J. Biochem. 132, 775–782 (2002)

Importance of a CDR H3 Basal Residue in V_H/V_L Interaction of Human Antibodies[1]

Takahide Aburatani,* Hiroshi Ueda,*,†,2 and Teruyuki Nagamune*

Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo; and †Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Bldg. FSB-401, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562

Received July 3, 2002; accepted August 29, 2002

Although the cooperativity of the V_H and V_L domains of an antibody in antigen binding has been extensively studied, the interaction between the V_H and V_L domains had not received sufficient attention. To systematically investigate the relationship between the amino acid sequence and V_H/V_L interaction strength, we here used a set of anti-bovine serum albumin antibodies having a single human framework for V_H (V3-23/DP-47 and JH4b) and Vk (O12/O2/DPK9 and Jk1), but with different V_H/V_L interaction strengths. By phage display of a V_H mini-library and analysis of the interaction of amino acids with immobilized V_L fragments, the residue at H95 (Kabat numbering) at the beginning of seven CDR H3 residues was found to play a key role in determining the V_H/V_L interaction. On saturation mutagenesis of H95, Gly showed the strongest interaction, while Asp, Asn, and Glu showed lesser interaction in that order. The generality of the rule was confirmed by the test with urine-derived human L chain instead of a particular V_L. The results demonstrate that H95 plays a central role in deciding the V_H/V_L interaction of human Fvs that have most commonly found frameworks.

Key words: antibody V region, combinatorial library, heterodimer, protein A, protein L.

Antibodies have been widely used to detect and analyze various substances because of the specificity and affinity of the interaction between an antibody and its antigen. The recognition of the antigen is accomplished by the antibody's V_H and V_L domains, which are located at the N termini of heavy and light chains, respectively. The use of these smaller antigen binding fragments has been exploited, such as single chain Fv (scFv) (*1, 2*) and Fab (*3*), which have been applied to various fields such as immunoassays.

Among the various immunoassays reported, the most widely used one is a sandwich immunoassay. This assay has several merits such as high sensitivity and specificity, a wide dynamic range, and a low signal background. However, it also has the weakness that a long and repetitive procedure due to several cycles of consecutive binding and washing steps is usually necessary. In addition, there is one fundamental limitation of the assay, the antigen to be measured requires at least two epitopes to be captured. As a way to circumvent these limitations, we recently developed a new immunoassay approach called the "open sandwich" (OS) immunoassay (*4*). This immunoassay utilizes the property of Fvs that the interaction between V_H and V_L is often weak in the absence of an antigen, and the unstable Fvs are markedly stabilized by the addition of an antigen. Compared with conventional two epitope sandwich immunoassays, this immunoassay based on antigen-driven association of V_H and V_L chains has proven to have several advantages such as the need of only a single Fv for the sandwich assay format, a wide dynamic range, a short measurement time (*5*), and potential for a homogeneous format (*6–8*). So far, several antibodies including anti–phosphorylcholine McPC603 (*9*), anti–hen egg lysozyme (HEL) HyHEL-10 (*4, 10*), and anti–4-hydroxy-3-nitrophenyl acetyl N1G9 (*8, 11*) are known to have such properties. On the other hand, several other antibodies including anti–hen egg lysozyme D1.3 (*12*) and anti Z-DNA Z22 (*13*) exhibit a stronger V_H/V_L interaction in the absence of an antigen, and thus are not readily suitable for this assay.

To explore the possibility and limits of the assay, it is obvious that knowledge on the V_H/V_L interactions of various antibodies is important. However, so far little has been discovered about the V_H-V_L amino acid sequence and the interaction between them. As a model system, we here used four anti-BSA antibodies that exhibit different strengths of interaction between V_H and V_L derived from a single human framework for V_H (V3-23/DP-47 and JH4b) and Vk (O12/O2/DPK9 and Jk1), both of which are most commonly used frameworks, and bind to protein A and protein L, respectively (*14*). By applying the mutational and phage display approaches, a residue that plays a key role in deciding the strength of the V_H/V_L interaction was identified, and its implication as to the CDR H3 structure will be discussed.

[1]This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (296-10145107) from MEXT, Japan, and a Grant-in-Aid for Scientific Research (C 12650782) from JSPS.

[2]To whom correspondence should be addressed at: Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Bldg. FSB-401, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562. Tel: +81-471-36-3644, Fax: +81-471-36-3642, E-mail: h-ueda@k.u-tokyo.ac.jp

Abbreviations: BSA, bovine serum albumin; Fv, antibody variable domains; V_H, antibody variable heavy chain domain; V_L, antibody variable light chain domain.

MATERIALS AND METHODS

Materials—The bacterial strains used were XL-1 Blue (*hsdR17*, *supE44*, *recA1*, *endA1*, *gyrA46*, *thi*, *relA1*, *lacI/F′* [*proAB*$^+$, *lacI*^{q}, *lacZΔM15* :: *Tn10* (*tet*^{r})]), TG1 (*supE*, *hsdΔ5*, *thi*, *Δ*(*lac-proAB*), */F′* [*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZΔM15*]), and HB2151 (*ara*, *Δ*(*lac-proAB*), *thi/F′* [*proAB*$^+$, *lacI*^{q}, *lacZΔM15*]). Four phagemids (pIT2) encoding anti-BSA scFvs (29LJ1, 29LJ2, 29LJ6, and 13CG2) selected from a synthetic human scFv phage library (*14*) were provided by I. M. Tomlinson, Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK. Phagemid vector pIT2 contains a *lac* promoter and a *pelB* leader sequence upstream of the scFv insert, which is then followed by His6 and myc tags, an amber stop codon and the gene encoding the pIII phage coat protein. After helper phage M13KO7 infection, scFv upstream of the amber stop codon is displayed on the phage particle with the use of a suppressor strain such as TG1. On the other hand, scFv with a His6-myc tag is secreted into the supernatant with the use of a non-suppressor strain such as HB2151.

