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Framework Residues 71 and 93 of the Chimeric B72.3 Antibody are Major Determinants of the Conformation of Heavy-chain Hypervariable Loops

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Structural analysis derived from the crystallographic study of the chimeric B72.3 antibody illustrated that some heavy-chain framework residues having atomic interactions with heavy-chain CDR residues may directly affect the conformation of CDR loops. For example, an alanine residue at H71 provides room for packing CDR2/CDR1 and lysine residues at H73 and H93 contribute a salt-bridge to aspartic acid at H55 in CDR2 and a hydrogen bond to the carbonyl group at H96 in CDR3, respectively. We have analysed the contribution of these framework residues to the TAG72-binding affinity. We altered these framework residues by site-directed mutagenesis, and determined the affinity of these mutant chimeric antibodies for the TAG72 antigen by solid phase radioimmunoassay. We found that a single amino acid substitution of alanine by phenylalanine at H71 or lysine by isoleucine at H93, significantly reduced the binding affinity for the TAG72 antigen by 12 and 20-fold, respectively, whereas the substitution of lysine by alanine at H73 reduced the binding affinity only two-fold. Our results indicate that heavy-chain framework residues alanine at H71 and lysine at H93 of the chimeric B72.3 antibody are the major determinants of the conformation of heavy-chain CDR2/CDR1 and CDR3 loops, whereas the salt-bridge between lysine at H73 and aspartic acid at H55 is less important. The hydrogen bond between two framework residues, glutamine at H5 and serine at H25 does not affect any CDR conformation. Our results will thus be of importance especially when the humanized B72.3 antibody is constructed by grafting the CDR loops to a human framework. The important framework region interactions must be maintained in the final humanized antibody.

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Keywords: FR residues; site-directed mutagenesis; CDR conformation; binding affinity; TAG72 antigen

The determination of the three-dimensional structure of antibody fragments by X-ray crystallography led to the realization that polypeptide chains of immunoglobulin molecules are folded into globular domains with four in the heavy (H) chain and two in the light (L) chain. These domains are connected by extended peptide segments like pearls on a string (Amzel & Poljak, 1979). All domains exhibit a similar polypeptide folding pattern characterized by two β-sheets. Sequence comparisons of heavy and light-chain variable (V) domains reveal that each domain has three complementarity-determining regions (CDR) flanked by four framework regions (FR) of less variable sequences (Kabat *et al*., 1987). The antigen combining-site is formed by the juxtaposition of six CDRs appearing as loops at one end of the β-sheet, three from V_H and another three from V_L. These CDR loops present a surface that interacts with the antigen. It has been reported that mutations in CDR can significantly alter both antibody specificity (Rudikoff *et al*., 1982; Ohno *et al*., 1985; Kussie *et al*., 1994) and antigen-binding affinity (Roberts *et al*., 1987; Allen *et al*., 1988; Sharon, 1990). The FRs comprise the conserved

Abbreviations used: H, heavy; L, light; CDR, complementarity-determining region; FR, framework region; V, variable.

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β-sheet framework and are responsible for the main-chain conformation of the CDRs and for the interchain interactions that bring domains together (Chothia *et al*., 1985). It has also been demonstrated that some framework residues may greatly affect the conformation of CDR loops (Kettleborough *et al*., 1991; Foote & Winter, 1992; Lavoie *et al*., 1992).

B72.3 is a murine monoclonal antibody with specificity for the mucin-like tumour-associated glycoprotein TAG72 (Thor *et al*., 1986). The chimeric cB72.3-1-3 V_H gene was previously cloned (Xiang *et al*., 1990). The amino acid sequence is numbered (Figure 1) according to Kabat's method (Kabat *et al*., 1987). Recently, crystallization of the chimeric B72.3 Fab' fragment has been reported (Brady *et al*., 1991) and its crystal structure has been analysed (Brady *et al*., 1992). The data illustrated that some atomic interactions of FR/CDR residues may directly affect the conformation of heavy-chain CDR loops (Figure 2). For example, the side-chain of alanine at H71 in FR3 provides room for packing CDR2/CDR1 (Figure 2A), whereas lysine residues at H73 and H93 in FR3 contribute a salt-bridge to aspartic acid at H55 in CDR2 (Figure 2B) and a hydrogen bond to the carbonyl group at H96 in CDR3 (Figure 2C), respectively.

