J. Biochem. 128, 449–454 (2000)

Modulation of the Peptide-Binding Specificity of a Single-Chain Class II Major Histocompatibility Complex[1]

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Received April 26, 2000; accepted June 20, 2000

We designed and expressed a single-chain class II major histocompatibility complex molecule capable of forming a stable complex with an antigenic peptide. The peptide-binding preference of the single-chain (sc) human leukocyte antigen derived from DRB5*0101 (DR51) was determined to be similar to that of the authentic one, which requires a bulky hydrophobic residue at position-1 (P1) as a primary anchor. For modulation of the peptide-binding affinity, we modified binding pocket 1 of sc DR51 by site-directed mutagenesis. The relative binding affinity of the engineered sc DR51 for several P1-substituted peptides was measured by competition assaying with a fluorescence labeled peptide. The sc DR51 molecule showed high affinity to the self-peptide derived from myelin basic protein, 87–98 with Phe as the P1 residue (F90F). While reduction of pocket 1 volume (βG86V) decreased the affinity of F90F, it rather increased the affinity of the Ala-substituted peptide as to the P1 residue (F90A). Through more extensive engineering in the peptide-binding groove of the sc DR51 molecule, it is expected that we can construct sc DR51 variants with various peptide ligand motifs.

Key words: class II MHC, HLA-DR, peptide-binding affinity, pocket engineering.

Major histocompatibility complex (MHC) proteins are heterodimeric glycoproteins that display a highly diverse set of peptides on the cell surface and serve as restricting elements for the cell-mediated immune system (*1*). Class II MHC proteins on the surfaces of B lymphocytes, dendritic cells, and macrophages present antigenic peptides to the T-cell receptor (TCR) of $CD4^+$ lymphocytes. Class II allelic variants can bind a wide variety of peptides, 10–34 residues with an average length of 15–18 amino acids (*2*). The crystal structures determined for different class II MHC proteins in complexes with defined peptides have led to a clear understanding of the mode of binding of high-affinity peptides (*3–6*). The conformations of bound peptides in class II MHC proteins are essentially identical in the various crystal structures. Peptides bind in an extended conformation similar to a polyproline type II helix, with several side chains in pockets within the site that determines the peptide sequence specificity (*7–9*). It was known that peptide-binding is accommodated by five polymorphic pockets on the surface of the HLA-DR1 molecule (*8*). Each of these pockets can accommodate a single amino acid residue when a particular peptide is bound. Accordingly, these pockets play a major role in determining the peptide-binding specificity of class II MHC molecules. In particular, pocket 1, the largest and deepest pocket, has been reported to play vital roles in peptide-binding and subsequent recognition by TCR.

Class II MHC molecules have been purified from detergent extracts of lymphocyte membranes (*10*) or as recombinant proteins using baculovirus and bacterial expression systems (*11–14*). These two-chain, four-domain molecular complexes, after loading with selected peptide epitopes, have been demonstrated to interact with T cells in an antigen-specific manner (*15–18*). However, due to their size, their heterodimeric structure, and the presence of multiple disulfide bonds, class II MHC molecules present an inherent difficult *in vitro* folding problem for large-scale production and further biochemical analysis. To overcome these obstacles, a trial at the construction of a single-chain class II MHC molecule with only an antigenic peptide-binding domain has been performed (*19*).

Efforts to determine class II allele-specific binding motifs have involved sequence alignment of eluted peptides (*20–22*), phage display libraries (*23, 24*), and extensive binding assays with synthetic peptides (*25–28*). The two HLA-DR alleles isotypically expressed on HLA-DR15Dw2-positive cells, DRB5*0101 (DR51) and DRB1*1501 (DR2) molecules, exhibit a number of differences in polymorphic residues of the β-chain, including the Gly-Val-dimorphism at position β86. The DR51 molecule requires a bulky hydrophobic residue (F or Y) at position-1 (P1) as a primary anchor. For the DR2 molecule, a nonaromatic, hydrophobic anchor (L, V, or I) at P1 was preferred (*29*). This different requirement as to the primary anchor is presumed to be due to the difference in the pocket 1 volume with the Gly-Val-dimorphism at position β86.