Vector Construction—To express either V_H or V_L of the four anti-BSA Fvs, eight phagemids were constructed. The V_H and V_L fragments were both separately amplified by PCR with four phagemids encoding anti-BSA scFvs as templates. The primers used for the amplification were as follows: VhITNcoRVBack (5′-CCGCCATGGCCGATATCCAGCTGTTGGAGTCTGGGGG-3′), and VhITHdNotFor (5′-GCCGCGGCCGCAAGCTTCGAGACGGTGACCAGGGTTC-3′) for the amplification of V_Hs; VkITNcoRVBack (5′-CCCGCCATGGCCGATATCCAGATGACCCAGTC-3′), and VkITHdNotFor (5′-GCCGCGGCCGCAAGCTTCCGTTTGATTTCCACCTTGG-3′) for the amplification of V_Ls. These primers were designed to incorporate *Nco*I and *Not*I sites into PCR fragments. The PCR products were digested with *Nco*I and *Not*I, and then inserted into phagemid vector pIT2.

Phage Display of V_H Fragments—TG1 cells carrying the V_H-encoding phagemids were incubated with shaking at 37°C in 2× TY containing 100 μg/ml ampicillin and 1% glucose (2TYAG) until the OD_{600} reached 0.5, and then helper phage M13KO7 was added with m.o.i of 20. The cells were incubated at 37°C without shaking for 30 min and then centrifuged at 3,300 ×*g* for 10 min. The cells were resuspended in 2× TY containing 100 μg/ml ampicillin and 25 μg/ml kanamycin, and then incubated with shaking at 30°C overnight. The overnight culture was centrifuged at 10,800 ×*g* for 10 min, and then PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) was added to the supernatant. After standing at 4°C for at least 1 h, the supernatant was centrifuged at 3,300 ×*g* for 30 min. The pellet was resuspended in TE (pH 8). The phage solution was centrifuged at 16,000 ×*g* for 10 min, and the supernatant containing the phage particle displaying the V_H fragment was recovered and stored at 4°C.

Soluble V_L Preparation—HB2151 cells carrying the phagemid encoding the V_L gene were incubated with shaking at 37°C in 2× TY containing 100 μg/ml ampicillin and 0.1% glucose until the OD_{600} reached 0.9, and then isopropyl β-D-thiogalactopyranoside was added to 1 mM. The cells were incubated at 30°C overnight and then centrifuged at 12,000 ×*g* for 20 min. Soluble V_L in the supernatant was precipitated by adding ammonium sulfate to 60% (w/w). After centrifugation at 5,000 ×*g* for 20 min, the pellet was resuspended in and dialyzed against sodium phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0). V_L was purified from the solution with Talon metal affinity resins (Clontech, Tokyo) according to the manufacturer's protocol. The purity of the resultant protein was confirmed by SDS-PAGE and CBB-R staining.

Chain Shuffling and ELISA—The strength of the V_H/V_L interaction of the sixteen combinations of V_H and V_L from anti-BSA antibodies was tested by phage ELISA. Falcon 3912 microplates (Becton Dickinson, Oxnard, CA) were coated overnight with 100 μl per well of 1 μg/ml Protein L (Actigen, Cambridge, UK), which specifically binds V_L, in 10 mM phosphate-buffered saline (PBS). After blocking at room temperature for 2 h with 2% skim milk in PBS (MPBS), and washing three times with PBS containing 0.1% Tween-20 (PBST), the plates were incubated at room temperature for 1.5 h with 100 μl per well of a 10 μg/ml V_L solution in PBS. The plates were washed three times with PBST and then incubated at room temperature for 1.5 h with V_H-phage (10^{10}–10^{11} cfu per well) in MPBS. The plates were washed 6 times with PBST and then incubated at room temperature for 1.5 h with 100 μl per well of 5,000-fold diluted HRP-conjugated sheep anti-M13 (Amersham-Pharmacia Biotech, Tokyo) in MPBS. The plates were washed 3 times with PBST and then developed with 100 μl/well substrate solution [200 μg/ml *o*-phenylenediamine (OPD, Wako), 0.04 μl/ml H_2O_2 in 50 mM sodium succinate, pH 5.0, or 100 μg/ml 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma) in 100 mM sodium acetate, pH 6.0]. After incubation for 5 to 30 min, the reaction was stopped with 50 μl per well of 1 M sulfuric acid and then the absorbance was read at 490 nm (OPD) or 450 nm (TMB).

Construction of a Mini-Library—A mini-library was constructed by introducing mutations into 29LJ2 V_H by overlap extension PCR. Two primers were designed to incorporate either Thr or Ser, Tyr or Thr, Ser or Ala, Asn or Asp, Tyr or Ser, and Gly or Ser at positions 50, 52a, 55, 56, 58 (CDR H2), and 95 (CDR H3), respectively, of 29LJ2 V_H. The DNA fragment encoding FR1, CDR H1, FR2, and CDR H2 (fragment 1) was amplified with primer M13RV (5′-CAGGAAACAGCTATGAC-3′), and equimolar mixture of 29-2h(2)FR1 (5′-CACGGAGTCTGCGTAADATGTATYAGMACCAGCAGTATAAATAGWTGAGACCCACTCCAG-3′) and 29-2h(2)FR2 (5′-CACGGAGTCTGCGTAADATGTATYAGMACCAGCATAATAAATAGWTGAGACCCACTCCAG-3′), which incorporates mutations into CDR H2 as above. The fragment encoding FR3 and CDR H3 (fragment 2) was amplified with primers 29-2h(2)RV (5′-TACGCAGACTCCGTGAAGGGCCGGTTC-3′) and 29-2h(3)FR (5′-TCCCTGGCCCCAGTAGTCAAAAGTATAATAACYTTTCGCACAGTAATATAC-3′), which incorporates mutations into CDR H3. The tail of fragment 1 overlaps the head of fragment 2. Fragment assembly was performed by PCR with an equimolar mixture of fragments 1 and 2, and primers M13RV and 29-2h(3)NotFR (5′-ATGATGATGTGCGGCCGCAAGCTTCGAGACGGTGACCAGGGTTCCCTGGCCCCAGTA-3′), which adds FR4 and a *Not*I restriction site to the PCR product. The PCR product was digested with restriction enzymes *Nco*I and *Not*I, and then inserted into the pIT2 phagemid. TG1 cells were transformed with the ligation product, and the resultant colonies were picked up and