Site-directed mutagenesis and affinity constants

In order to confirm these observations, we performed site-directed mutagenesis at four FR positions (H25, H71, H73 and H93) with six oligonucleotides (oligo 17 to 22) resulting in six mutant chimeric antibodies cB72.3m17-22, respectively. These four mutation sites in the context of the entire Fv are shown in Figure 3. The affinity constants of these six mutant antibodies for the TAG72 antigen determined in a solid-phase radioimmunoassay (Xiang *et al*., 1990) were compared to that of the original cB72.3-1-3 antibody. The cB72.3m17 antibody with only one amino acid substitution of alanine at H71 by phenylalanine showed a reduced binding reactivity for the TAG72 antigen. Its affinity constant K_a for the TAG72 antigen is only 0.57×10^8 M^{-1}, which is 12-fold lower than that of the original cB72.3-1-3 antibody (6.80×10^8 M^{-1}) (Table 1). The cB72.3m18 antibody with one amino acid substitution of lysine at H73 by arginine with a similar basic side-chain showed the same TAG72-binding reactivity as the original cB72.3-1-3 antibody, suggesting that the salt-bridge between H73 and H55 was maintained. However, the cB72.3m19 antibody with the amino acid substitution of lysine by alanine at H73 showed slightly decreased binding reactivity for the TAG72 antigen. Its affinity constant K_a is 3.38×10^8 M^{-1}, which is about twofold less compared to that of the cB72.3-1-3 antibody. Interestingly, the amino acid substitution of lysine at H93 by the hydrophobic isoleucine significantly decreased the TAG72-binding reactivity of cB72.3m21 (K_a, 0.33×10^8 M^{-1}), which is 20-fold less compared to that of the cB72.3-1-3 antibody. On the other hand, the TAG72-binding reactivity of cB72.3m20 with the amino acid substitution of lysine at H93 by arginine with a similar basic side-chain was almost the same as the original cB72.3-1-3 antibody; the corresponding affinity constants for the TAG72 antigen were approximately 6×10^8 M^{-1} (Table 1). The cB72.3m22 antibody with the amino acid substitution of serine at H25 by alanine showed no difference in its TAG72-binding reactivity compared to the cB72.3-1-3 antibody. Overall, these results indicate that two framework residues, namely H71 and H93, are major determinants of the conformation of CDR loops of the chimeric B72.3 antibody. Furthermore, the results show that the salt-bridge formed between H55 and H73 is less important in the conformation of the CDR2 loop, and that the hydrogen bond formed between the glutamine residue at H5 and the serine side-chain at H25 does not affect any CDR loop conformation at all.

In the analysis of immunoglobulins of known structure, Tramontano *et al*. (1990) defined residues

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|FR1
|Q V Q L Q Q S D A E   L V K P G A S V K I   S C K A S G Y T F T
                   10                      20                      30

|CDR1      |FR2                            |CDR2
|D H A I H |W A K Q K   P E Q G L E W I G |Y   I S P G N D D I K Y
                   40                      50     52a              59

             |FR3
N E K F K G |K A T L   T A D K S S S T A Y   M Q L N  S L T S E D
                   69                      79       82a b c       86

                 |CDR3        |J2
S A V Y F C K R |S Y   Y G H |W G Q G T T L   T V S S
                   96        103           109       113
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Figure 1. Amino acid sequences of the cB72.3-1-3 V_H region. The one-letter amino acid code is used. Amino acids are numbered sequentially according to Kabat's method. Demarcated are framework regions (FR), complementarity-determining regions (CDR) and joining segment (J).