For the production of a simple MHC class II molecule

[1] This work was supported by a grant for Brain Korea 21 founded by the Ministry of Education of Korea.

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Abbreviations: HLA, human leukocyte antigen; MHC, major histocompatibility complex; TCR, T-cell receptor; PBS, phosphate-buffered saline, 10 mM phosphate, 150 mM NaCl (pH 7.4 if not otherwise indicated); PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography.

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capable of forming a stable complex with an antigenic peptide, we have manipulated DR51 (DRA, DRB5*0101) into a single polypeptide consisting of only α1 and β1 domains by using a peptide linker. Using the constructed sc DR51 molecule, we analyzed the peptide-binding preference by competitive binding assaying. To modulate the binding affinity of a P1 residue–substituted peptide we engineered binding pocket 1 of the sc DR51 molecule, intensively at the position β86 residue.

MATERIALS AND METHODS

Cloning, Construction, and Expression of DR51 Variants—Total cellular mRNA was isolated from human lymphoblastoid cell line GMO6825A (Coriell Institute of Medical Research, NJ), and cDNA was synthesized by standard protocols. Oligonucleotide primers (DRα-forward, 5′-TCT GTG CAT ATG ATC AAA GAA GAA CAT GTG ATC ATC-3′, and DRα-reverse, 5′-TCA CTA AGC TTA GTT CTC TGT AGT CTC TGG GAG-3′ for the α chain, and DRβ-forward, 5′-TCT GTG CAT ATG GGG GAC ACC CGA CCA CGT TTC TTG-3′, and DRβ-reverse, 5′-TCA CTA AGC TTA CTT GCT CTG TGC AGA TTC AGA-3′, for the β chain) for a polymerase chain reaction were used for subcloning into expression plasmid pET21a from Novagen Inc. Restriction sites for *Nde*I and *Hin*dIII are denoted by underlining, respectively. sc DR51 was constructed by inserting the synthetic oligonucleotide coding the peptide linker (GGGGSIQGRISGGGGS, 16 amino acids) between the C-terminus of the β1 domain and the N-terminus of the α1 domain. The constructed plasmid expressing the sc DR51 molecule was transfected into the expression host, *E. coli* BL21 (DE3). A culture was grown at 37°C in Luria-Bertani broth containing 100 μg/ml ampicillin. Protein expression was induced in mid-log growth by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. After 3 h culture cells were harvested for inclusion body preparation.

Mutagenic Oligonucleotides and Site-Directed Mutagenesis—The mutagenic oligonucleotides used to generate the mutant constructs are shown in Table I. Site-directed mutagenesis was performed using a QuickChange™ site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). In brief, a nonidentical duplicate of the original vector is produced by polymerase chain reaction-like amplification using *Pfu* polymerase and primers containing the desired mutation. The parental template is then digested specifically by the restriction enzyme *Dpn*I, which only cuts dam-methylated DNA (target sequence, 5′-Gm6ATC-3′). The nicked vector DNA incorporating the desired mutations is transformed into *E. coli*. Reaction parameters were chosen according to the manufacturer's recommendations. All mutant constructs were confirmed by sequencing with the Sanger dideoxy chain termination reaction for double-stranded DNA.

Purification of Recombinant sc DR51s—Cell pellets were resuspended in ice-cold PBS containing 20 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid, followed by sonication for 3 × 20 s of the cell suspension cooled in a salt/ice/water bath. The cell suspension was then centrifuged and the supernatant fraction was poured off. The inclusion bodies of the recombinant proteins were dissolved in 20 mM ethanolamine, pH 10, containing 6 M urea and 20 mM dithiothreitol overnight. The recombinant proteins of interest were purified and concentrated by FPLC ion-exchange chromatography using Q-Sepharose Fast Flow media (Pharmacia Biotech, Piscataway, NJ) and a gradient of NaCl. All purified proteins were stored at –70°C before *in vitro* folding.

In Vitro Folding of sc DR51s—The purified sc DR51 molecules were thawed, and then dialyzed against 20 mM ethanolamine, pH 10, containing 5 mM reduced glutathione and 0.5 mM oxidized glutathione for *in vitro* folding at a final concentration of 50 μM. After *in vitro* folding for 48 h at 4°C, the folding mixture was dialyzed against PBS at 4°C and then concentrated by centrifugal ultrafiltration with Centricon-10 (Amicon, Bervery, MA). For purification to homogeneity, a final step was included involving gel filtration chromatography on a Superose 12 HR 10/30 column (Pharmacia Biotech).