shaken for 6–8 h at 37°C in 100 μl 2TYAG in a 96-well plate. By M13KO7 infection, the phage displaying V_H was produced as described above. The interaction between V_H mutants and V_L was measured by ELISA, and clones were sequenced with a DNA sequencer SQ-5500 (Hitachi, Tokyo).

Saturation Mutagenesis—Saturation mutagenesis of 29IJ2 V_H at position 95 was performed by a PCR megaprimer method (*15*). For the first PCR, the DNA fragment from FR1 to CDR H3 was amplified with primers M13RV and 29-2h(sat)FR (5′-GTAGTCAAAAGTATAATAMNNTTTCGCACAGTAATATAC-3′), which introduced saturation mutations at position 95. The PCR product was purified by agarose gel electrophoresis and used as megaprimers. For the second PCR, the megaprimer, a DNA fragment including FR4 of 29IJ2 V_H, and primer ReverseSEQ′ were used to amplify a DNA fragment including the 29IJ2 V_H gene, with a mutation at position 95, and then the PCR product was further amplified by PCR with primers M13RV and ReverseSEQ′ (5′-CCCTCATAGTTAGCGTAACGA-3′). The PCR product was digested with restriction enzymes *Nco*I and *Not*I, and then inserted into pIT2. The ligation product was used to transform the TG1 cells and the phage displaying V_H was produced as described above. The interaction between V_H mutants and V_L was measured by ELISA. To normalize the V_H/V_L interaction as to the expression level of each V_H mutant on the surface of the phage, the interaction between each V_H-phage and protein A, which specifically binds the correctly folded human V_H3 family (*16, 17*), was evaluated by ELISA. Among the 19 mutants produced, a mutant V_H with Cys95 was poorly displayed, as judged on protein A binding. This was probably because of its poor folding/secretion, so it was omitted from the following experiment.

BIAcore Analysis—Soluble V_H fragments were prepared as described for V_L fragment preparation. Purified 29IJ2 V_L was biotinylated with sulfo-NHS-biotin (Pierce, Rockford, IL), and then immobilized (~1,000 RU) on a SA5 sensor chip (Biacore, Tokyo) as specified by the manufacturer. Measurements with BIAcore 2000 were performed with a continuous flow of 30 μl/min of HBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.05% Tween-20) at 25°C. Kinetic analysis was performed with BIAevaluation 2.1 software (Biacore), and the equilibrium dissociation constant, K_d, was obtained by dividing k_{off} by k_{on}.

RESULTS

Analysis of V_H/V_L Interaction by Chain Shuffling—The four human anti-BSA antibodies used in this study recognize an identical epitope of BSA (data not shown), and exhibit identical amino acid sequences for FR1/2/3, CDR1, and a part of CDR2/3. They were selected by bio-panning against BSA from a phagemid library displaying single human frameworks for V_H (V3-23/DP-47 and JH4b) and Vk (O12/O2/DPK9 and Jk1), with side chain diversity incorporated at CDR2 and CDR3 of both chains (*14*). Their amino acid sequences differ by 18 residues, *i.e.* in parts of CDR2 and CDR3 (V_H: 11 residues, V_L: 7 residues) (Fig. 1). The antigen binding affinity of each scFv was examined by ELISA using phage-displayed scFv with the same titer, and confirmed to be similar (Fig. 2). To determine whether or

(a)

```
                 1    1    2    2    3    3    4    4    5     5    6    6
      1    5     0    5    0    5    0    5    0    5    0  a  5    0    5
29IJ1 EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSNIYASGDSTAYADSVKGR
29IJ2 EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSIYTAGADTSYADSVKGR
29IJ6 EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSHISPYGANTRYADSVKGR
13CG2 EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTIYYAGSNTYYADSVKGR
                                      CDRH1                CDRH2

                                                    1    1    1
      7    7    8         8    9    9    0    0    1
      0    5    0  abc    5    0    5    0    5    0
FTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGYYTFDYWGQGTLVTVSS
FTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSYYTFDYWGQGTLVTVSS
FTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGLRAFDYWGQGTLVTVSS
FTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGYYTFDYWGQGTLVTVSS
                                      CDRH3
```

(b)

```
                 1    1    2    2    3    3    4    4    5    5    6    6
      1    5     0    5    0    5    0    5    0    5    0    5    0    5
29IJ1 DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYYASKLQSGVPSRFSGSG
29IJ2 DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYSASYLQSGVPSRFSGSG
29IJ6 DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYRASLLQSGVPSRFSGSG
13CG2 DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYYASNLQSGVPSRFSGSG
                               CDRL1                CDRL2

                                              1    1
      7    7    8    8    9    9    0    0
      0    5    0    5    0    5    0    5
SGTDFTLTISSLQPEDFATYYCQQAGYSPTTFGQGTKVEIKR
SGTDFTLTISSLQPEDFATYYCQQADTSPTTFGQGTKVEIKR
SGTDFTLTISSLQPEDFATYYCQQGRDRPATFGQGTKVEIKR
SGTDFTLTISSLQPEDFATYYCQQSDTSPTTFGQGTKVEIKR
                               CDRL3
```

Fig. 1. **Sequences of four anti-BSA antibodies.** (a) and (b) represent the V_H and V_L sequences of the four anti-BSA antibodies (29IJ1, 29IJ2, 29IJ6, and 13CG2), respectively. Eighteen bold letters represent residues that are different among the four anti-BSA antibodies.

not the 18 amino acid difference influences the interaction between V_H and V_L, the V_H/V_L interaction of the four antibodies was evaluated by ELISA employing soluble V_L proteins immobilized *via* protein L, and phage-displayed V_H fragments. As shown in Fig. 3, one (29IJ2) of the four Fvs showed significantly lower V_H/V_L affinity, while the other three showed strong affinity both in the absence and presence of BSA.

