Allele Specificity of Structural Requirement for Peptides Bound to HLA-DRB1*0405 and -DRB1*0406 Complexes: Implication for the HLA-associated Susceptibility to Methimazole-induced Insulin Autoimmune Syndrome

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Summary

Self-peptides bound to HLA-DR4 (DRA–DRB1*0405 complex) were eluted from the purified DR4 complex, fractionated on reverse-phase HPLC, and subjected to NH_2-terminal sequencing. Seven independent sequences were obtained, and all putative peptides synthesized bound to DRB1*0405 as well as DRB1*0406 complex, which differ only at DRβ residues 37, 57, 74, and 86. Binding assay using analogue peptides of a DR4 binder GSTVFDNLPNPE revealed that FxxLxN is an important anchor motif necessary for binding (where x is any amino acid), which was common to DRB1*0405 and 0406. Determination of the binding affinity of 60 synthetic AAFAALANAA-based analogue peptides showed that substituting F to W or C; L to F, W, or Y; and N to Q or S on AAFAALANAA changed the affinity substantially between DRB1*0405 and DRB1*0406. It is noteworthy that all patients with methimazole-induced insulin autoimmune syndrome are positive for DRB1*0406 and negative for DRB1*0405. Interestingly, the quantitative structural motif identified in this study predicted that ^{8}TSICSLYQLE^{17} of human insulin α chain may bind specifically to DRB1*0406 using its ^{10}IxxLxQ^{15} motif. Indeed, DRB1*0406 complex bound ^{8}TSICSLYQLE^{17} with a high affinity, and in striking contrast, DRB1*0405 complex did not. Furthermore, a short-term T cell line specific to human insulin established from a DRB1*0406-bearing individual did show reactivity with a peptide fragment containing the ^{10}IxxLxQ^{15} motif. Although this fragment probably exists at a very low level under normal physiological conditions due to the disulfide bond between flanking cysteine residues (^{6}Cys-^{11}Cys), a reducing compound such as methimazole may cleave the disulfide bond in vivo and allow DRα–DRB1*0406 complex on antigen-presenting cells to bind much of the linear fragment of insulin α chain, which may lead to the activation of self-insulin–specific T-helper cells.

Recognition of peptide fragments in the context of class II MHC molecules by T cells is a central event in the development of immune responses. Proteolytic fragments of peptides processed by APC that match the physicochemical character of the peptide-binding grooves formed by α and β chains of class II MHC molecules are expressed on the surface of APC and are recognized by T cells (1). However, the peptide-binding grooves of most stable MHC molecules are occupied by self-peptides (2), and most T cells are tolerized or ignorant against these peptides except in autoimmune conditions.

The amino acid residues in the grooves of the HLA class II molecules are highly polymorphic, indicating that genetic polymorphism accounts for the wide spectrum of peptides capable of binding. Among the HLA class II genes, DRB1 coding for DRβ chains has the highest degree of polymorphism, and appears to be responsible for variations in the immune responses of different individuals to different antigens. Among these, DR4 has 14 subtypes designated DRB1*0401 through DRB1*0414 with slight differences in the structure of the peptide-binding groove, thus allowing detailed analyses of peptide–DR interactions.

DRB1*0405 is the most common DR4-associated subtype (30% in antigen frequency) among Japanese, yet it is also unique to Orientals. In Caucasians, the most common DR4 subtype is DRB1*0401 which differs from DRB1*0405 at

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only two residues (57th and 71st) on the β chain, and is strongly associated with rheumatoid arthritis (RA)[1] among both Japanese and Caucasians. However, in the Japanese, the relative risk of RA associated with DRB1*0405 is higher than with DRB1*0401. Furthermore, DRB1*0401, but not DQ genes in linkage disequilibrium, reportedly play a major genetic role in the development of RA (3). On the contrary, DRB1*0406, another DR4 subtype unique to Orientals that differs from DRB1*0405 at four residues, does not confer susceptibility to RA but rather to insulin autoimmune syndrome (IAS) (4).