Synthesis and Fluorescence Labeling of Binding Peptides—The HLA-DR51 binding peptide, MBP fragment (Ac-VHFFKINVTPRT, myelin basic protein 87–98), and F90 substituted peptides (Table II) were synthesized by the standard solid phase method using side chain-protected Fmoc [*N*-(9fluorenyl)methoxycarbonyl] amino acids with an automated peptide synthesizer, Applied Biosystems 431A (Applied Biosystems, Foster City, CA). The deprotected crude peptides were purified by reverse-phase HPLC (Thermo Separation Products, San Jose, CA). The homogeneity and identity of the purified peptides was confirmed by mass spectrometry (PerSeptive Biosystems, Foster City, CA). The synthesized peptides were labeled at the N-terminus with the fluorescence dye, fluorescein (PanVera, Madison, WI).

Size-Exclusion Chromatography for the Competitive Peptide-Binding Assay—The folded sc DR51s (1 μM) were coincubated with the fluorescein-labeled peptide (10 μM) and the competitor peptide (100 μM) in PBS with a protease inhibitor mixture (7 μM pepstatin, 200 μM PMSF, and 5 μM leupeptin). After incubation for 24 h at 37°C, size exclusion chromatography was performed on a Superose 12 HR 10/30 column. The fluorescence intensity at the sc DR51 and peptide complex peak (excitation at 490 nm and emis-

TABLE I. **Mutagenic oligonucleotides for site-directed mutagenesis.**

Name	Nucleotide sequence
βG86A-Forward	5′-AAC TAC GGG GTT GcT GAG AGC TTC ACA-3′
βG86A-Reverse	5′-TGT GAA GCT CTC AgC AAC CCC GTA GTT-3′
βG86W-Forward	5′-AAC TAC GGG GTT tGG GAG AGC TTC ACA-3′
βG86W-Reverse	5′-TGT GAA GCT CTC CCa AAC CCC GTA GTT-3′
βV85G-Forward	5′-CAC AAC TAC GGG GgT GGT GAG AGC TTC-3′
βV85G-Reverse	5′-GAA GCT CTC ACC AcC CCC GTA GTT GTG-3′

Mismatches with the template are indicated by lowercase letters.

TABLE II. **Amino acid sequences of binding peptides and side chain volumes at the P1 residue.**

Peptide	Amino acid sequence	P1 side chain volume (Å³)
F90F[a]	VHF**F** KNIVTPRT	189.9
F90A	VHF**A** KNIVTPRT	88.6
F90W	VHF**W**KNIVTPRT	227.8
F90H	VHF**H** KNIVTPRT	153.2

[a]An eluted self-peptide derived from myelin basic protein (87–98). The substituted residue at P1 is shown in bold type.

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sion at 516 nm) was detected with a fluorescence de-tection system (Waters, Milford, MA). Competition was calculated from fluorescence signals in absence (F_0) and presence (F_c) of the competitor: % competition = $(F_0 - F_c)/F_0 \times 100\%$.

Fluorescence Polarization for the Peptide-Binding Assay—Serially diluted sc DR51s were co-incubated with the fluorescein-labeled peptide (1 nM) in PBS with a protease inhibitor mixture (7 μM pepstatin, 2 μM PMSF, and 5 μM leupeptin). After incubation for 24 h at 37°C, the fluorescence polarization was measured three times with Beacon® 2000 (PanVera).

RESULTS AND DISCUSSION

Design of sc DR51—Cloned DRA and DRB5*0101 were conformed by sequencing with the Sanger dideoxy chain termination reaction for double-stranded DNA, respectively. For the construction of an sc DR51, we selected the site between the C-terminus of the β1-domain and the N-terminus of the α-domain for fusion. The distance between the two termini was about 19 Å. No steric hindrance was expected on linking with the designed peptide linker. The three-dimensional structure of the designed sc DR51 was predicted using the DR1 (DRA and DRB1*0101) crystal structure (Protein Data Bank code, 1aqd) as a template (*30*). When the primary sequences of DRB1*0101 and DRB5*0101 were aligned, 86 of 96 amino acids within the β1-domain were found to be identical (89.6%). These modeling studies and the nature of the side-chain interaction predicted that the antigen-binding domain would remain stable in the absence of the α2 and β2 domains. Figure 1 shows the full amino acid sequence of the designed sc DR51.