To determine which of V_H and V_L of 29IJ2 is responsible for the weaker interaction, chain shuffling of the four pairs was performed. All sixteen pairs each comprising four V_H and V_L chains were examined as to the interaction between V_H and V_L by phage ELISA. As shown in Fig. 4, the pairs involving 29IJ1 V_H, 29IJ6 V_H, and 13CG2 V_H exhibited high affinity with any of the four V_Ls. On the other hand, the pairs having 29IJ2V_H exhibited weaker affinity with all V_Ls. Since V_H phages poorly interacted with protein L-coated wells without immobilized V_L, the interaction was confirmed to be specific. This suggests that the strength of the V_H/V_L interaction of these four Fvs depended solely on V_H, *i.e.* not on V_L. This led us to the assumption that the V_H amino acid sequence has a close relationship to the V_H/V_L interaction.

Determination of a Key Residue Responsible for the V_H/V_L Interaction—To identify the residue(s) that affects the V_H/V_L interaction, we focused on the difference in the V_H amino acid sequence between 13CG2 and 29IJ2, represen-

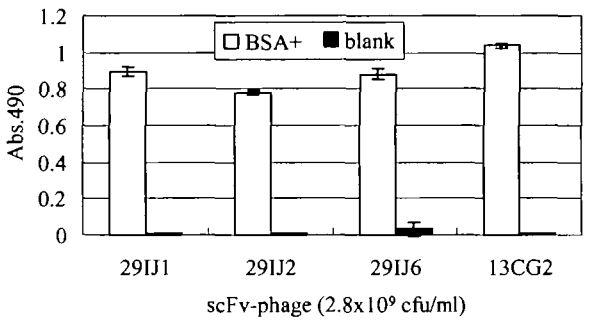

Fig. 2. **Binding of scFv-phages (2.8 × 10^9 cfu/ml) to BSA-plates measured by ELISA.** scFv-phages, 2.8 × 10^9 cfu/ml, were incubated with BSA immobilized on a microplate. *o*-Phenylenediamine was used for development.

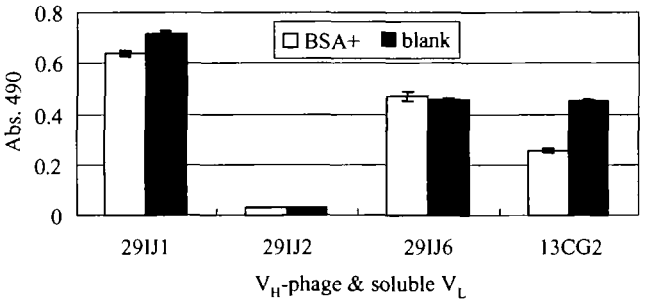

Fig. 3. **V_H/V_L interaction of four anti-BSA antibodies (29IJ1, 29IJ2, 29IJ6, and 13CG2).** Four V_H-phages, 1.5 × 10^{10} cfu/ml, were incubated with V_Ls of the original combination immobilized on a microplate precoated with protein L in the presence (100 μg/ml) or absence of BSA. *o*-Phenylenediamine was used for development.

