectors. The pTZ18U phagemid was obtained from Bio-Rad Laboratories (Richmond, CA). The genomic clone of the S107 V_H region gene and the pSV2neoVκ22Cκ construct were kindly provided by Dr. S. L. Morrison (University of California, Los Angeles, CA) (25). The pSV2gptS107γ2b vector was constructed by inserting the S107 (T15) V_H region gene into the pSV2gptγ2b plasmid (Fig. 1), which was a kind gift from Dr. J. Sharon (Boston University School of Medicine) (26) and which has been engineered in our laboratory as described previously (27).

Synthesis of Mutant Oligonucleotides. Synthesis was performed in Dr. J. P. Adelman's laboratory (Oregon Health Sciences University) in a DNA synthesizer (391; Applied Biosystems, Inc., Foster City, CA). The total length of the oligo is 75 nucleotides with the central 57 nucleotides being randomly substituted while leaving nine unmutated nucleotides at each end. Standard synthesis procedures were used except that each of the four parental nucleotides was doped with 3% of each of the three "mutant" nucleotides to give rise to a maximum yield of double mutations in the target sequence (28). The oligonucleotides were purified and phosphorylated by standard methods (29).

Generation of Mutant Library. Mutagenesis was performed according to the instruction manual of the Muta-Gene kit (Bio-Rad Laboratories) as depicted in Fig. 1. Briefly, the mixture of mutant oligonucleotides was hybridized to the single-stranded, uracil-containing pTZ18US107 plasmid that contains the genomic S107 V_H region gene. The complementary strands were then synthesized by T4 DNA polymerase using the oligonucleotide as primer. Ligase was added to seal the new strand to the 5′ end of the oligo. The double-stranded DNA was transformed into *Escherichia coli* XL-1 blue strain, which has functional uracil *N*-glycosylase and thus selects against the uracil-containing parental strand. The transformants were stored as a mutant library at 4°C.

DNA Sequencing. Sequencing was performed as described in the instruction manual of the UBA sequenase kit (United States Biochemical Corp., Cleveland, OH) using double-stranded plasmid DNA as template. A 17-nucleotide primer, 5′ TTCATCTGAAGGTAGAG 3′, complementary to the framework 3 region of the S107 V_H gene, was used to sequence the CDR2 mutations. A JH1 primer, 5′ GAGGAAACGGTGACCGT 3′, plus the above framework 3 primer were used to sequence the entire S107 V_H region. The complete V region of each of the mutants was sequenced after introduction into the pSV2 expression vector to ensure that no other changes were introduced in addition to the desired CDR2 mutations.

Cell Culture and Transfection. SP2/0 myeloma cells were cultured in IMDM plus 20% FCS. Transfection was performed by the lipofectin method as described previously (27). Briefly, cells were grown in six-well tissue culture plates to ~50% confluence and transfected with 10 μg DNA plus 30 μl lipofectin (Bethesda Research Laboratories, Gaithersburg, MD). After 24 h, selection drugs (see below) were added and the cells were transferred to a 96-well plate. Stable transfectants could be seen microscopically in ~7 d and the supernatants screened for antibody in ~2 wk. Usually 10–20 drug-resistant transfectants were obtained from each transfection and three clones that secreted antibody were saved for further analysis. Transfection was done in either of two ways: (*a*) cotransfection of pSV2neoVκ22Cκ and pSV2gptS107 DNA and selection with G418 (1 mg/ml); (*b*) SP2/0 cells were first transfected with pSV2neoVκ22Cκ and selected with G418; the stable transfectant of SP2/0-Vκ22Cκ was then transfected with pSV2gptS107 and selected with mycophenolic acid (30).

Antibody Purification. Antibody secreted by transfectants was purified from tissue culture supernatants by affinity chromatography on either PC-Sepharose 4B or protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) as described previously (27, 31).

ELISA. Direct binding and solid phase inhibition ELISA as well as the various antigens used were described previously (32). Supernatants from 96-well plates were first screened with goat

[1] *Abbreviations used in this paper:* GPC, L-α-glycerophosphocholine; NPPC, *p*-nitrophenyl phosphocholine; PC, phosphocholine.

anti-mouse IgG, goat anti-mouse κ (Southern Biotechnology Associates, Birmingham, AL), and PC-histone. Transfected cells that secreted approximately the same amount of γ and κ chains were expanded and saved. Binding to R36a, PC-histone, PC-BSA, and PC-KLH was tested by direct binding ELISA (31, 32) with various concentrations of affinity-purified antibody. Expression of the T15 idiotype was tested with two anti-T15 antibodies. Goat anti-T15 antiserum was kindly provided by Dr. J. Kenny (National Cancer Institute, Frederick, MD). This antiserum is specific for T15 V_H and V_L regions, and its binding can be inhibited by PC-BSA but not by free PC or BSA as tested in this laboratory. mAb B36-82 (γ1, κ) (33, 34) was a kind gift from Dr. J. Cerny (University of Maryland, Baltimore, MD) and also reacts with T15 V_H-V_L. The recognition of T15 by B36-82 is not inhibited by free PC and is only marginally inhibited by PC-BSA (34, 35). Purified anti-T15 antibody was coated on the plates and then overlaid with purified mutant T15 antibodies at 100 ng/ml. Binding was detected by further incubation with rabbit anti-mouse γ2b antibody coupled to alkaline phosphatase and developed with the substrate, nitrophenyl phosphate. Wells receiving buffer instead of T15 antibody were used as blanks. Mutant antibodies were also tested for binding to various PC-unrelated antigens, including BSA, histone, KLH, albumin, ubiquitin, actin, FITC-BSA, DNP-BSA, oxazolone-BSA, soman-BSA, and DNA as described previously (32). To maximize our ability to detect anti-DNA activity, single-stranded DNA (ssDNA) was used in the binding tests since, except in a few cases (12, 36), most DNA-reactive antibodies have been shown to bind ssDNA (36-38), whereas a considerable proportion of the latter do not bind dsDNA (36-38). The I_{50} values for the inhibitors PC, *p*-nitrophenyl PC (NPPC), L-α-glycero-PC (GPC), and choline were determined as detailed previously (32).