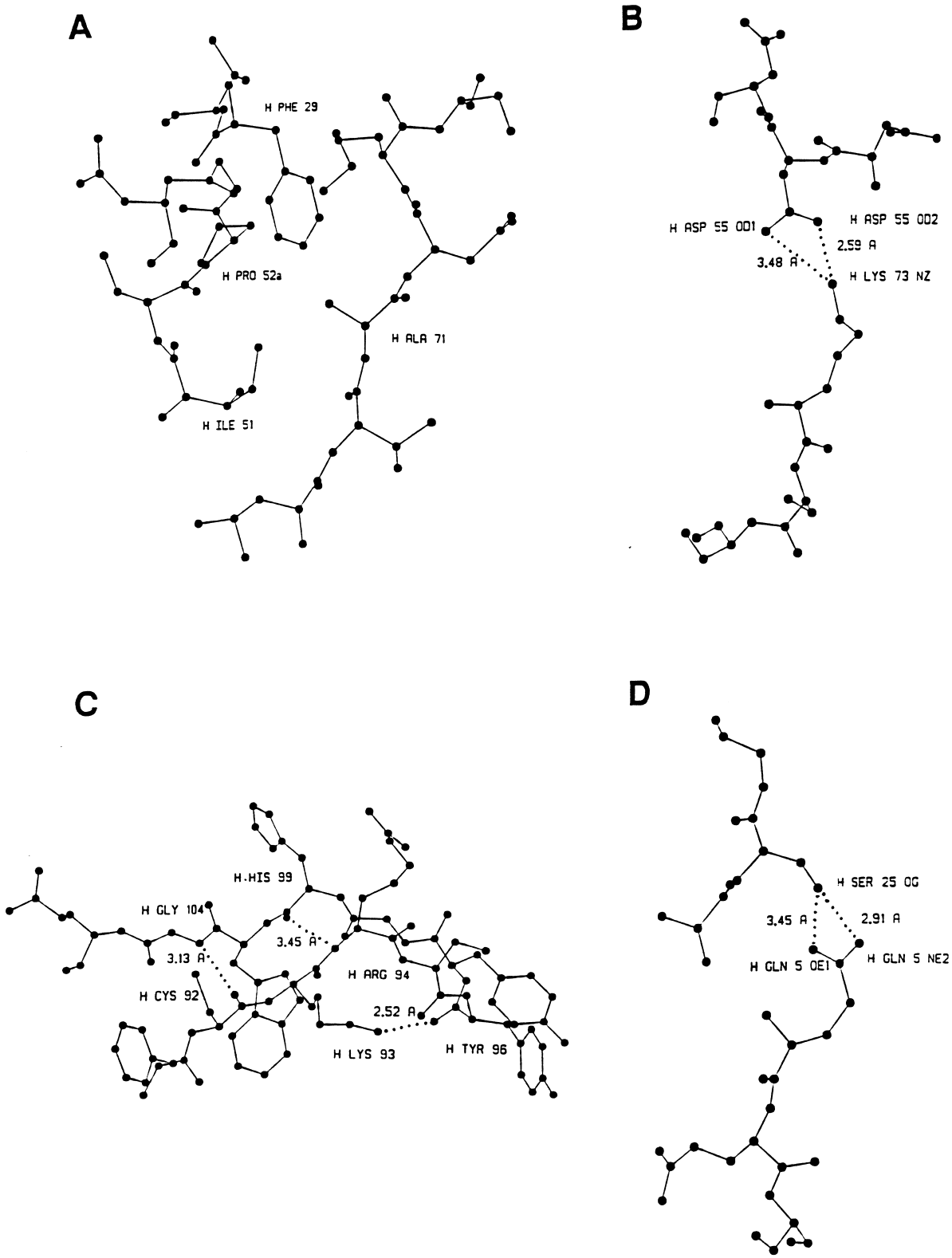

Figure 2. Diagrams of some FR residues with atomic interaction with CDR residues from the co-ordinates of crystallographic analysis of chimeric B72.3 Fab' deposited in the Brookhaven Data Bank. A, Alanine at H71 in FR3 provides room into which packs CDR2 residues isoleucine of H51 and proline of H52a, and the FR1 residue phenylalanine at H29. B, The salt-bridge formed between lysine of H73 in FR3 and aspartic acid of H55 in CDR2. C, The hydrogen-bond formed between the side-chain of lysine at H93 in FR3 and the main-chain carbonyl group at H96 in CDR3. D, The hydrogen-bond formed between serine of H25 and glutamine of H5 in FR1. The α-carbon trace and the amino acid side-chain conformation are marked respectively. Putative hydrogen bonds and salt-bridge are shown as broken lines.

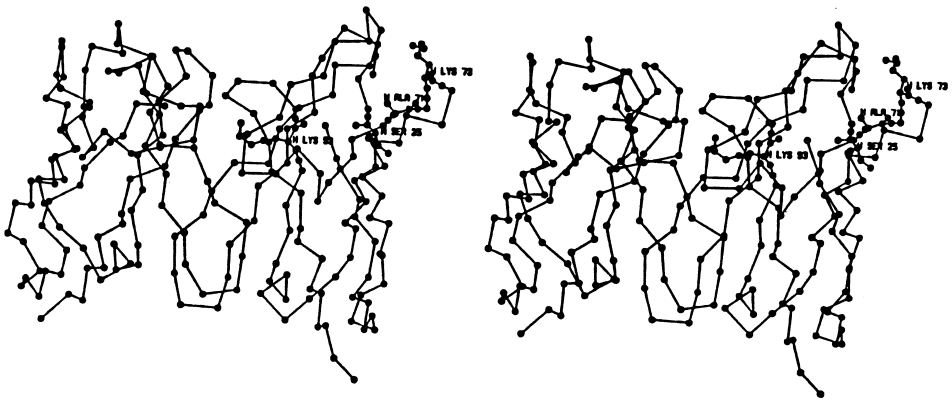

Figure 3. Stereo diagram of the α-carbon trace of the Fv region with mutation sites displayed.