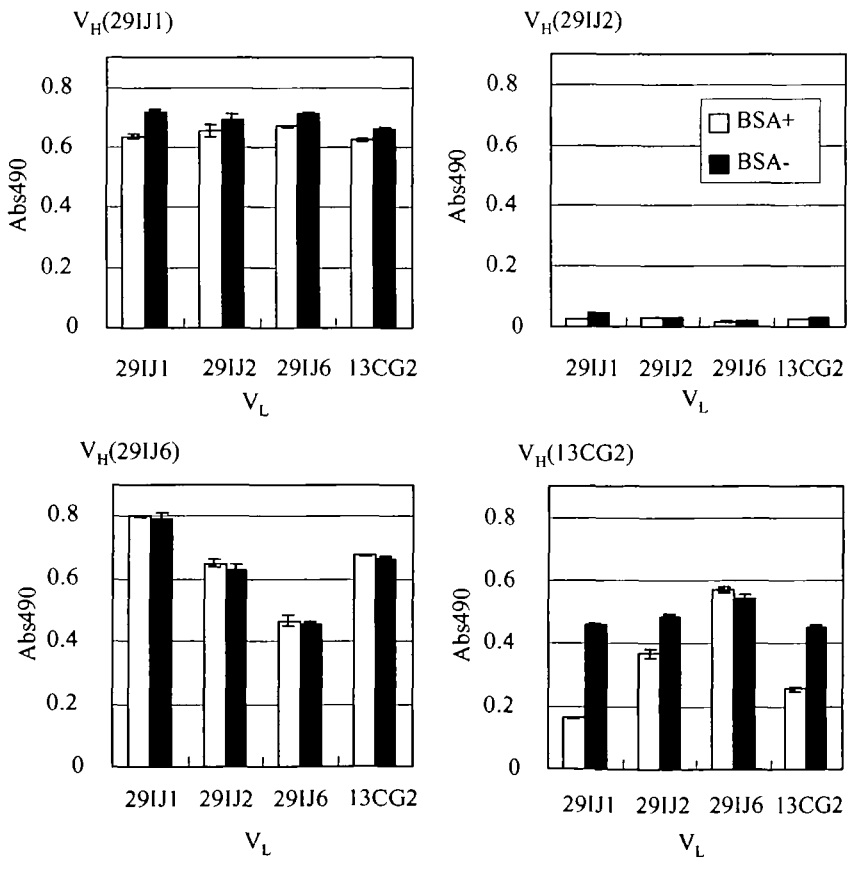

Fig. 4. **Binding of V_H-phages (1.5 × 10^{10} cfu/ml) to V_L-plates measured by ELISA.** Four V_H-phages were incubated with four V_Ls in all the combinations. Other conditions were the same as in Fig. 3.

tative V_Hs having high and lower affinity with V_L, respectively. The two V_Hs have different amino acids at a total of six positions, namely positions 50, 52a, 55, 56, and 58 in CDR H2, and position 95 in CDR H3, according to the Kabat numbering (*18*) (Fig. 1). To identify the residue(s) that affects the V_H/V_L interaction strength, a V_H-phage mini-library, which was designed to encode either a 13CG2 V_H-type or 29IJ2 V_H-type amino acid at every sixth position, was constructed.

Clones of phages displaying a mutant V_H protein were prepared, and the interaction of every V_H and V_L (of 29IJ2 and 13CG2) was evaluated by ELISA, bound phages being detected. Display of a V_H fragment was confirmed by control ELISA with immobilized protein A, which specifically binds human V_H3 (data not shown). Out of 19 samples whose nucleotide sequences were determined, 14 exhibiting absorbance values of less than 0.1 were classified as V_H with a weak V_H/V_L interaction, and the other 5 samples exhibiting ones of more than 0.1 classified as V_H with a strong interaction.

Table I summarizes the interaction strength, and amino acid residues at positions 50, 52a, 55, 56, 58, and 95 of every V_H mutant. All the mutants that have Gly at position 95, which is the 13CG2 V_H type, and exhibit a strong V_H/V_L interaction, and the types of residues at 50, 52a, 55, 56, and 58 have no apparent relationship to the V_H/V_L interaction. This clearly indicates that the residue at H95 determines the strength of the V_H/V_L interaction of these Fvs.

Saturation Mutagenesis of H95—To investigate the role of H95 in the V_H/V_L interaction in more detail, saturation mutagenesis of H95 was performed in the context of the weaker binder 29IJ2 V_H. Each phage-displayed V_H mutant was evaluated as to its interaction with 29IJ2 V_L by ELISA, along with its binding to protein A, as a control (Fig. 5). From the absorbance of wells with protein A, all the mutants other than S95C were confirmed to be displayed and correctly folded on the surface of the filamentous phage (data not shown). V_Hs that have Gly at position 95, 29IJ2 V_H (S95G) (G) and 13CG2 V_H (13G), exhibited the strongest interaction with 29IJ2 V_L. After Gly, V_Hs with Asp (D), Asn (N), and Glu (E) showed stronger interaction in that order. In addition, the decreased affinity of 13CG2 V_H with the

TABLE I. **Relationship between the V_H/V_L interaction and the amino acid sequences of V_H mutants.**

V_H/V_L interaction	Clone	50	52a	55	56	58	95
	13CG2 V_H	Thr	Tyr	Ser	Asn	Tyr	Gly
Strong	A1	...	Thr	Ala	Asp	...	...
	A3	Ser	...	Ala	Asp	...	...
	A12	...	Thr	Ala	...	...	...
	B2	...	...	Ala	...	...	...
	B5	Ser	...	...	...	...	...
	B9	Ser	Thr	...	...	...	...
	C1	Ser	Thr	...	Asp	...	...
	C3	...	Thr	...	...	...	...
	I1	...	...	...	...	Ser	...
	29IJ2 V_H	Ser	Thr	Ala	Asp	Ser	Ser
Weak	A7	...	Thr	...	Asp	...	Ser
	D7	...	Thr	...	...	...	Ser
	I2	...	...	...	...	...	Ser

Binding of V_H mutants to V_L was measured by ELISA. Only the residues different between 29IJ2 V_H and 13CG2 V_H (positions 50, 52a, 55, 56, 58, and 95) are presented. The V_H mutants were produced as described under "MATERIALS AND METHODS." "..." means the same amino acid as in 13CG2 V_H.

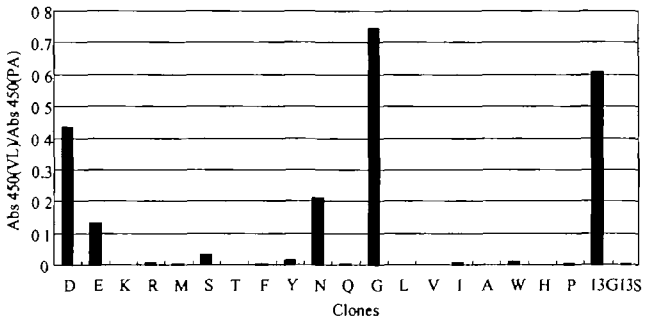

Fig. 5. **Normalized binding of 29IJ2 V_H mutant-displaying phages to 29IJ2 V_L as to its binding to Protein A measured by ELISA.** Each 29IJ2 or 13CG2 V_H mutant-displaying phage was incubated with 29IJ2 V_L or protein A immobilized on a microplate, and then their interaction was measured by phage ELISA. The data were normalized by dividing the Abs450 of a 29IJ2 V_L-coated plate by the Abs450 of a protein A-coated plate. Each letter (X) at the bottom represents the phage displaying the 29IJ2 V_H(S95X) mutant. '13G' and '13S' denote 13CG2 V_H and 13CG2 V_H(G95S), respectively. TMB was used for development.