The structural motif of peptides capable of binding to HLA-DR have been determined based on non-self-peptide sequences capable of stimulating T cells in the context of DR, and on self-peptide sequences eluted from purified DR complexes with (5–8) or without (9, 10) peptide–DR binding assay. Phage peptide libraries have also been used to identify HLA-binding motifs (11, 12). Some of these reports suggested that only certain residues are involved in allele-specific peptide binding, whereas the other residues are promiscuous (9, 12).

In the current study, we not only detail the elution, purification, and sequencing of naturally processed self-peptides bound to purified DR4 (DRB1*0405) complex, but also quantitatively demonstrate a structural motif of the DR4 (DRB1*0405 and DRB1*0406)-binding peptides. Moreover, through binding assays using a series of polyalanine-based peptides with single amino acid substitutions, we identified the conserved residues for the allele-specificity and promiscuity of peptide–DR4 interactions. Based on these data, allele-specific interactions between DR4 subtypes and peptide fragments derived from human insulin, an autoantigen recognized by T cells in IAS are analyzed.

Materials and Methods

Cells and HLA Typing. HLA–DR4 complexes were isolated from EBV-transformed human B lymphoblastoid cell lines EBWa and KT13 (13) homozygous for HLA-DRB1*0405 or DRB1*0406, respectively. The cell line was maintained in vitro at 37°C, in 5% CO_2 by culture in RPMI 1640 (GIBCO BRL, Gaithersburg, MD), containing 10% heat-inactivated FCS, 2 mM glutamine, and antibiotics. HLA–DR alleles of the B lymphoblastoid cells as well as PBMC donors used for a generation of an insulin-specific T cell line were determined by investigating hybridization between HLA-DR genes amplified by polymerase chain reaction and sequence specific oligonucleotide probes, as described elsewhere (14).

Isolation of HLA-DR4. Cells were lysed in 1% NP-40/PBS, containing 5 mM sodium orthovanadate, 25 mM iodoacetamide, and 1 mM PMSF as described elsewhere (15). The supernatants of the resulting homogenate were precleared first by normal mouse γ-globulin–bound Sepharose and second by protein A–bound Sepharose. The effluent was applied for affinity chromatography with a mAb HU-4 (anti-DR IgG2a; 13, 16), which was previously shown to precipitate DRαDRβ(B1) but not DRαDRβ(B4) complex (13). The HU-4 column was made by using ImmunoPure IgG Orientation Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. The column was washed sequentially with 30 bed volumes of 0.5% NP-40, 0.1% SDS/PBS, and 15 bed volumes of 1% *n*-octyl-glucoside/PBS. The DR–peptide complex was eluted with 1% *n*-octyl-glucoside, 50 mM diethylamine, and 0.15 M NaCl, at pH 10.5, immediately neutralized with half volume of 0.1 M Tris and 0.15 M NaCl at pH 6.8 (17), and then precipitated by acetonitrile at a final concentration of 80%. Seven cycles of purification with a total of 20 g (10^{10} cells) of EBWa cells yielded a total of 900 μg of purified DR4 molecules as determined by SDS-PAGE. These DR preparations did not exhibit visible bands at dye fronts on 11% SDS-PAGE. The DRB1*0406 complex was purified from KT13 cells in the same manner.

Separation of Self-peptides and DR Molecules. Dried acetonitrile precipitates were dissolved in 2.5 M acetic acid and incubated for 30 min at 37°C. The solution was centrifuged in a Centricon-10 (10-kD cut-off; Amicon Corp., Danvers, MA) to separate DR4 and oligopeptides. Centrifugation was repeated twice with graded additions of 2.5 M acetic acid to increase the yield of self-peptides, and the filtrate was concentrated on a Speed Vac (Savant Instrument Inc., Farmingdale, CA) to 100 μl. The buffer of the acid-treated DR fraction was exchanged to 1 mM PMSF, 0.05% NP-40, 5% DMSO, and 0.15 M NaCl in 50 mM phosphate buffer, pH 7.0, by repeated addition of the buffer and centrifugation, until the pH value of the filtrate remained at 7.0. The DR preparation in a neutral buffer thus obtained was either immediately used for DR–peptide binding assay in the presence of protease inhibitor cocktail or kept frozen at −80°C until assay.