Expression and Purification of sc DR51—All sc DR51 constructs showed a good yield with the T7-driven expression system, being found exclusively in insoluble fractions (approximately 70–100 mg/liter). The insoluble inclusion bodies were washed extensively, dissolved in 6 M urea, and then further purified by denaturing anion exchange chromatography. The isolated proteins were substantially pure, as judged on SDS-PAGE (*31*).

In Vitro Folding and Biochemical Characterization—After removing urea by dialysis and gel filtration, we obtained a soluble and homogeneous form of the sc DR51 molecule. The final yield of sc DR51 molecules was approximately 7–10 mg/liter. The presence of the native disulfide bond between cysteines β15 and β79 was demonstrated by a gel shift assay in which identical samples with and without the reducing agent, dithiothreitol, were boiled 5 min prior to SDS-PAGE. In the absence of dithiothreitol the disulfide bond is retained and proteins typically move through acrylamide gels faster due to their more compact structure (Fig. 2).

Peptide-Binding to sc DR51 Molecules—Antigenic peptides are presented to CD4⁺ T cells by MHC class II molecules *via* a highly polymorphic peptide–binding groove. It was reported that a class II MHC molecule undergoes a defined conformational change upon binding to a peptide, which can be observed experimentally as a decrease in

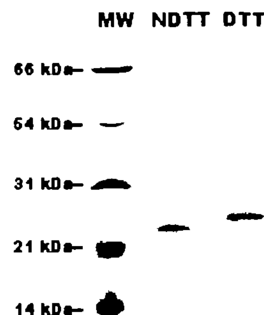

Fig. 2. **Detection of the native disulfide bond between cysteines β15 and β79.** The formation of a disulfide bond in the folded and purified sc DR51 molecules was confirmed by SDS-PAGE, with (DTT) or without (NDTT) the addition of a reducing agent, dithiothreitol, before gel analysis.

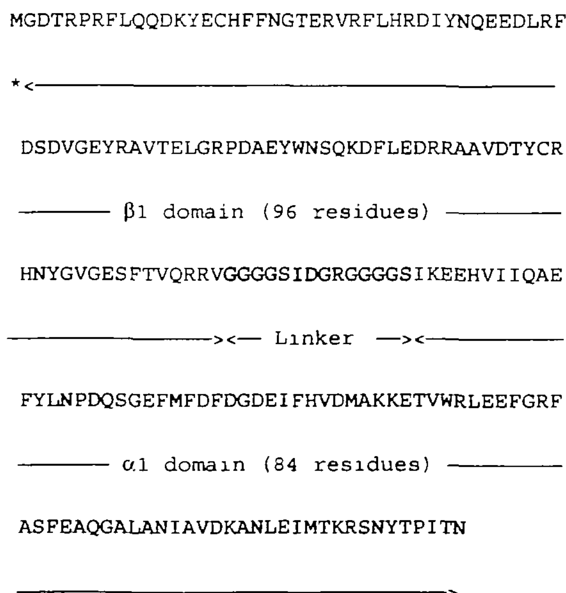

Fig. 1. **Construction of the sc DR51 molecule.** The first residue denoted by an asterisk originated from the expression vector.