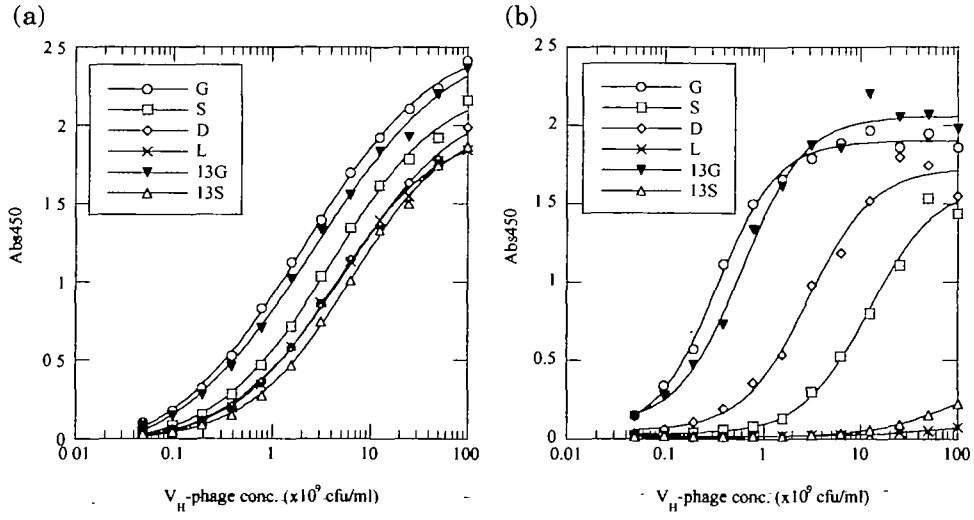

Fig. 6. **Dose-dependency of the interaction between V_H mutants and protein A (a) or 29IJ2 V_L (b).** Binding of the six V_H-phages (G, S, D, L, 13G, and 13S) to protein A or 29IJ2 V_L was evaluated by phage ELISA. Serially diluted V_H-phages were incubated with either immobilized protein A or 29IJ2 V_L immobilized through protein L on a microplate. Other conditions were the same as in Fig. 5.

TABLE II. **Relative affinity of selected V_H fragments displayed on a phage.**

Ligand		G	S	D	L	13G	13S
Protein A	ED_{50}	2.0	3.9	5.6	4.2	2.6	6.2
	R	0.9996	0.9991	0.9995	0.9996	0.9987	0.9995
29IJ2 V_L	ED_{50}	0.33	12	2.9	460	0.59	40
	R	0.9977	0.9953	0.9933	0.9948	0.9950	0.9999
	K_d (nM)	5.68 ± 0.20	—	—	—	9.43 ± 0.19	—

The concentration corresponding to 50% specific binding (ED_{50}) in 10^9 cfu/ml, and the corresponding R value according to curve fitting are shown. The curves were fitted to a four parameter logistic equation ($y = (a - d)/(1 + (x/c)^b) + d$) using Kaleida Graph 3.5 (Synergy Software, Reading, PA), where $y = A_{450}$; $a = A_{450}$ at low asymptote; b = slope factor; $c = ED_{50}$, and $d = A_{450}$ at high asymptote. The K_d value was obtained with BIAcore using a soluble V_H fragment. (—) not performed.

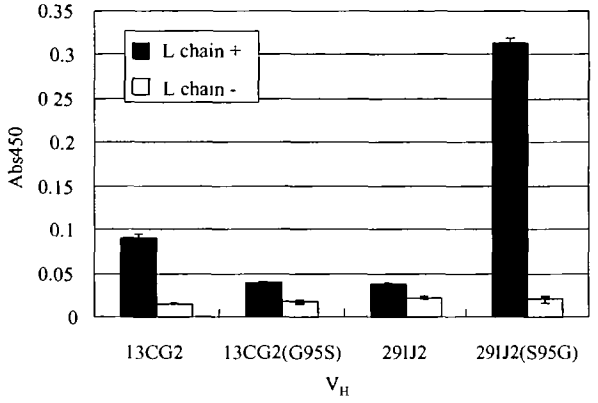

Fig. 7. **Binding of V_H-phages (1×10^{12} cfu/ml) to the human urine L chain measured by ELISA.** V_H-phages (13CG2 and 29IJ2, and their mutants as to position 95), 1×10^{12} cfu/ml, were incubated with urine-derived human L chain immobilized on a microplate precoated with protein L. TMB was used for development.

G95S mutation (13S) confirmed that the H95 also affects the V_H/V_L interaction in a different context. Also, analysis of the interaction between each 29IJ2 V_H mutant and 13CG2 V_L gave essentially the same result as with 29IJ2 V_L (data not shown).

While normalized as to protein A binding, the observed values might be influenced by the amounts and stability of the displayed fragments. To evaluate the effects of the mutation on these factors more precisely, the dose-dependency of the binding of selected V_H mutant phages to protein A and 29IJ2 V_L was investigated by ELISA (Fig. 6). The dose-dependent curves were fitted to a four parameter logistic equation [Howes, 1996 #33], and the titers of the phage corresponding to 50% specific binding (ED_{50}) were calculated. In Fig. 6a, some difference in signal probably due to that in the display level and the affinity to protein A among V_H mutants was observed. However, the ED_{50} of the interaction between each V_H phage and protein A was within threefold in titer for all the mutants, indicating a limited effect of the mutations on protein A binding (Table II).