Purification of Self-peptides on HPLC. The acid-eluted crude self-peptide fraction obtained from DRB1*0405 complex was loaded onto a 2.1 × 150 mm C18 reverse-phase HPLC column (Waters Assoc., Milford, MA) equilibrated with 0.06% TFA and 5% acetonitrile in water. The column was eluted at a flow rate of 0.25 ml/min at room temperature in a gradient of increasing acetonitrile concentration in 0.052% TFA with the absorption at 214 and 280 nm (A_{214} and A_{280}) continuously monitored.

Sequence Analyses and Peptide Synthesis. Sequencing by Edman degradation was performed in a pulsed liquid sequencer (477A) equipped with an on-line phenylthiohydantoin amino acid analyzer (120A; both models from Applied Biosystems, Foster City, OR). All peptides used for the binding assay were synthesized by a solid-phase simultaneous multiple peptide synthesizer (PSSM-8; Shimadzu Corp., Kyoto, Japan) based on the Fmoc strategy. All peptides were purified by C18 reverse-phase HPLC. Purified peptide 0405BP3 was sequenced to confirm its structure.

DR–Peptide Binding Assay. The DR–peptide binding assay was performed as described elsewhere with minor modifications (18). Briefly, purified DR was incubated for 48 h with 20 nM ^{125}I-labeled peptides in a total volume of 50 μl, and in some experiments, with various doses of various unlabeled peptides, in the presence of a protease inhibitor cocktail. Purified peptides were radioiodinated using Iodobeads (Pierce Chem. Co.,). The solution used for incubation was: 1 mM PMSF, 1.3 mM 1.10 phenanthroline, 73 μM pepstatin A, 8 mM EDTA, 6 mM *N*-ethyl maleimide, 200 μM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 0.05% NP-40, 5% DMSO, and 0.15 M NaCl in 50 mM phosphate buffer at pH 7.0. The DR–peptide complexes were separated from free peptides by gel filtration on a Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ) column (4 × 120 mm) equilibrated with 0.5% NP-40, 0.1% NaN_3/PBS, and the fractions were assayed for ra-

[1] *Abbreviations used in this paper:* IAS, insulin autoimmune syndrome; IC_{50}, concentration for 50% inhibition; MIF, migration inhibitory factor; PKM2, pyruvate kinase, M2 isozyme; RA, rheumatoid arthritis; 0405BP, DRB1*0405 complex binding peptide.

dioactivity. The fraction of peptide bound to DR molecules was calculated as the ratio of peptide in the void volume to the total peptide recovered, as described by O'Sullivan et al. (18).

Binding Inhibition Assay. Peptide inhibitors were added to DR molecules at the same time as radioiodinated 0405BP3. Peptide inhibitors were typically tested at concentrations ranging from 500 μM to 50 nM. The data were then plotted, and the concentration which yielded 50% inhibition (IC_{50}) of binding was determined.

Generation of a Human T Cell Line Specific to Insulin. PBMC (5×10^5/ml) of a DRB1*0406-bearing individual were pulsed with 50 μM of human recombinant insulin (I-0259; Sigma Chemical Co., St. Louis, MO) in the presence of 5×10^{-5} M of 2-mercaptoethanol overnight at 37°C in 5% CO_2/95% of air with humidified atmosphere. The stock solution of insulin was prepared first by adding 5 mM of 2-mercaptoethanol to reduce insulin. These cells were then washed and incubated (1×10^5/well) in a 96-well flat-bottomed culture plate. 7 d after stimulation, cells were fed with insulin-pulsed, irradiated autologous PBMC (1×10^5/well) in the presence of recombinant human IL-2 (50 U/ml) and IL-4 (10 U/ml). Cells were incubated for 7 d after feeding and the T cell blasts were transfered to a 24-well plate to be expanded by feeding and incubation for 7 d. To investigate a proliferative response to insulin peptides of the T cell line, viable T cell blasts were separated by Ficoll-Paque (Pharmacia Fine Chemicals) density gradient and incubated (3×10^4/well) for 72 h with irradiated autologous PBMC (1.0×10^5/well) and soluble synthetic peptides (5 μM) in a 96-well plate, in the presence of [^{3}H]thymidine during the final 16-h period. Cells were then harvested and subjected to liquid scintillation counting.