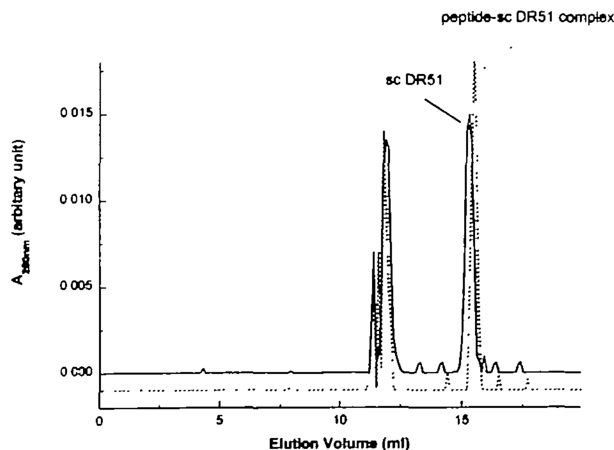

Fig. 3. **Gel filtration traces of empty sc DR51 and the peptide-sc DR51 complex.** sc DR51 and a 50-fold molar excess of a binding peptide (F90F) were incubated at 37°C for 48 h in PBS with a protease inhibitor mixture. Gel filtration was performed on a Superose 12 HR column in PBS. Empty sc DR51 and the peptide-sc DR51 complex are denoted by solid and dotted lines, respectively.

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hydrodynamic radius (*32*). It was known that DR51 exhibits high affinity to myelin basic protein fragment 87–98 (F90F). Through alanine substitution analysis it was demonstrated that DR51 requires a bulky hydrophobic residue, F90, as the P1 residue for binding with myelin basic protein fragment 87–98 (*29*). We assumed that our engineered sc DR51 molecule should have a similar peptide-binding groove to that of the authentic DR51 molecule. We examined the binding peptide (F90F) as to its ability to induce this conformational change in sc DR51 using a gel filtration assay. Complexes of the peptide (F90F) and sc DR51 exhibited decreased apparent molecular weights and a sharpened elution profile relative to the empty protein (Fig. 3). Some aggregates were detected after incubation. This strongly supported that the F90F peptide binds to a sc DR51 molecule in a specific manner like the authentic DR51 molecule. The increase in conformational compactness was very similar to that induced by peptide binding to the complete soluble domain of class II MHC (*32*). Furthermore, it suggests that a major conformational change through peptide-binding had occurred within the peptide binding domain of the DR51 molecule.

Engineering for Alteration of the Peptide-Binding Affinity—One of the requirements we deduced for peptide-binding to DR51 molecules is the existence of a bulky hydrophobic residue at P1 as a primary anchor. It is presumed that glycine at position 86 of the β-chain endows pocket 1 with a deep and wide space. For modulation of the peptide-binding affinity, we introduced a mutation into the peptide-binding groove of sc DR51, which is expected to change the pocket 1 volume. The three-dimensional model of each mutant was built using program Insight II (Molecular Simulation, San Diego). Table III shows the variation of the pocket 1 volume on modification in the mutant sc DR51 molecules, which was calculated with CAST, www server (*33*).

Peptide-Binding Affinity of sc DR51s—For each sc DR51 molecule, we measured the relative binding affinity as to the synthesized peptides, including myelin basic protein fragment (F90F) and P1-substituted peptides, by size-exclusion chromatography. The binding proportion of the wild sc DR51 molecule was about 55% when a 10-fold molar excess of fluorescence labeled F90F was added. The binding proportion was calculated as the intensity of fluorescence using the fluorescence labeled peptide as a standard. Through competitive binding assaying, it was found that the wild-type sc DR51 has a similar peptide binding preference to that demonstrated for the authentic DR51 molecule. The wild-type sc DR51 showed the strongest affinity

TABLE III. **Expected change of the pocket 1 volume.**

sc DR51	Pocket 1 volume variation ($Å^3$)
Wild-type	0
βG86A	−28.5
βG86V	−79.9
βV85G	+79.9

A model of each mutant was built using program Insight II (Molecular Simulation, San Diego) The pocket 1 volume of each mutant was calculated with www server, CAST (http://sunrise.cbs.umn.edu/cast/).