On the other hand, the ED_{50} of the binding to 29IJ2 V_L was markedly influenced by the mutations (Fig. 6b and Table II). Compared with G, which showed the minimum ED_{50}, D, S(29IJ2 wt), and 13S showed 8.8-, 36-, and 121-fold increases in ED_{50}, respectively. Mutant L [29IJ2V_H(S95L)] showed a 1,400-fold increase, while a modest 2.1-fold increase was observed for protein A binding. These data again showed the remarkable effect of the H95 mutation on the V_H/V_L interaction, but much lower ones on V_H expression and folding.

The affinities of the two strongly interacting V_H fragments (G and 13G) to 29IJ2 V_L were determined by BIAcore analysis. Association rates, k_{on}, of 2.00 ± 0.10 and 0.81 ± 0.03 ($\times 10^5$/Ms), and dissociation rates, k_{off}, of 1.14 ± 0.02 and 0.76 ± 0.03 ($\times 10^{-3}$/s) were obtained for G and 13G, respectively. The ratio of the two equilibrium dissociation constants, K_d, obtained for G and 13G was in good agreement with the ED_{50} ratio, suggesting the reliability of the assays (Table II).

Interaction with the Human L Chain—To determine whether or not V_H with Gly95 exhibits high affinity also with other V_Ls, its interaction with the polyclonal human κ chain was examined. The four kinds of V_H fragments, 29IJ2 V_H(S95G), 13CG2 V_H(wt), 29IJ2 V_H(wt), and 13CG2 V_H(G95S), were used, the former two having Gly at position 95, and the latter two having Ser there. The human κ chain was immobilized on an ELISA plate precoated with protein L. When 100 μl each of V_H-phage (1×10^{12} cfu/ml) was added to the corresponding well and analyzed as to the interaction, V_Hs that have Gly at position 95 exhibited strong interaction with the human κ chain, compared to the V_Hs that have Ser at position 95, as in the case of 29IJ2 V_L (Fig. 7). This indicates that V_Hs that have Gly at position 95 with a framework (V3-23/DP-47 and JH4b) not only interact strongly with a particular V_L, but also with V_Ls with other framework/CDR sequences.

DISCUSSION

The aim of this study was to identify the residue(s) that determines the strength of the V_H/V_L interaction in a defined system. The four anti-BSA scFvs used here share one of the most commonly used framework sequences of human antibodies, and all exhibited the same specificity with similar affinity to the antigen, but with markedly different V_H/V_L affinities. Because the result obtained with monoclonal V_L was consistent with polyclonal L chains, the importance of the heavy chain CDR3 sequence, especially H95, in the V_H/V_L interaction is probably generalized. This was rather unexpected because in all the crystal structures of antibodies solved to date, H95 does not face the V_L interface, but is rather buried in the V_H or its sidechain interacting with the antigen. The preferred residues for the strong interaction observed here were compact residues, *i.e.* Gly, Asp, Asn, and Glu. However, a minimum alteration of Gly to Ala at this position markedly affected the interaction.

Kim *et al.* recently described that Gly at H95 caused structural diversity in the N-terminal strand of CDR H3 and afforded a variety of CDR H3 conformations (*19*). On

the other hand, in the immune response of C57BL/6 mice to (4-hydroxy-3-nitrophenyl)acetyl, antibodies with Gly at H95 exhibited consistently higher affinity with the antigen, which is probably related to their flexibility in the CDR H3 structure in the antigen-combining site and its influence on the angle between the V_H/V_L domains (*20, 21*). Considering the close location of CDR H3 and CDR L3, it is natural to think that the flexibility of CDR H3 influences not only the antibody/antigen interaction but also that of V_H/V_L. It is possible that the flexibility of a CDR-H3 loop caused by Gly at position 95 makes the loop come into contact more tightly with V_L, which in turn results in higher affinity to the antigen.

Smith and Xue pointed out that the first residue of the CDR H3 is predominantly Gly, while the amino acid at this position is inherently variable because of the junctional diversity that arises during V-D-J rearrangement (*22*). They also stated that the amino acid at position 95 second to Gly is Asp. In our study, Asp at position 95 also strengthened the interaction between V_H and V_L. For the development of the B cell lineage, whether or not the newly generated μ H chains in pre-B cells can associate with the surrogate L chain, which is composed of VpreB and λ5, is significant (*23*). Possibly, there is a mechanism by which B cells expressing V_H and exhibiting stronger interaction with V_L have advantages for the development of B cells over B cells expressing V_H and exhibiting weaker interaction with V_L, because of the structural similarity of surrogate and mature light chains. If so, further elucidation of a relationship between affinity maturation and the strength of interaction between V_H and V_L is certainly of interest.

In the case of a murine anti-HEL antibody, HyHEL-10, a very weak V_H/V_L interaction is markedly strengthened in the presence of the bridging antigen HEL (*4*). In the crystal structure of the HyHEL-10 Fv-HEL complex, the interaction across the imperfect V_H-V_L interface is mediated by 11 water molecules, thus these waters are thought to mediate a stabilized V_H/V_L interaction in the presence of HEL (*24*). On the other hand, in the crystal structure of stable D1.3 Fv or D1.3 Fv-HEL complex at 1.8 Å resolution, no water molecule is found in the V_H-V_L interface, while 23 and 48 water molecules are found in the free antigen combining site of Fv and the Fv:HEL interface, respectively (*25*). These results suggest the possibility that the degree of the perfection of V_H/V_L complementarity well correlates with the affinity between them. This in turn supports our hypothesis of increased CDR3 flexibility as a source of increased affinity. However, our hypothesis does not preclude the importance of other residues, especially those at the V_H/V_L interface. Further studies are needed to determine the relative importance of residues other than those in CDR2 or 3, especially framework residues. By investigating the V_H/V_L interaction of antibodies with other framework pairs, the relationship between the V_H/V_L interaction and their amino acid sequences will become clearer, and through analysis of V_H/V_L pairs at different B cell developmental stages, their immunological meaning will also be elucidated.