Results

*Purification and Sequencing of Self-peptides Eluted from HLA-DR4 (DRB1*0405).* Self-peptides eluted from 900 μg of purified DR4 (DRB1*0405) were separated by reverse-phase HPLC. The resulting chromatogram (Fig. 1) showed marked clustering of signals between 60 and 80 min, which correspond to 22 and 32% acetonitrile concentrations in the eluates, respectively. The experiment was repeated with another 900 μg of purified DR preparation, and a similar pattern was obtained. Edman degradation of these fractions yielded eight definitive sequences long enough for a homology search (Table 1). Of these eight sequences, peaks 4, 5, 6, 7, and 8 were identified as fragments of CD23, CD20, migration inhibitory factor (MIF), pyruvate kinase, and CD23, respectively, by homology search with the SWISS-PROT database R26.0 (Table 1). Peak 8 was presumably derived from CD23 closer to the amino terminus than peak 4. The origins of the other three peptides were not identified. Because of limited quantity of peptides used for sequencing, all eight peptides tended to show unidentifiable low signals towards the COOH termini; therefore, it seems likely that all peptides were longer than shown in Table 1.

Detection of Peptide–DR Binding by Inhibition Assay. Three peptides (PKM2p99-117 [SDPILYRPVAVALDTKGPE], 0405BP3 [GSTVFDNLPNPEIDGDYYGW], and 0405BP1 [VPIQRAVYQNVVVNN]) were selected for the direct binding assay because of the presence of Tyr residues for radioiodination, and the following results were obtained (data not shown): (*a*) all three peptides were tested for binding with DRB1*0405 and DRB1*0406 complexes, and exhibited 10–20% of DR-binding activity at pH 7.0, whereas an irrelevant peptide (NELSGEAHKDALGKLY) did not show any significant binding; (*b*) the binding affinity of these peptides to DR4 complexes at pH 4 was lower than that at pH 7; and (*c*) of the three synthetic peptides, 0405BP3 exhibited the highest percent binding to both the DRB1*0405 and DRB1*0406 complexes. To this binding assay system, various concentrations of either unlabeled 0405BP3 peptide or an irrelevant peptide were added and the percent binding inhibition (percent inhibition) was determined. As shown in Fig. 2, the coexistence of unlabeled 0405BP3 competitively inhibited the binding of iodinated 0405BP3 to the DR4 complexes in a dose-related manner, whereas the irrelevant peptide did not.

*Eluted Self-peptides All Bind to the DRB1*0405 and DRB1*0406 Complexes.* By using the competitive inhibi-