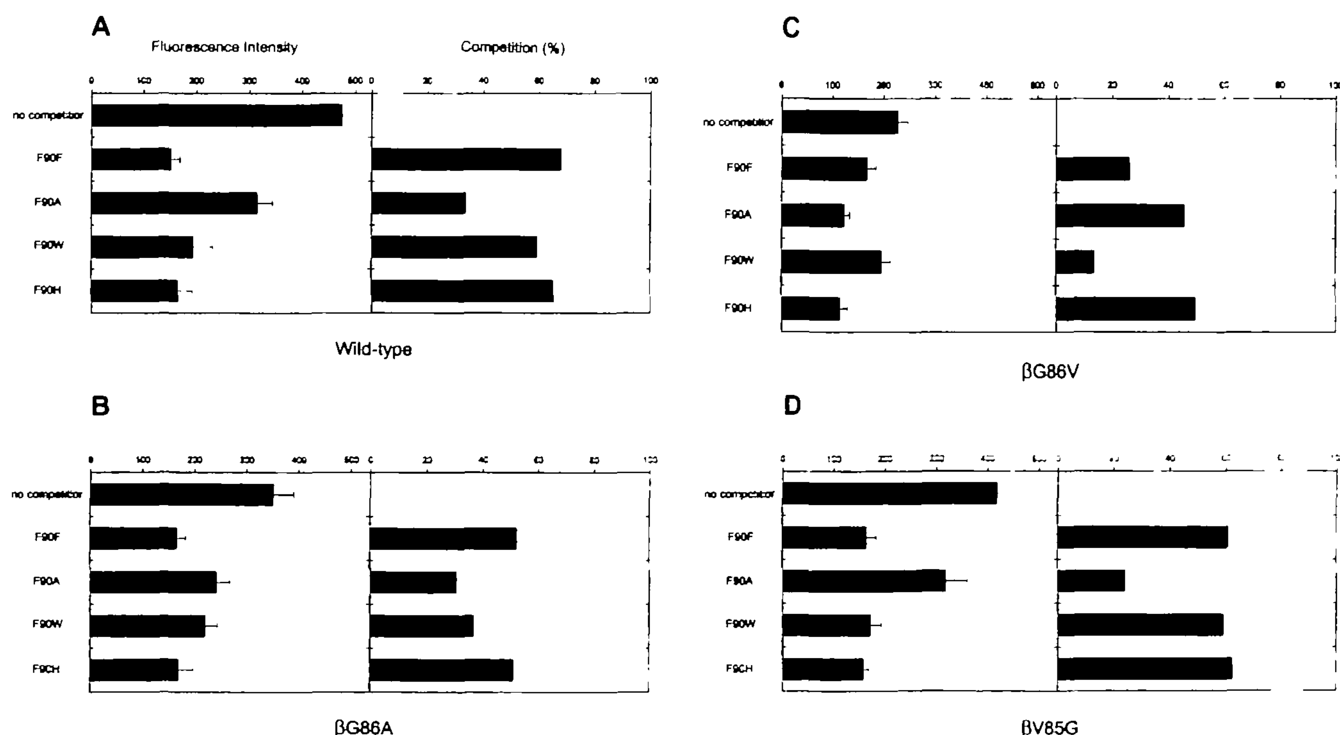

Fig. 4. **Analysis of the peptide-binding to each sc DR51 mutant through size-exclusion chromatography using fluorescein-labeled myelin basic protein fragment (85–98) as a competitor peptide.** (A) Wild-type, (B) βG86A, (C) βG86V, and (D) βV85G. Competition was calculated from fluorescence signals in the absence (F_o) and presence (F_c) of the competitor: % competition = $(F_o - F_c)/F_o \times 100\%$. Three independent experiments for each peptide were performed. The results of the competition assay are presented as the arithmetic mean.

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to the F90F peptide, which is an eluted self-peptide in HLA-DR51 (Fig. 4A). This preference supports that the peptide-binding groove of the sc DR51 molecule is similar to that of the authentic DR51 molecule. The F90A peptide, which has a small aliphatic residue, Ala, as the P1 residue, showed a drastic decrease in affinity to the sc DR51 molecule. The slight decrease in affinity to the sc DR51 molecule of the F90W peptide suggests that there is conformational flexibility to accommodate a larger side chain than that of Phe for the P1 residue. The affinity of the F90H peptide showed no significant decrease compared to that of F90F. It is expected that for F90H binding to the sc DR51 molecule the H90 residue may be used as the P1 residue because the other anchoring residues, I93 and R97, must also contribute to the peptide-binding. In particular, the positively charged R97 residue may form a salt bridge with D at position 30 in the β chain. These results suggest that the P1 side chain volume and pocket 1 space are major keys for DR51 and peptide-binding.