Table 1. Affinity constants of mutant cB72.3m antibodies

Antibody	Amino acid substitution	Affinity constant (K_a)
cB72.3-1-3	—	6.80
cB72.3m17	Alanine by phenylalanine (H71)	0.57
cB72.3m18	Lysine by arginine (H73)	6.42
cB72.3m19	Lysine by alanine (H73)	3.38
cB72.3m20	Lysine by arginine (H93)	6.24
cB72.3m21	Lysine by isoleucine (H93)	0.33
cB72.3m22	Serine by alanine (H25)	6.61

Six oligonucleotides (oligo 17 to 22) complementary to different areas of the V_H region of the chimeric cB72.3-1-3 antibody were designed and synthesized for site-directed mutagenesis. Since alanine at H71 provides room into which packs CDR2 residues isoleucine at H51 and proline at H52a as well as the FRl residue phenylalanine at H29 (Figure 2A), oligo 17 (5' gggca aggcc acact gactt ttgac aaatc ctcca gcac 3') complementary to FR3 of the V_H region was synthesized for substitution of alanine at H71 by phenylalanine. Due to the large size of the side-chain in this amino acid substitution at H71, the CDR1/CDR2 conformation is altered. Since lysine at H73 forms a salt-bridge to aspartic acid at H55 in CDR2 (Figure 2B), oligo 18 (5' ctgac tgcag acaga tcctc cagc 3') and 19 (5' ccaca ctgac tgcag acgca tcctc cagca ctgc 3') were synthesized for substitution of lysine at H73 by arginine with a similar basic side-chain to examine if the salt-bridge between H73 and H55 would be maintained; substitution by alanine was also examined to see if the loss of the salt-bridge would affect its binding affinity for the TAG72 antigen. Lysine at H93 forms a hydrogen bond to the carbonyl group at H96 in CDR3 such that the CDR3 loop is bent and lies at the base of the pocket formed by the CDR loops (Figure 2C). Oligo 20 (5' gtgta tttct gtaga agatc gtac 3') and 21 (5' gtgta tttct gtata agatc gtac 3') were synthesized for substitution of lysine at H93 by arginine with a similar basic side-chain for maintenance of this hydrogen-bond, and by isoleucine for inducing the loss of this hydrogen-bond. For completeness and for comparison purposes, we also selected to alter the hydrogen-bond formation between two FR residues, serine at H25 and glutamine at H5 (Figure 2D) to examine if FR/FR interaction might affect CDR loop conformation. Amino acid substitution of serine by alanine at H25 was made by using oligo 22 (5' tcctg caagg cttct ggcta cacc 3'). All of these amino acid substitutions were conducted by site-directed mutagenesis using oligo 17 to 22 primers and verified by the dideoxy nucleotide-sequencing method (Sanger *et al*., 1977). Expression vectors mpSV2neo-EP1-$V_{m17\text{-}22}C_{\tau 1}$ used for production of mutant chimeric antibodies cB72.3m17-22 were derived from the mpSV2neo-EP1-$C_{\tau 1}$ (Xiang *et al*., 1990). The mutant $V_{m17\text{-}22}$ region fragments (*Ecor*I/*Sal*I) containing an *Eco*RI site at the 5' end and the RNA splice junction consensus sequence (5' caggtaagt 3') at the 3' end were purified from M13mp19-$V_{m17\text{-}22}$ and ligated into the mpSV2neo-EP1-$C_{\tau 1}$ (*Eco*RI/*Sal*I-digested) to form six expression vectors including mpSV2neo-EP1-$V_{m17}C_{\tau 1}$, mpSV2neo-EP1-$V_{m18}C_{\tau 1}$, mpSV2neo-EP1-$V_{m19}C_{\tau 1}$, mpSV2neo-EP1-$Vm_{20}C_{\tau 1}$, mpSV2neo-EP1-$V_{m21}C_{\tau 1}$ and mpSV2neo-EP1-$V_{m22}C_{\tau 1}$, respectively. The DNA of these expression vectors was purified in quantity by standard methods (Maniatis *et al*., 1982), and then transfected into the heavy-chain-loss mutant cell line B72.3Mut(K) (Xiang *et al*., 1990), which expresses only the cytoplasmic K light-chain, by electroporation (Potter *et al*., 1984). The transfected cells were selected for growth in the presence of geneticin at 2.0 mg/ml. The growth transfectants for each mutant antibody showing binding reactivities in a human τ chain capture ELISA (Xiang *et al*., 1990) were selected and sub-cloned. Mutant antibodies were then purified from their culture supernates by protein A affinity chromatography (Xiang *et al*., 1990). Protein concentrations were determined using a BioRad protein assay kit (BioRad, CA, USA) according to the method described in the manual. Affinity constants (K_a, $\times 10^8$ per mol per litre) were determined from Scatchard plots of the binding of different mutant chimeric antibodies cB72.3m17-22 to the TAG772 antigen in a solid-phase radioimmunoassay as described (Xiang & Chen, 1992). Bovine mucin (Sigma Chemical Co., MO, USA), which contains a large amount of the TAG72 epitope (Xiang *et al*., 1990), was used as the TAG72 antigen source in this study. ELISA, enzyme-linked immunosorbent assay.