Results

Generation of the T15 H Chain CDR2 Mutant Library. Oligonucleotides encoding the CDR2 of T15 (S107) V_H region were synthesized with random incorporation of point mutations and annealed to the wild-type S107 V_H1 gene present in the pTZ vector (Fig. 1). After strand extension, the pTZ/S107 constructs were transformed into the XL-1 blue strain of *E. coli* to produce a mutant library. Mutations were determined by direct sequencing of individual transformants. 76 of 289 clones sequenced had point mutations and retained the correct reading frame. Out of the 76 clones, 12 had stop codons, seven had silent mutations, and 57 had mutations that caused amino acid substitutions. As listed in Fig. 2, there were 51 unique mutants: 18 have a single substitution, 13 have two, 10 have three, nine have four, and one has five substitutions. The mutations are randomly distributed throughout the whole CDR2 region, and every position has at least two different amino acid substitutions. There is a total of 115 substitutions representing 19 different amino acids. Tryptophan was not found as a substitution and is not present in the germline H chain CDR2 of S107.

Binding of Mutant Antibodies to R36a and PC-Protein. Wild-type and 46 of the mutated S107 V_H region genes shown in Fig. 2 were linked to the mouse γ_{2b} constant region gene (Fig. 1) and expressed in SP2/0 myeloma cells (SP2/0 itself does not express mature H or L chains) together with germline Vκ22Cκ L chain gene. Transfectants expressing both V_H S107 and Vκ22 were selected and their binding features ana-

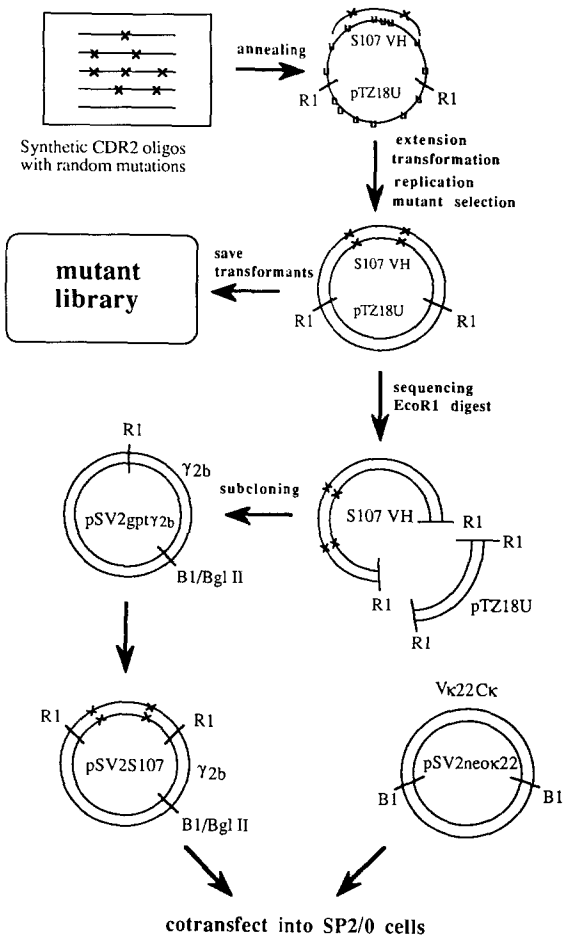

Figure 1. Strategy of random mutagenesis and transfection. The whole V_H CDR2 sequence (57 nucleotides) of the T15 antibody was synthesized in a DNA synthesizer. During automated synthesis, random mutations were introduced into the DNA by "doping" each of the four dNTP with 3% each of the other three. The synthetic mutant oligonucleotides were annealed to the single-stranded, uracil-containing pTZ18US107 phagemid, which contains the wild-type S107 V_H gene. The complementary strand was then extended with DNA polymerase using the oligo as primer. The mutant pTZS107 phagemids were transformed into *E. coli* XL-1 blue strain, which was able to select against the uracil-containing wild-type strand; the transformants were saved as a mutant library. Individual transformants from the library were sequenced to determine the mutations. The mutant variable region genes were removed from the pTZ18U vector and inserted into a mammalian expression vector, pSV2gpt, which carried a mouse Ig γ_{2b} constant region gene. The pSV2gptS107 construct was transfected into the myeloma cell line SP2/0 together with a mouse Vκ22Cκ L chain gene, which had been cloned into the pSV2neo vector. R1, EcoRI; B1, BamHI. The various gene segments are not drawn to scale.

lyzed. Purified antibodies at various concentrations were tested for binding to *S. pneumoniae* R36a and to PC-protein. In R36a, PC is linked to the bacterial cell wall polysaccharides via teichoic acid. Fig. 3 shows the results for several representative antibodies that have comparable (M20, M67), lower (M135, M296), or undetectable (M28, M111) binding to R36a and PC-histone as compared to wild-type T15 antibody. In addition to R36a and PC-histone, all 46 mutant antibodies were also examined for binding to two other PC-protein conjugates, PC-KLH and PC-BSA, and the results were com-