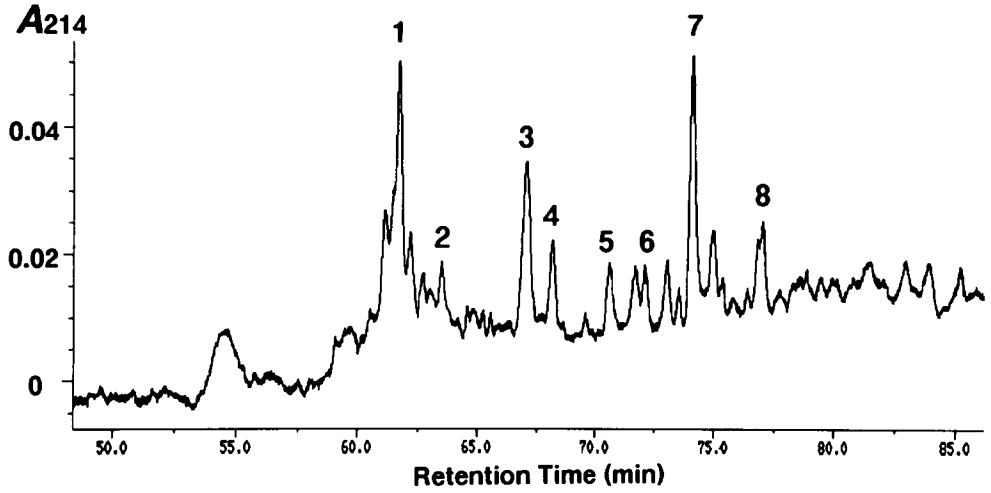

Figure 1. HPLC chromatogram with crude mixture of DRB1*0405-binding peptides. Acid-eluted small peptide fraction was separated on C18 reverse-phase HPLC with A_{214} continuously monitored. Retention times of 60 and 80 min correspond to 22 and 32% acetonitrile in eluates, respectively.

Table 1. *Amino Acid Sequences of Eluted Peptides and Their Homology*

Peak number	Sequence* (upper) and homologous peptide (lower)	
1	VP I QRAVYQNVVVNNPXD	
2	SPGTGAYYVLLN	
3	GSTVFDNLPNPE I DGDYYGW	
4	XGQLVS I NN	
	E------HSPEEQDFLTKHA	(CD23p195-214)
5	GPKPLFRRM	
	---------SSLVGPTQSFF	(CD20p26-45)
6	XXXQY I AVHVVPDQT	
	GKPP----------LMAFG	(MIFp32-51)
7	SDP I LYRPVAVALD	
	----------TKGPE	(PKM2p99-117)
8	XEGQLVS I	
	-------HSPEEQDFLTKHA	(CD23p195-214)

* Eight peaks shown in Fig. 1 were sequenced by Edman degradation. Peak numbers correspond to those in Fig. 1. Lower line of each peak number indicates the most homologous sequence in the SWISS-PROT database R26.0 followed by a protein name and residue number. (-) Indicates a residue identical with that obtained by sequencing.

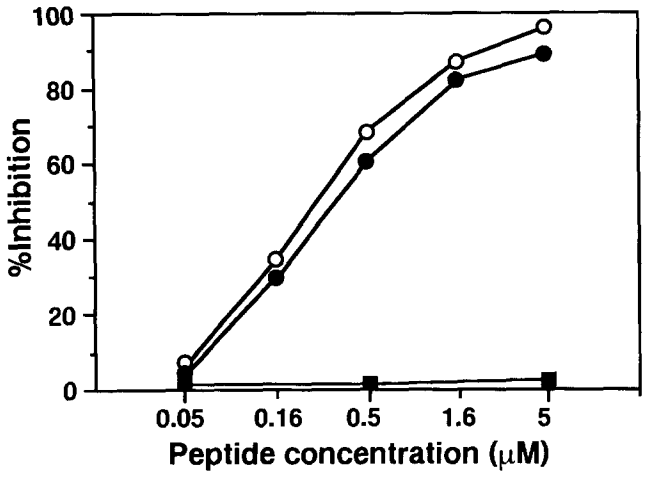

Figure 2. Detection of peptide-DR4 binding by inhibition assay. The DRB1*0405 complex (*open circle*) or DRB1*0406 complex (*closed circle*) were incubated for 48 h with various concentrations of unlabeled 0405BP3 (GSTVFDNLPNPEIDGDYYGW) in the presence of radioiodinated 0405BP3 (20 nM). Peptides bound to DR4 complexes were quantitated as the total radioactivity in the void volume on a Sephadex G-50 column. Percent inhibition relative to the radioactivity obtained in the absence of competitor peptide was determined. An irrelevant peptide (NELSGEAHKDALGKLY) tested as a competitor revealed nearly identical results either with DRB1*0405 or with DRB1*0406 (*closed square*).