We wish to thank Ian Tomlinson for kindly providing the anti-BSA clones.

REFERENCES

1. Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., and Crea, R. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **85**, 5879–5883
2. Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.M., Lee, T., Pope, S.H., Riordan, G.S., and Whitlow, M. (1988) Single-chain antigen-binding proteins. *Science* **242**, 423–426
3. Better, M., Chang, C.P., Robinson, R.R., and Horwitz, A.H. (1988) *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041–1043
4. Ueda, H., Tsumoto, K., Kubota, K., Suzuki, E., Nagamune, T., Nishimura, H., Schueler, P.A., Winter, G., Kumagai, I., and Mohoney, W.C. (1996) Open sandwich ELISA: a novel immunoassay based on the interchain interaction of antibody variable region. *Nat. Biotechnol.* **14**, 1714–1718
5. Suzuki, C., Ueda, H., Tsumoto, K., Mahoney, W.C., Kumagai, I., and Nagamune, T. (1999) Open sandwich ELISA with V(H)-/V(L)-alkaline phosphatase fusion proteins. *J. Immunol. Methods* **224**, 171–184
6. Ueda, H., Kubota, K., Wang, Y., Tsumoto, K., Mahoney, W., Kumagai, I., and Nagamune, T. (1999) Homogeneous non-competitive immunoassay based on the energy transfer between fluorolabeled antibody variable domains (Open sandwich FIA). *Biotechniques* **27**, 738–742
7. Arai, R., Nakagawa, H., Tsumoto, K., Mahoney, W., Kumagai, I., Ueda, H., and Nagamune, T. (2001) Demonstration of a homogeneous noncompetitive immunoassay based on bioluminescence resonance energy transfer. *Anal. Biochem.* **289**, 77–81
8. Yokozeki, T., Ueda, H., Arai, R., Mahoney, W., and Nagamune, T. (2002) A sensitive homogeneous noncompetitive immunoassay for the detection of small haptens. *Anal. Chem.* **74**, 2500–2504
9. Glockshuber, R., Malia, M., Pfitzinger, I., and Pluckthun, A. (1990) A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* **29**, 1362–1367
10. Ueda, Y., Tsumoto, K., Watanabe, K., and Kumagai, I. (1993) Synthesis and expression of a DNA encoding the Fv domain of an anti-lysozyme monoclonal antibody, HyHEL10, in *Streptomyces lividans*. *Gene* **129**, 129–134
11. Suzuki, C., Ueda, H., Mahoney, W., and Nagamune, T. (2000) Open sandwich enzyme-linked immunosorbent assay for the quantitation of small haptens. *Anal. Biochem.* **286**, 238–246
12. Ito, W. and Kurosawa, Y. (1998) Characterization of Fv fragments expressed on phage surface. *J. Biochem.* **123**, 832–838
13. Polymenis, M. and Stollar, B.D. (1995) Domain interactions and antigen binding of recombinant anti-Z-DNA antibody variable domains. The role of heavy and light chains measured by surface plasmon resonance. *J. Immunol.* **154**, 2198–2208
14. de Wildt, R.M., Mundy, C.R., Gorick, B.D., and Tomlinson, I.M. (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat. Biotechnol.* **18**, 989–994
15. Sarkar, G. and Sommer, S.S. (1990) The "megaprimer" method of site-directed mutagenesis. *BioTechniques* **8**, 404–407
16. Potter, K.N., Li, Y., and Capra, J.D. (1996) Staphylococcal protein A simultaneously interacts with framework region 1, complementarity-determining region 2, and framework region 3 on human VH3-encoded Igs. *J. Immunol.* **157**, 2982–2988
17. Roben, P.W., Salem, A.N., and Silverman, G.J. (1995) VH3 family antibodies bind domain D of staphylococcal protein A. *J Immunol.* **154**, 6437–6445
18. Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., and Foeller, C. (1991) *Sequences of Proteins of Immunological Interest*, fifth ed., U.S. Government Printing Office, Bethesda
19. Kim, S.T., Shirai, H., Nakajima, N., Higo, J., and Nakamura, H. (1999) Enhanced conformational diversity search of CDR-H3 in antibodies: role of the first CDR-H3 residue. *Proteins* **37**, 683–696

20. Furukawa, K., Akasako-Furukawa, A., Shirai, H., Nakamura, H., and Azuma, T. (1999) Junctional amino acids determine the maturation pathway of an antibody. *Immunity* **11**, 329–338
21. Furukawa, K., Shirai, H., Azuma, T., and Nakamura, H. (2001) A role of the third complementarity-determining region in the affinity maturation of an antibody. *J. Biol. Chem.* **276**, 27622–27628
22. Smith, D.K. and Xue, H. (1997) Sequence profiles of immunoglobulin and immunoglobulin-like domains. *J. Mol. Biol.* **274**, 530–545
23. Melchers, F., Karasuyama, H., Haasner, D., Bauer, S., Kudo, A., Sakaguchi, N., Jameson, B., and Rolink, A. (1993) The surrogate light chain in B-cell development. *Immunol. Today* **14**, 60–68
24. Kondo, H., Shiroishi, M., Matsushimai, M., Tsumoto, K., and Kumagai, I. (1999) Crystal structure of anti-hen egg white lysozyme antibody (HyHEL-10) Fv-antigen complex. *J. Biol. Chem.* **274**, 27623–27631
25. Bhat, T., Bentley, G., Boulot, G., Greene, M., Tello, D., Dall'Acqua, W., Souchon, H., Schwarz, F., Mariuzza, R., and Poljak, R. (1994) Bound water molecules and conformational stabilization help mediate an antigen-antibody association. *Proc. Natl. Acad. Sci. USA* **91**, 1089–1093