	50	51	52	a	b	c	53	54	55	56	57	58	59	60	61	62	63	64	65	# of mut.
WT	A	S	R	N	K	A	N	D	Y	T	T	E	Y	S	A	S	V	K	G	0
M20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	1
M22	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	2
M28	-	-	-	-	-	S	-	-	-	-	-	-	-	R	-	T	-	T	-	4
M32	-	-	-	K	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	2
M34	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	P	-	N	-	3
M41	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
M54	-	-	-	-	-	T	-	-	-	-	-	-	-	-	P	-	G	-	S	4
M66	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	1
M67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-	-	1
M72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	1
M85	-	-	I	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	2
M102*	-	-	-	-	-	-	-	Y	-	A	P	Q	-	-	-	T	-	-	-	5
M106	-	-	-	C	-	-	K	-	-	-	-	-	-	-	-	-	-	-	R	3
M107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	1
M111	-	-	S	-	-	-	-	-	H	-	-	-	-	-	-	-	-	N	-	3
M113	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	1
M127	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	1
M134*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	D	2
M135	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	1
M142	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	1
M143	-	-	-	-	-	-	-	-	-	-	R	Q	-	-	-	P	-	N	-	4
M145	-	-	-	-	-	-	-	H	-	-	-	Q	-	-	-	-	G	-	-	3
M148	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	1
M150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	1
M152	-	-	S	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	2
M153	-	-	-	-	-	-	-	-	-	-	-	-	-	T	V	-	M	T	-	4
M154	-	-	-	K	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	2
M159	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	M	R	-	3
M160	-	-	-	-	-	-	-	-	H	-	-	-	-	R	-	-	-	-	-	2
M161	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	1
M164	-	-	I	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	2
M166	-	-	-	K	-	-	-	-	-	-	-	G	-	-	-	Y	M	-	-	4
M171	-	-	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
M173	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
M175	-	-	-	-	-	-	-	-	H	S	-	-	-	-	-	-	-	-	-	2
M183	-	-	-	-	E	-	-	Y	-	-	-	Q	S	-	-	-	-	-	-	4
M201*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	1
M205	-	-	-	D	-	-	-	-	-	-	-	A	-	-	-	-	N	-	-	3
M229	-	G	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	2
M240	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	N	-	2
M241	-	R	-	-	-	-	-	E	-	-	-	-	S	-	-	-	-	-	-	3
M242	-	-	S	-	-	V	-	-	-	-	-	-	-	K	-	-	-	-	-	3
M244	-	-	-	T	-	-	-	-	-	-	-	-	-	R	-	-	-	-	A	3
M255	-	-	-	-	Q	-	-	-	F	-	-	V	-	-	-	-	-	M	-	4
M257	-	-	-	-	-	-	K	Y	-	-	-	K	-	R	-	-	-	-	-	4
M260	Q	C	-	-	L	-	-	-	-	-	-	-	-	-	-	T	-	-	-	4
M275*	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
M276	-	G	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	2
M289	-	-	-	-	-	V	S	E	-	-	-	-	-	-	-	-	-	-	-	3
M296	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
M306*	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1

Figure 2. Amino acid sequences of the wild-type (germline) and mutant S107 CDR2 regions. The wildtype (WT) sequence is shown on the top with the single-letter code. Mutant clone names are shown on the left. The number of substitutions in each clone is shown on the right. Substitutions are indicated by single-letter code, and a dash indicates sequence identity. The distribution of mutations is random throughout the CDR2 region according to χ^2 analysis ($\chi^2 = 19.82$, df=18, $p > 0.25$). Mutants with an asterisk are not included in further analysis due to failure of transfection.

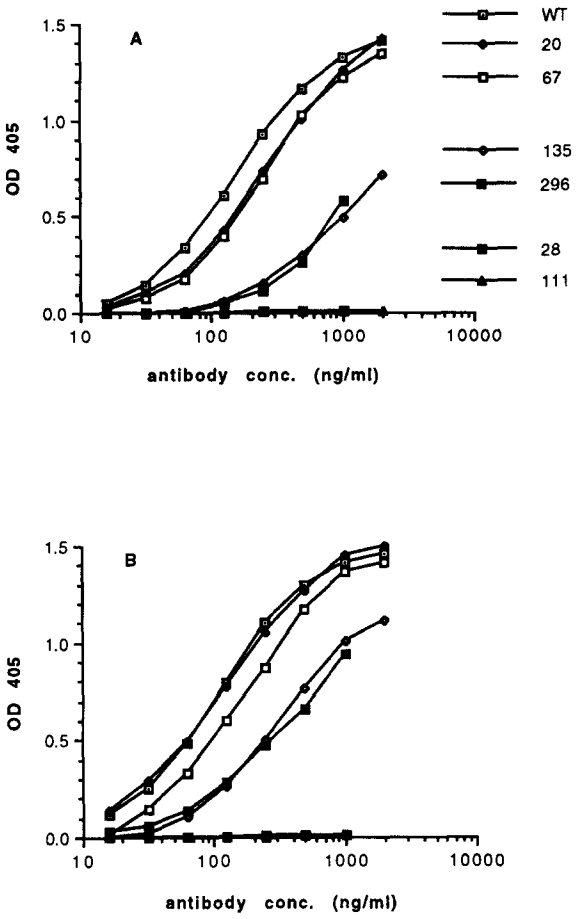

Figure 3. Binding of wild-type and mutant T15 antibodies to carbohydrate (*A*) and protein (*B*) conjugated forms of PC. Affinity-purified antibodies at various concentrations were tested by ELISA for binding to R36a (*A*) and PC-histone (*B*). Each OD_{405} point represents the mean of triplicate determinations.

parable to those for PC-histone (data not shown). Binding activity was calculated based on the concentration of antibody that gave an OD value of 0.5 in ELISA. Mutant antibodies that differed from wild-type by more than threefold were considered to have decreased or increased binding activity. As shown in Table 1, 20 antibodies (43%) lost their ability to bind R36a; of these 20, 17 (37%) also lost the ability to bind PC-protein. In addition, six antibodies (14%) had decreased binding for R36a and seven antibodies (15%) had decreased binding for PC-protein. Thus, the majority of mutant antibodies either lost or had decreased binding, particularly for the carbohydrate form of PC present in R36a, which is commonly perceived as the environmentally important form of PC-antigen (39). In contrast, none of the mutant antibodies displayed increased binding activity and only 20 mutant antibodies retained binding to R36a and PC-protein that was comparable to wild type.

The correlation of loss of binding with the level of mutation was analyzed to determine whether the frequency of nonbinding mutants would increase as the level of mutation increased. Table 2 lists the percentage of nonbinding mutants containing one to four mutations. Only 1 of 15 mutant antibodies lost the ability to bind PC among those that had a single substitution. Loss of binding increased dramatically to ~50% when two, three, or four mutations were present. As a consequence of random mutagenesis, most of the 15 single mutations were unique and did not appear in other mutant antibodies. However, four of them, Ser62→Pro in M20, Asn-53→Ser in M135, Asp-54→Val in M142, and Glu-58→Gln in M161, did occur in antibodies with multiple mutations. Two of the four, M20 and M142, had multi-mutated counterparts (M34 and M152) that lost PC binding ability. This suggests that the increase in nonbinding mutants having two or more mutations may partly be a consequence of cooperative effects between different mutations.