tion assay thus established, we tested if isolated self-peptides bind to the DR4 complexes. In addition to the 0405BP3 peptide shown in Fig. 2, 0405BP1, PKM2p99-117, CD20p26-45, CD23p195-214, MIFp32-51, and 0405BP2 (SPGTGAYYVLLN) were incubated with the DRB1*0405 complex in the presence of radioiodinated 0405BP3. The results shown in Table 2 clearly show that all seven peptides competitively inhibited the binding of labeled 0405BP3 peptide to the DRB1*0405 complex (59–103% inhibition), whereas the irrelevant peptide did not (1% inhibition). Thus, these data strongly suggest that all the peptides shown in Table 1 were natively bound to the peptide-binding groove of the DRB1*0405 complex, and were not co-purified contaminants. Furthermore, these peptides showed affinity not only to the DRB1*0405 from which they were originally derived, but also to the DRB1*0406 complex. However, the affinity of 0405BP1, 0405BP2, PKM2p99-117, and MIFp32-51 to the DRB1*0406 complex were significantly lower than that to the DRB1*0405 complex, suggesting heterogeneity in peptide binding between these two DR4 complexes.

*Binding of Single Amino Acid–substituted DR4 Binder to the DRB1*0405 and 0406.* We then truncated the carboxy terminus of the 0405BP3 peptide and identified by titration a 12 mer (GSTVFDNLPNPE) that binds to the DRB1*0405 complex as efficiently as the original 0405BP3 peptide (titration shown in Fig. 4). This also indicates that iodinated Tyr residues on 0405BP3 are not involved in binding to the DR4 complex. To identify the structural motif of the DR4-binding peptides, we synthesized a series of nonconservatively modified peptides of the truncated 0405BP3 with the hydrophilic residues on ^{1}GSTVFDNLPNPE^{12} substituted to Ala, and at position 2, hydrophilic Ser was substituted to Val because of the structural similarity between Ala and Ser. To avoid introducing side chains too large to fit in the groove or charged side groups generating repulsive interactions, hydrophobic residues were substituted to small neutral hydrophilic Ser residues. As shown in Fig. 3 *a*, only two nonconservative substitutions (F to S and L to S) completely eliminated peptide binding to the DRB1*0405 complex. In addition, while substitution of 10th N to A inhibited binding by 70%, the adjacent substitution of P to S inhibited the binding only slightly. Furthermore, exactly the same pattern was obtained by using the DRB1*0406 complex (Fig. 3 *b*). These results revealed a putative structural "backbone" of - - - -F- - L-N(P)-, which plays a crucial role in the binding of ^{1}GSTVFDNLPNPE^{12} to the DR4 complexes.

Competition Assay with Polyalanine Design Peptides. To determine which residues are required for high affinity binding to the DR4 complexes, we synthesized a polyalanine-based 10-mer analogue (AAFAALANAA) of 0405BP3, and tested its affinity to the DRB1*0405 and the DRB1*0406 complexes. As shown in Fig. 4, the polyalanine-based peptide affinity to both DRB1*0405 (Fig. 4 *a*) and DRB1*0406 (Fig. 4 *b*) complexes was equal to that of the truncated 0405BP3 (GSTVFDNLPNPE). The same results were obtained with a 12-mer analogue, AAAAFAALANAA (not shown). The results confirmed that the affinity of these peptides are the same as the original 0405BP3 (GSTVFDNLPNPEIDG-

Table 2. *Binding of Eluted Self-peptide to DRB1*0405 and DRB1*0406 Complexes*

Unlabeled synthetic peptide as an inhibitor*	Percent inhibition for binding to DRB1*0405	Percent inhibition for binding to DRB1*0406
Irrelevant peptide (NELSGEAHKDALGKLY)	1	2
0405BP1 (VP I QRAVYQNVVVNN)	103	70
0405BP2 (SPGTGAYYVLLN)	93	85
0405BP3 (GSTVFDNLPNPE I DGDYYGW)	97	96
PKM2p99-117 (SDP I LYRPVAVALDTKGPE)	90	71
CD20p26-45 (GPKPLFRRMSSLVGPTQSFF)	90	96
CD23p195-214 (EGQLVS I HSPEEQDFLTKHA)	59	63
MIFp32-51 (GKPPQY I AVHVVPDQTMAFG)	94	69

* Purified DR4 complexes were incubated for 48 h with 20 nM of ^{125}I-0405BP3 with or without various unlabeled peptides (20 μM). The DR–peptide mixtures were then separated on a Sephadex G-50 column. Total radioactivity of peptide bound to DR molecules was obtained by subtracting the corresponding chromatography fractions run in the absence of DR. Percent inhibition was calculated based on the total radioactivity obtained in the absence of competitive inhibitors.