from H52a to H55 as the H2 region; this region links two strands of the β-hairpin, V_H CDR2. They further divided this H2 region into three groups, namely, the three, four and six-residue H2 regions (Tramontano *et al*., 1990). Examination of the interactions of the CDR2 loop with the rest of V_H domains shows that the determinants of conformation are not entirely within the sequence of the loop itself, but involve packing of the loop against the rest of the structure. Tramontano *et al*. have also illustrated that the framework residue 71 is a major determinant of the conformation of V_H CDR2 (Tramontano *et al*., 1990). The B72.3 heavy-chain belongs to the family with a four-residue H2 loop. Residues at H52a/H53 and H29 pack against the residue at H71; the latter is alanine with a small side-chain that provides a cavity for bringing the CDR1 and CDR2 loops close together. In order to examine the importance of alanine at H71 in CDR loop conformation, alanine at H71 was substituted by phenylalanine. A single amino acid substitution of alanine by phenylalanine significantly reduced the binding affinity of the mutant cB72.3m17 antibody for the TAG72 antigen by 12-fold; this is possibly due to a major conformational change of the CDR1/CDR2 loops. Therefore, our study provides an additional evidence that the FR residue alanine at H71 is a major determinant of the conformation of CDR loops.

The B72.3 heavy-chain CDR3 loop is short and comprises only five residues, which adopts a sharp hairpin turn (Figure 2C). The amino acid residue at H93 in FR3 is usually an alanine which packs at the H/L chain interface, but is a lysine in the B72.3 antibody, and makes a charged hydrogen-bond to the carbonyl group of tyrosine at H96 in CDR3. From an examination of the Fab' crystal structure, we found that the hydrogen-bond formed between lysine at H93 and tyrosine at H96 is the only significant atomic interaction between the side-chain of lysine at H93 and the rest of other V_H residues (data not shown). This atomic interaction gives the CDR3 loop an unusual conformation. The CDR3 loop forms part of the bottom of the antigen combining-site as well as a side of the pocket. The amino acid substitution of lysine at H93 by hydrophobic isoleucine which lacks the capability of hydrogen-bond formation, significantly decreased the binding affinity of mutant cB72.3m21 antibody for the TAG72 antigen by 20-fold, indicating the framework residue H93 is a major determinant of the conformation of the CDR3 loop.

Recently, a number of mouse/human chimeric B72.3 antibodies have been constructed by recombinant DNA technology in order to reduce the human anti-mouse antibody response (Whittle *et al*., 1987; Xiang *et al*., 1992). However, clinical studies suggested that an anti-idiotypic immune response to the variable region was still present (Khazaeli *et al*., 1991). In order to minimize this response, the approach of genetically engineering a humanized antibody was taken, in which mouse CDR loops were grafted directly onto a human framework (Jones *et al*., 1986). In this communication we report experimental proof of the involvement of FR residues (H71 and H93) in the conformation of B72.3 CDR loops. Therefore, our results will be of great importance when the humanized B72.3 antibody is constructed. The important framework region interactions must be maintained in the final humanized antibody.

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