Recognition of Mutant Antibodies by Antiidiotypic Reagents. To investigate whether the loss of binding was due to major conformational changes in the antibody combining site, expression of the T15 idiotype by the mutant antibodies was tested with a polyclonal anti-T15 antiserum and the anti-T15 mAb B36-82 (33, 34). These antiidiotypic antibodies (anti-Id) recog-

Table 1. *Binding Features of T15 Heavy Chain CDR2 Mutant Antibodies*

Mutants[§]	Binding activity* R36a	Binding activity* PC-histone	Recognition by anti-T15‡ Antiserum	Recognition by anti-T15‡ B36-82
	%			
S107 wt	100	100	+ + +	+ + +
M22	0[‖]	0	+ + +	+
M28	0	0	+ + +	+ + +
M34	0	0	+ + +	+ + +
M54	0	0	+ + +	+ +
M66	0	0	+ + +	+ + +
M85	0	0	+ + +	+ + +
M111	0	0	+ + +	+ + +
M152	0	0	+ + +	+ + +
M153	0	0	+ + +	+ + +
M154	0	0	+ + +	+ + +
M164	0	0	+ + +	+ + +
M166	0	0	+ + +	+ + +
M171	0	0	+ + +	+ + +
M229	0	0	+ + +	+ + +
M241	0	0	+ + +	+ + +
M242	0	0	+ + +	+ + +
M260	0	0	+ + +	+ + +
M32	0	**12**	+ + +	+ + +
M106	0	**7**	+ + +	+ + +
M183	0	58	+ + +	+ + +
M135	**10**	**27**	+ + +	+ + +
M173	**7**	39	+ + +	+ + +
M244	**9**	**29**	+ + +	+ + +
M289	**12**	**31**	+ + +	+ + +
M296	**14**	**19**	+ +	+ +
M41	**16**	**20**	+ + +	+ + +
M20	74	100	+ + +	+ + +

continued

Table 1. (*continued*)

Mutants[§]	Binding activity* R36a	Binding activity* PC-histone	Recognition by anti-T15‡ Antiserum	Recognition by anti-T15‡ B36-82
M67	57	65	+ + +	+ + +
M72	52	63	+ + +	+ + +
M107	71	77	+ + +	+ + +
M113	73	58	+ + +	+ + +
M127	114	114	+ + +	+ + +
M142	77	76	+ + +	+ + +
M143	57	78	+ + +	+ + +
M145	46	34	+ + +	+ + +
M148	50	57	+ + +	+ + +
M150	64	73	+ + +	+ + +
M159	117	160	+ + +	+ + +
M160	59	50	+ + +	+ + +
M161	78	126	+ + +	+ + +
M175	47	74	+ + +	+ + +
M205	161	160	+ + +	+ + +
M240	40	44	+ + +	+ + +
M255	117	110	+ + +	+ + +
M257	109	126	+ + +	+ + +
M276	42	58	+ + +	+ + +

* Binding activity was calculated based on the antibody concentration (ng/ml) that gives an OD of 0.5 in ELISA: binding activity = 100 × (WT conc. giving OD 0.5/mutant conc. giving OD 0.5). Those binding activity values that show a threefold difference from the wildtype T15 antibody are highlighted in boldface print.

‡ Binding to various anti-T15 antibodies was tested by direct-binding ELISA as described in Materials and Methods. Purified antibodies were added to the anti-T15-coated plates at a concentration of 100 ng/ml. The OD readings of triplicates were averaged and are expressed in a simplified scale: + + + >0.8; + + = 0.2–0.8; + = 0.01–0.2.

§ The T15 wild-type and VH CDR2 mutant antibodies are all IgG2b and were expressed in myeloma cell line SP2/0, which had been cotransfected with the wild-type Vκ22 light chain gene. The corresponding CDR2 sequences for these antibodies are shown in Fig. 2.

‖ These antibodies gave no binding at concentrations of 2,000 ng/ml.

nize epitopes that are close to the antigen-binding site and require the presence of both the T15 H and L chain variable regions (35, 40). As shown in Table 1, all of the mutant antibodies were able to react with both antiidiotypic reagents to approximately the same degree as the parental T15 antibody. The only exception is M22, which showed a weaker reactivity to B36-82 than to the wild-type antibody. These results suggest that there is little alteration in the overall conformation of the V regions of these mutant antibodies.

Reactivity with PC Analogues and PC-unrelated Antigens. To test whether the H chain CDR2 mutations have an influence on fine specificity, the mutants that retained binding activity were examined for their ability to bind PC, NPPC, GPC, and choline. NPPC resembles PC when it is coupled to a

Table 2. *Correlation between the Level of Mutation in CDR2 and the Frequency of Occurrence of Nonbinding Antibodies*

No. of mutations	No. of antibodies	No. of nonbinders	Percent of nonbinders
1	15	1	6.7
2	12	7	58
3	10	4	40
4	9	5	56

Table 3. *Fine Specificity of the T15 V_H CDR2 Mutants that Retain Binding Activity for PC Protein*

Mutants‡	I_{50} (10^{-5} M)*			
	PC	NPPC	GPC	Choline
Wildtype	0.08	0.36	0.43	62
M20	0.05	0.30	0.26	42
M41	0.10	0.73	0.85	78
M67	0.06	0.25	0.18	40
M72	0.07	0.43	0.29	63
M107	0.09	0.58	0.68	81
M113	0.07	0.78	0.17	57
M127	0.13	0.65	0.74	71
M135	0.06	0.22	0.16	18
M142	0.22	1.74	0.97	179
M143	0.07	0.86	0.76	120
M145	0.48	8.65	7.74	630
M148	0.08	0.17	0.62	81
M150	0.05	0.28	0.46	39
M159	0.09	0.31	0.89	50
M160	0.09	1.19	0.70	83
M161	0.09	0.97	0.91	144
M173	0.12	0.48	0.13	31
M175	0.08	0.52	0.37	76
M205	0.08	0.16	0.23	33
M240	0.09	0.11	0.82	80
M244	0.21	0.47	1.34	50
M255	0.06	0.23	0.17	34
M257	0.11	0.96	0.88	123
M276	0.10	0.55	0.35	69
M289	0.07	0.26	0.38	23
M296	0.07	1.00	0.72	62

* The I_{50} value is defined as the concentration (10^{-5} M) of the haptens required to inhibit by 50% the binding of antibody to PC histone-coated plates. Each sample was tested two to three times; the differences in I_{50} values between tests were less than twofold. The mean values are shown.