DYYGW) shown in Fig. 2. These results indicate that: (*a*) the FxxLxN (where x is any amino acid) motif plays a crucial role in the binding of 0405BP3 peptide to both the DRB1*0405 and DRB1*0406 complexes; and (*b*) the possible minor compartment of 11th proline (Fig. 3) could be replaced by alanine.

To identify other structural motifs, 60 peptides with substitutions of the F, L, or N residues of AAFAALANAA to R, K, H, E, D, Q, N, T, S, C, G, A, V, L, I, F, M, W, Y, P, or hydroxyproline were synthesized and evaluated by binding inhibition assay with DRB1*0405 and 0406 complexes. For quantitative evaluation, the IC50 for each peptide was determined, where a lower value indicates better binding. As shown in Fig. 5 *a*, substitutions of the first anchor caused marked variation in IC50 values. Thus, only hydrophobic residues showed detectable binding to the DRB1*0405 or 0406 complex. With Trp in this position, the IC50 for DRB1*0405 was less than 10% of that for DRB1*0406. Conversely, hydrophilic residues at the first anchor could not bind to either DR4 complex except for Cys which had a weak affinity to the DRB1*0405 complex. Although substitutions to hydrophobic residues exhibited the highest affinity at the second anchor, certain nonbasic hydrophilic amino acids also showed affinity (Fig. 5 *b*). Among these nonbasic hydrophilic residues in the second anchor, neu-

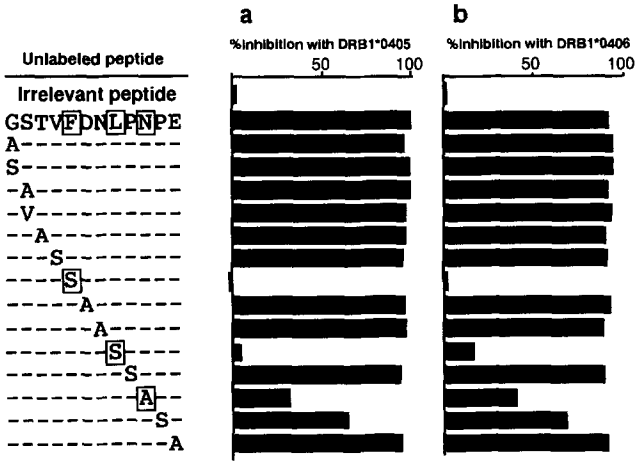

Figure 3. Binding of single amino acid-substituted DR4 binder to the DRB1*0405 (*a*) and DRB1*0406 (*b*) complexes. Peptides with single amino acid substitutions based on truncated 0405BP3 (GSTVFDNLPNPE) were synthesized and tested on a binding inhibition assay at 5 μM. (–) Indicates a residue without substitution. Larger percent inhibition indicates better binding to DR4. Substitutions yielding less than 50% inhibition (i.e., IC_{50} more than 5 μM) and corresponding residues on the original peptide are boxed.

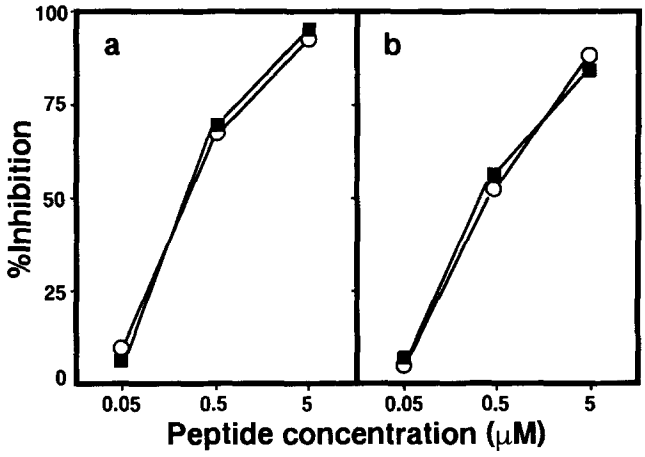

Figure 4. Competition assay with polyalanine design peptides. DRB1*0405 (*a*) and DRB1*0406 (*b*) were incubated with various concentrations of GSTVFDNLPNPE (*closed square*) and AAFAALANAA (*open circle*) in the presence of radioiodinated 0405BP3, and percent inhibition was determined.