The relative binding affinities of βG86A and βV85G mutants for all tested peptides were slightly decreased. However, their preference-patterns were similar to that of the wild-type. Although we expected that the βV85G mutant would have a deeper and wider pocket 1, and increased affinity to F90W, there was no significant change in the binding affinity of F90W. In the case of βG86V, while the relative binding affinities for peptides with a bulky side chain at P1 (F90F and F90W) were decreased, the affinities for peptides with a small side chain at P1 (F90A and F90H) were rather increased. These alterations of binding affinity were presumed to be due to the reduction of the pocket 1 volume (Table III). This ligand motif difference between the wild-type and βG86V is similar to that experimentally detected between two-chain class II molecules derived from DRB5*0101 and DRB1*1501, in which the Gly-Val-dimorphism at position β86 gave a different requirement for a primary anchor (*29*). These results strongly support that an acceptable space in pocket 1 for the P1 side chain volume is critical in binding between a peptide and the DR51 molecule. Through more extensive engineering in the peptide-binding groove of sc DR51, it is expected that we can construct sc DR51 variants with various peptide ligand motifs.

For quantitative analysis of binding affinity, we used the fluorescence polarization technique. Saturation for peptide-binding was not fully achieved due to protein aggregation at high concentration. Bovine serum albumin (BSA) was used as a negative control to demonstrate that this assay system actually detects specific binding. The calculated dissociation constant (K_d) for the wild-type sc DR51 and F90F binding was determined to be 166 nM by fitting a single site, sigmoidal curve to the data shown in Fig. 5. The dissociation constant for the full domain of DR1 and the tightly binding peptide was in a similar range to our result (*34*). This indicates that the sc DR51 molecule retains its high-affinity to F90F. The binding affinity of sc DR51 and F90A (K_d = 353 nM) was decreased compared to that of sc DR51 and F90F, an original self-peptide. The binding affinity of βG86V for F90F (K_d = 467 nM) was drastically decreased compared to that of the wild-type sc DR51. K_d for βG86V and F90A binding was 258 nM. The binding preference of βG86V was changed, that is, βG86V preferred F90A which had a small residue at P1. These results well agreed with those of competitive binding assaying involving size-exclusion chromatography.

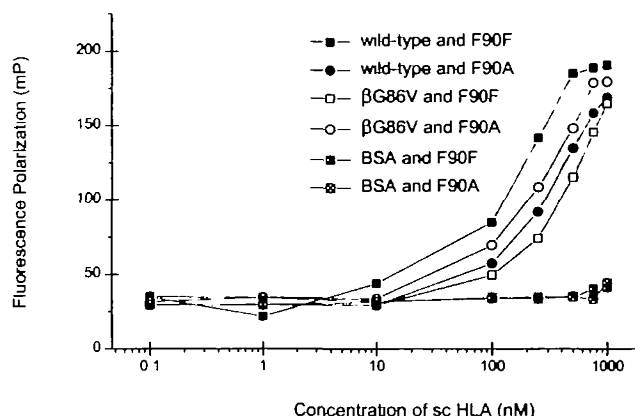

Fig. 5. **Binding affinity between sc DR51s and peptides, determined using fluorescence polarization.** The polarization value *versus* the concentration of sc DR51 was plotted using Beacon®2000 (Panvera). 31.7 mP for all free peptides and 191.2 mP for all bound peptides were used for all calculations. The dissociation constant (K_d) was calculated by fitting a single site, sigmoidal curve to the experimental data.

From the above results, we could deduce that the constructed sc DR51s have similar binding groove and peptide-binding conformations to those of the whole DR51 molecule. Through engineering of pocket 1 of sc DR51, it was found that an acceptable space in pocket 1 for the P1 side chain is critical in binding between a peptide and the DR51 molecule. It has been reported that the soluble MHC class II molecules in association with an antigenic peptide can recognize TCRs of $CD4^+$ T cells and induce antigen-specific apoptosis in a T cell clone (*35*). Thus, it is presumed that our designed sc DR51 molecule can recognize T cells in an antigen-specific manner. Furthermore, it is expected that exchange of the binding peptide in sc DR51 would make it possible to construct a modulator for autoimmune responses.

The authors wish to thank the Korea Basic Science Institute (KBSI) for the FP analysis.

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