‡ Only antibodies that bound to both R36a and PC protein are shown.

protein via diazophenyl linkage, whereas GPC more closely resembles PC-polysaccharide. Binding to various hapten analogues was examined by inhibition ELISA. I_{50} values are summarized in Table 3. All mutant antibodies that bound R36a and PC-protein also retained their ability to bind the PC analogues. Moreover, the overall fine specificity patterns of mutant antibodies as determined by I_{50} values were similar to that of the wild type, namely, lowest I_{50} for PC, intermediate for NPPC and GPC, and much greater for choline.

To investigate whether the mutations introduced in the V_H CDR2 of T15 antibody would create new antigen binding specificities, the mutants were tested for their ability to bind to 11 different non-PC antigens, including BSA, histone, KLH, albumin, ubiquitin, actin, ssDNA, DNP-BSA, FITC-BSA, oxazolone-BSA, and soman-BSA. None of the mutant antibodies showed detectable reactivity to any of these antigens (data not shown).

Discussion

Effects of Random Mutations of H Chain CDR2 of T15 Antibody on PC Binding. In this report, we have used a mutagenesis strategy that allowed rapid generation and isolation of random point mutants. Within the 57-nucleotide target sequence of the T15 V_H CDR2, point mutations were introduced into every position and all 12 types of nucleotide substitutions were observed (data not shown). The distribution of mutations was statistically random although some positions appeared to accumulate mutations more frequently than others. This approach has previously been used in other systems for structure-function analysis and has generated important information not easily obtainable by other mutagenesis procedures (41–44). For example, Murray et al. (42) applied this approach to the α1 domain of a mouse MHC class I molecule and found that a mutation at conserved residue Tyr-27→Asp destroyed its recognition by alloreactive CTL; prior information had not implicated Tyr-27 as a critical residue for allorecognition. This strategy is particularly suitable for antibody studies because the essentially random mutations generated by this technique provide a useful in vitro model system for examination of important aspects of antibody somatic hypermutation.

57% (26/46) of the mutant T15 antibodies either lost or had reduced binding to PC antigens. In contrast, none of the 46 mutants showed increased binding. The results are consistent with previous findings showing that there is little affinity maturation in the anti-PC response (45, 46) and that mutated T15 antibodies have less than optimal binding to *S. pneumoniae* (22). Gearhart and colleagues (6, 46, 47) examined the affinity of four IgG T15 antibodies with mutations in V_H and/or V_L. Three of these antibodies had a modestly increased affinity (1.6-fold) for *N*-2,4-dinitrophenyl-*p*-aminophenyl-PC (DPPC), and one had no obvious change in affinity. Two were also tested for binding to PC, with one having no change and the other having a 3.3-fold decrease (47). More recently, Levy et al. (18) reported that T15 V_H region genes isolated during the immune response to PC-KLH had a low level of mutation compared with other antibody genes isolated during the same response. Such results suggest that T15 antibodies may be particularly susceptible to binding loss or binding decrease due to somatic mutation. Our studies here represent a direct test of the susceptibility of the T15 V_H gene to functional damage by somatic mutation in CDR2 and, as such, the results provide support for the hypothesis.

Recent studies from Foote and Milstein (48) suggest that association rates rather than affinity provide the selection pressure in memory responses. Although few T15 antibodies display increased affinity during immune responses, some of them may have improved kinetics. It would be of great interest

to test whether our mutant T15 antibodies display increased association rates.

In addition to mutations known to increase binding, mutations that do not affect binding or do not cause amino acid replacement (silent or nonselectable mutations) have also been frequently observed in secondary antibodies in various immune responses (49–51), implying that somatic mutations may also generate mutants that no longer bind antigen. Based on the number of invariant and conserved residues compiled by Kabat et al. (52), Shlomchik et al. (37) estimated that half of the mutations in the framework regions would be deleterious. Others have analyzed the antibodies derived from particular immune responses and estimated that 25–50% of randomly acquired mutations would be damaging (17, 53). However, these calculations were based on the comparison of antibody sequences and represent only theoretical figures. Our finding that 43% of randomly mutated, unselected antibodies lost their antigen-binding ability provides the first direct evidence to support the prediction that many B cells in each mutating clone may be lost to the response. If one considers that ~40% of the CDR mutants may lose antigen binding ability, that 50% of the framework mutations could be structurally nonpermissive (37), and that ~5–10% of random mutations can result in a termination codon (17, our data), the wastage during the diversification of B cells via somatic mutation would be surprisingly high. However, as mentioned above, T15 antibodies are unique in that they completely dominate the primary anti-PC response and have little affinity maturation. Thus, they may not be representative of the majority of antibody responses. The issue of whether the frequent loss of binding is a unique feature of T15 antibodies or if it is a common consequence of somatic mutation in other antibodies is currently under investigation.

Among the 15 single mutants, only one lost PC binding, indicating that in T15 antibodies, one mutation in V_H CDR2 is tolerated well. However, two mutations sharply increased the frequency of binding-loss mutants to 58%. This high frequency cannot be solely attributed to the higher probability of hitting crucial positions; rather, in some cases, two or more mutations may have a cumulative effect (see discussion below regarding M154). It is not clear at present why increasing the number of mutations to three or four did not further increase the fraction of nonbinding mutants. Some mutations may provide compensatory changes; or, the sample size in this instance may be too small to draw a conclusion. Experiments to assess the effects of individual as well as combinations of mutations are in progress.

Do V_H CDR2 Mutations Generate New Specificities? It was reported previously that a single mutation in V_H CDR1 of the T15 antibody not only resulted in loss of binding to PC but also generated new specificities for DNA, cardolipin, and some phosphorylated proteins (11, 12). Other evidence also suggests that mutants with different antigen binding specificities exist in the memory response (9). Changes in fine specificities after somatic mutations are known to occur in antiinfluenza HA responses (49, 54). Others have reported (37, 38) that somatic mutations in antibodies that bound only ssDNA gave them the added ability to bind dsDNA. Furthermore, Fish et al. (55) found that boosting with different analogues of arsonate elicited antibodies with different fine specificities. It has therefore been hypothesized that the somatic mutation mechanism not only serves to increase the efficiency of the organism to deal with the eliciting antigen, but also may act as a means of increasing the overall diversity of the antibody repertoire (9). On the other hand, Malipiero et al. (6) analyzed the affinity of 12 mutated anti-PC antibodies (including the T15, McPC167, and McPC603 families) and found that eight had higher affinity than their unmutated counterparts, two had the same affinity, and two had lower affinity. They therefore argued that the major role of somatic mutation was to increase affinity, not to create new specificities. However, the polyreactivity of those mutants was not examined. To test this hypothesis, we examined the 46 mutants for their reactivity to 11 PC-unrelated antigens, including haptens, proteins, and DNA. None of the mutants gained reactivity to these new antigens and few had obvious changes in fine specificities, consistent with the notion that the major effect of somatic mutation on antibody function is to decrease or increase affinity for the primary antigen rather than to generate new specificities. It is worth noting, however, that we selected the V_H CDR2 for mutational analysis because it is a central feature of the T15 active site. We cannot exclude the possibility that regions other than CDR2, if subjected to this type of analysis, would provide evidence for increased affinity and/or altered specificities.

Structural Basis for Loss of Binding in One- and Two-Site Mutants. Analysis of the known antibody structures (56, 57) shows that H chain CDR2 contains two adjacent antiparallel β strands connected by main-chain hydrogen bonds between residues 50 and 58 and between residues 52 and 56. The loop of residues 52A–54 connects the two strands at the top of the antigen binding pocket and varies in length and conformation. A second loop made up of residues 61–65 varies in sequence and conformation among the known structures and is distant from the antigen-binding site. The V_H chain of the T15 antibody has very high homology to that of the related anti-PC antibody McPC603, differing by just five amino acids: four replacements and one insertion. Because of this high homology, the model of the binding site of the T15 antibody (13) derived from the crystal structure of the antibody McPC603 (20) can be reliably used to examine the structural basis for loss of binding in our CDR2 mutants. The positions of specific CDR2 residues are shown in the context of the V region structure and bound PC in Fig. 4.

Our collection of a large number of CDR2 mutants provides an excellent opportunity to investigate the contribution of individual residues to antigen binding. Although most of our mutants have more than one amino acid substitution, those residues most responsible for the altered binding phenotypes can be estimated by subtractive comparisons. For example, if a nonbinding antibody having two substitutions shares one with another antibody that retains binding, the shared mutation may not affect binding but the unique mu-

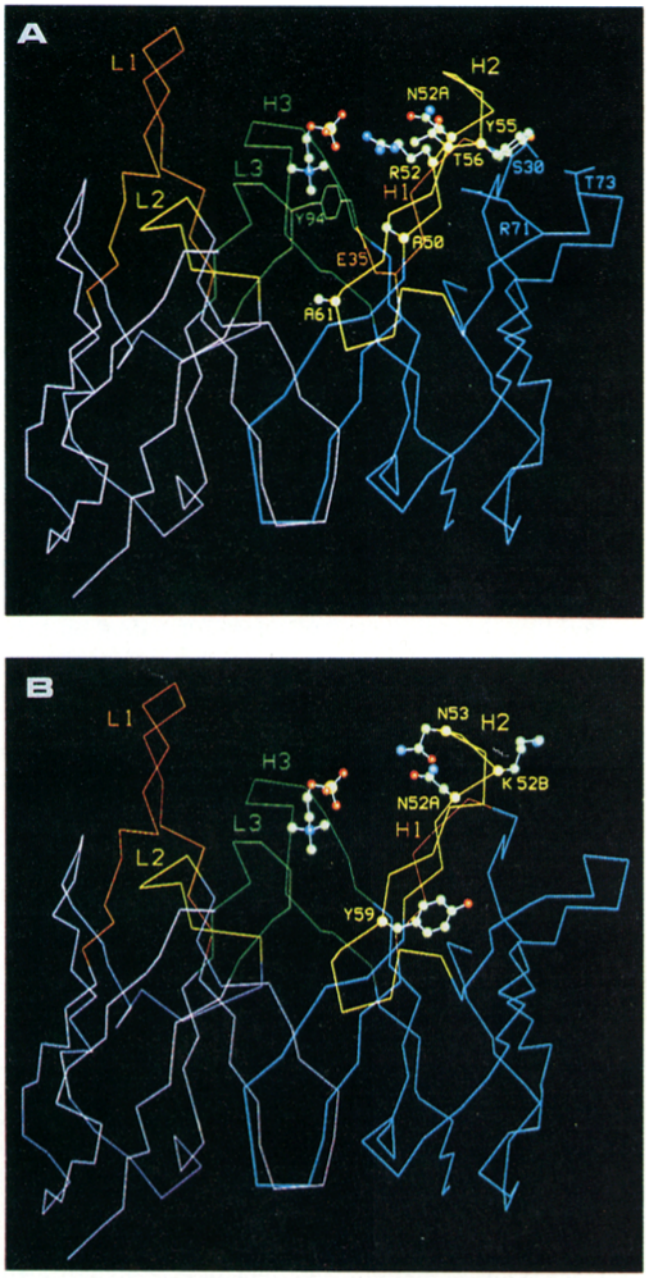

Figure 4. Computer graphics model of the T15 (S107) variable region (13). (*A*) Location of the V_H CDR2 residues critical for PC binding. The α-carbon backbones for the V_H (*blue*, *right*) and V_L (*purple*, *left*) chains are shown with red CDR1 (H1 and L1), yellow CDR2 (H2 and L2), and green CDR3 (H3 and L3) α-carbon atoms and labels. Bound PC (*top*, *center*) is shown as a ball-and-stick model color coded by atom type with green carbon, blue nitrogen, red oxygen, and yellow phosphorus atoms. Critical residues are colored by atom type and have yellow labels with one-letter amino acid codes. Ala-50 lies at the bottom of the antigen binding pocket adjacent to the important hydrogen bond (green line) between the hydroxyl of L chain Tyr-94 (green side chain and label) and the carboxylate group of H chain Glu-35 (red side chain and label). The side chains of Arg-52, Asn-52A, and Thr-56 extend into the antigen binding site. Tyr-55 lies between the backbone of V_H CDR2 and the side chains of framework residues Ser-30, Arg-71, and Thr-73 (blue side chains and labels). Ala-61 extends towards the V_L chain, but is distant from the antigen-binding site. (*B*) Location of residues that appear to be involved in binding to the carbohydrate form of PC present in R36a; highly conserved residues are also displayed. The side chains of residues Asn-52A and Asn-53 lie at the top of the binding pocket, extending into the antigen binding site, and may influence the binding of the large carbohydrate carrier of R36a. Although residues Lys-52B and Tyr-59 are highly conserved, they do not appear to be essential for antigen binding. Their side chains extend away from the antigen binding site and lie on the surface of the antibody.

tation may be essential. Fig. 5 lists several sets of mutants. Within each set, the residue estimated to be crucial for PC binding is printed in bold face and underlined.

In M22, the Ala-50→Thr change is most likely responsible for the loss of antigen binding. The other mutation present in M22, Val-63→Leu, is probably not critical because the three single-site mutants at Val-63, M67, M72, and M150, retain binding (Fig. 5). Even the nonconservative change of Val-63 to a negatively charged Glu in M150 does not appear to weaken PC binding. In addition, three multi-site mutants with mutations at Val-63 also retain binding: M145, M159, and M205. On the other hand, although Ala-50 does not directly contact PC, its side chain contacts several residues known to be critical for PC binding. Ala-50 contacts the side chain of Arg-52, the side chains of H chain Glu-35 and L chain Tyr 94, and the structurally conserved framework region of the V_H chain (Fig. 4 *A*). The Ala-50 side chain forms part of the surface upon which lie the side chain atoms of H chain Glu-35 and L chain Tyr 94 that make a hydrogen bond between the V_H and V_L chains. This hydrogen bond appears to be important for maintaining the shape of the antigen binding pocket (20). Our results show that even a modest increase in the size of the residue 50 side chain (Ala→Thr) can not be tolerated in this tightly packed region. The only other mutant with a change at position 50 is M260, which also has lost its ability to bind antigen, but this mutant has other changes that may contribute to the loss of binding.

In the two-site mutants, M85 and M152, the Arg-52→Ser and Arg-52→Ile changes are most likely responsible for the loss of antigen binding because of the importance of the salt bridge between the side chain of Arg-52 and the phosphate group of PC (20, 58). In fact, all seven mutants that have a substitution at Arg-52 failed to bind PC. This is consistent with a recent mutagenesis study of the McPC603 antibody in which even the conservative Arg-52→Lys exchange abolished PC binding (59). These results indicate that the precise geometry in this region is necessary for a favorable interaction between Arg-52 and the phosphate group of PC. Comparison of the McPC603 structure with sequence data (58) and examination of the model of the T15 antibody (13) shows that there are three charged side chains in the T15 binding site that electrostatically complement bound PC. Mutations of any one of these residues, Arg 52, Glu 35 (11), or Asp 95 (14) of the V_H, abolish binding. These findings emphasize the importance of electrostatic interactions in binding PC.

The only single-site mutant that has lost PC binding is the Tyr-55→Ser mutant M66. Inspection of the sequences compiled by Kabat et al. (52) revealed that Tyr-55 is very highly conserved in antibodies with V_H CDR2 sequences of 19 amino acids, which includes the T15 antibody. Tyr-55 has

	50	51	52	a	b	c	53	54	55	56	57	58	59	60	61	62	63	64	65	PC	R36a
GL	A	S	R	N	K	A	N	D	Y	T	T	E	Y	S	A	S	V	K	G	+	+
M22	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-
M67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-	-	+	+
M72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	+	+
M150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	+	+
M85	-	-	I	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-
M160	-	-	-	-	-	-	-	-	H	-	-	-	-	R	-	-	-	-	-	+	+
M175	-	-	-	-	-	-	-	-	H	S	-	-	-	-	-	-	-	-	-	+	+
M255	-	-	-	-	Q	-	-	-	F	-	-	V	-	-	-	-	-	M	-	+	+
M152	-	-	S	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-
M142	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	+	+
M66	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-
M154	-	-	-	K	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-
M175	-	-	-	-	-	-	-	-	H	S	-	-	-	-	-	-	-	-	-	+	+
M32	-	-	-	K	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	+	-
M160	-	-	-	-	-	-	-	-	H	-	-	-	-	R	-	-	-	-	-	+	+
M257	-	-	-	-	-	-	K	Y	-	-	-	K	-	R	-	-	-	-	-	+	+
M229	-	G	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-
M276	-	G	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	+	+
M296	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
M107	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	+	+

Figure 5. Comparison of sequences of mutant antibodies showing residues critical for antigen binding. Six sets of antibodies are included. Each set has one nonbinding antibody that is compared with one or more antibodies that retained binding activity. The amino acid substitutions that may be responsible for the altered binding phenotypes are indicated as bold face letters and underlined.

been implicated as a key residue for maintaining the type 4 canonical structure found for V_H CDR2s that contain 19 amino acids (56). The side chain of Tyr-55 does not contact PC in the McPC603 crystal structure. Instead, it points away from the antigen binding site, filling the region between the backbone atoms of CDR2 residues 52, 52a, and 52b, and the V_H framework region made up of residues Ser-30, Arg-71, and Thr-73 (Fig. 4 *A*). Thus, an appropriately sized residue may be essential for maintaining the proper geometry of the backbone of CDR2 by providing contacts between CDR2 and the V_H region framework. Consistent with this, conservative replacements of Tyr-55 by the aromatic side chains of His in M160 and M175 and Phe in M255 did not significantly affect binding (Fig. 5).

The source of the loss of binding in the M154 mutant (Asn-52A→Lys and Thr-56→Ile) is not clear. Neither of these two residues directly contacts bound PC (20). The Asn-52A→Lys mutation in M32 resulted in a lower binding for PC than seen for the T15 antibody and abolished R36a binding (Table 1). Although M32 has a Ser-60→Arg mutation, this change is probably not critical because two other antibodies (M160 and M257) had the same mutation but retained full binding. The change of Thr56→Ile also does not appear to be entirely responsible for loss of binding because M175 with Thr-56→Ser retained full binding. In addition, a single-site mutant Thr-56→Ile made by site-directed mutagenesis (M. Brown et al., Oregon Health Sciences University, unpublished results) shows only a slightly reduced binding. Therefore, both changes may be required for loss of binding.

The two-site mutant M229, with Ser-51→Gly and Ala-61→Pro, shares the Ser-51 change with M276, which retains binding. Therefore, the loss of binding in M229 would appear to be due to the Ala-61→Pro mutation. This result is unexpected because residue 61 is distant from the antigen-binding site (Fig. 4 *A*). Pro-61 is present in two antibodies with known structure, HyHEL-10 and J539 (21), which both show a very different conformation for the loop made up of residues 60–65 compared with the McPC603 antibody. This change in conformation, however, does not appear to perturb the structure of the region of V_H CDR2 involved in antigen binding (residues 50–58), nor have residues 60–65 been implicated as being important for contacts between the V_L and V_H chains in the McPC603 antibody (20). It is interesting that Pro-61 does not occur in any of the V_H CDR2 sequences of murine antibodies with 19 amino acid listed by Kabat et al. (52), although it frequently appears in CDR2s having 16 or 17 amino acids. Ala-61 can be replaced by other amino acids, as shown by the single-site mutant M107 with Ala-61→Gly, which does retain binding. This suggests that the conformational change of residues 60–65 induced by the conformationally constrained Pro-61 side chain may be responsible for the loss of binding in M229, although the precise mechanism for this loss is not clear.

These critical residues show a variety of different mechanisms for affecting antigen binding. Ala-50 and Arg-52 are essential for maintaining specific interactions between the antibody and PC, with even conservative changes abolishing binding. Examination of the crystal structure of the related antibody McPC603 has suggested the importance of these two residues (20). Tyr-55 and Ala-61 are not in the binding pocket, but are important for maintaining the conformation of the V_H chain CDR2 loop. Our mutagenesis experiments suggest the functional importance of these residues, which is not obvious from the crystal structure alone. In the double-mutant M154 with Asn-52A→Lys and Thr-56→Ile, neither mutation alone is sufficient to cause loss of binding, but both mutations together do, suggesting an additive and/or cooperative effect between these two mutations.

Several mutant antibodies (M32, M106, M135, M173, M183, and M289) lost or greatly reduced their binding to R36a while retaining considerable binding to free PC and PC-protein. This finding suggests that although these T15 antibodies are PC specific, the actual ligand they recognize when PC is presented on a carrier contains a contribution from the carrier itself. Indeed, Claflin et al. (60) have found that the consequence of an antibody response to PC in the context of *S. pneumoniae* or *Proteus morganii* was determined by the carrier; the former was dominated by T15 antibodies whereas the latter was dominated by the McPC603 family although both families of antibodies bind free PC. In McPC603 antibodies, the region proposed to interact with *P. morganii* carrier determinants is on the surface area of the inner lip of the binding site, whereas the region contacting PC itself is at the bottom of the cavity and is preserved in all anti-PC-*morganii* antibodies (23, 24). Positions that are most important for differential recognition of *P. morganii* include 52A and 53 of the V_H CDR2 (22, 23). Interestingly, five of the six mutant antibodies (except M183) that differentially recognize R36a and PC-protein have a mutation at these positions (Fig. 5), which are on the inner lip of the binding cavity based on the computer derived model of T15 antibody (Fig. 4 *B*). The Lys-52B→Glu change of M183 also occurs at the top surface of the V_H CDR2. Although this residue extends away from the binding pocket, it might contact R36a car-

rier, with the large change in its electrostatic characteristics resulting in loss of binding to R36a. It will thus be of great interest to test whether our R36a nonbinding T15 mutants would bind *P. morganii* and to characterize the structural changes introduced by the corresponding CDR2 mutations.

Mutations of Sequentially Conserved Residues in V_H CDR2. About 20% of the residues in antibody variable regions are invariant or susceptible mainly to conservative substitutions (52). It is assumed that such residues are important for maintaining the overall antibody structure rather than for determining the binding specificity (61). Nonconservative substitutions of such residues would thus result in structurally nonfunctional antibody presumably due to improper folding and assembly or inability to be secreted. While most of these residues are located in framework regions, two of them, Tyr-59 and Lys-52B (Fig. 4 *B*), are found in the H chain CDR2. Among the total mouse Ig sequences compiled by Kabat et al. (52), Tyr-59 has been found in 1,091/1,127 (97%) and Lys-52B in 165/171 (96%). Phenylalanine is the major substitution for Tyr-59 and asparagine for Lys-52B. In addition, in the mouse V_H subgroup IIIA to which V_H S107 is assigned, Tyr-59 occurs in 137/138 and Lys-52B in 126/130 reported sequences (52). Several nonconservative substitutions were found at these residues in our 46 mutants. Serine (M164, 183, and 241) and aspartic acid (M240) were able to substitute for Tyr-59 without affecting the overall structure of the antibody V region as assessed by their reactivity with antiidiotypic reagents (Table 1). Thus, loss of the spatial relationships contributed by the bulky side chain of Tyr-59 does not appear to be an important consideration, although in the case of the serine substitution, this might be rationalized by the addition of a water molecule. Likewise, Lys-52B was substituted by glutamic acid (M183), glutamine (M255), and leucine (M260), and the resultant mutant clones were able to produce structurally functional antibody. Moreover, several of these antibodies (M183, M240, and M255) were also able to bind PC. These results nevertheless stress that substitution of highly conserved residues even by markedly different amino acids need not be deleterious to antibody structure. Although we do not formally exclude the possibility that other mutations elsewhere may compensate for the mutations at the conserved positions, we consider this possibility unlikely because each of the seven mutant antibodies has different substitutions at different positions and it seems unlikely that each of these could compensate for the various substitutions at Tyr-59 or Lys-52B. In agreement with our results is the finding that replacement of two other invariant V_H residues, Cys-92 and Trp-36, by site-directed mutagenesis had no influence on antibody function (62, 63). Thus, the significance of the invariant residues in the variable region needs to be interpreted carefully; they may be conserved for reasons other than maintaining the antibody structure, such as regulation of antibody repertoire development via idiotypic interactions or interactions with T cells; or they may play a role in signal transduction.

In this report, we tested the hypothesis that T15 anti-PC antibodies are highly susceptible to loss of antigen binding upon somatic mutation. The finding that >55% of the T15 antibodies with random mutations in their H chain CDR2 lost or reduced their ability to bind PC whereas none had increased binding activity supports this hypothesis. Although many mutations had a profound influence on antibody binding avidity, they did not create new binding specificities, neither did they change the overall conformation of the V region. Structural analysis on several of the nonbinding mutant antibodies revealed a variety of structural mechanisms underlying the loss of binding. Some mutations directly affect the antigen-antibody interaction while others may affect the conformation of the CDR2 loop. Thus, our examination of a large number of mutants in a small region of the antibody combining site clarifies the roles of specific residues and provides fundamental information that will be useful for redesigning antibodies (64, 65).

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