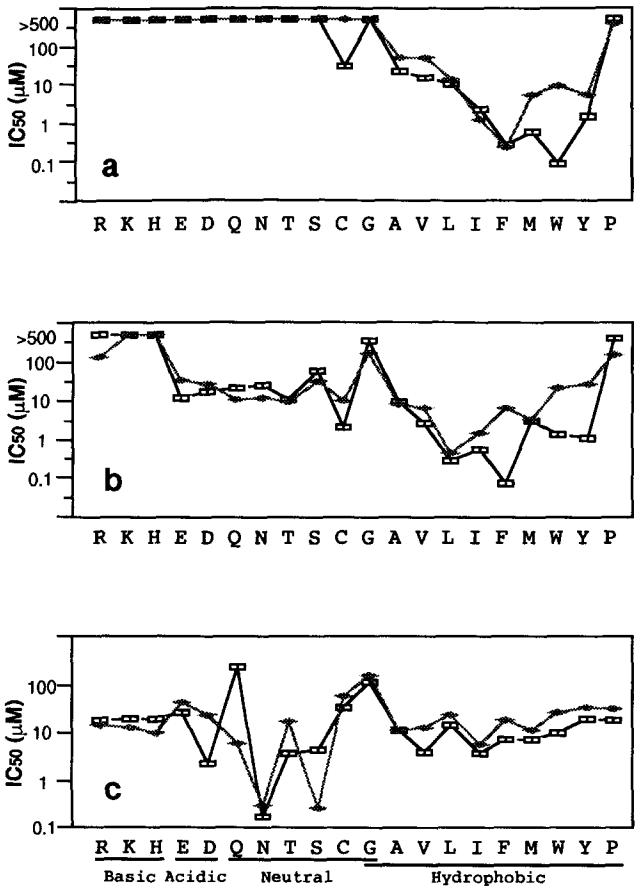

Figure 5. Binding spectrum of AAFAALANAA-based single amino acid–substituted peptides. (*a*) The first anchor "F" of AAFAALANAA was substituted to various amino acid residues (*abscissa*) and their IC_{50} values (μM) against the binding of ^{125}I-0405BP3 with DRB1*0405 (*open rectangle* with *solid line*) and DRB1*0406 (*shaded diamond* with *shaded line*) complexes were determined. A smaller IC_{50} indicates better binding. (*b*) The second anchor "L" of AAFAALANAA was substituted and the same assay was performed. (*c*) The third anchor "N" of AAFAALANAA was substituted and the same assay was performed.

tral Ser had the lowest affinity, which was comparable to ^{8}Leu to ^{8}Ser substitution of ^{1}GSTVFDNLPNPE^{12} shown in

Table 3. *Binding of Polyalanine-based Peptides to DR4*

Synthetic peptide as a competitor*	IC50 with DRB1*0405	IC50 with DRB1*0406
AAFAAMASAA	3.6	1.1
AALAAIASAA	11.0	8.8
AAYAALANAA	1.4	5.0
KAAYAAVAVAA	5.1	12.2
AAKAAKAGAA	>500	>500

* Polyalanine-based peptides were incubated at various concentrations with DR4 complexes in the presence of ^{125}I-0405BP3 (20 nM) and IC_{50} values were determined. KAAYAAVAVAA was derived from 0405BP1, MIFp32-51, and PKM2p99-117. AAFAAMASAA, AALAAIASAA, and AAYAALANAA were derived from CD20p26-45, CD23p195-214, and 0405BP2, respectively.

Fig. 3. Phe, Trp, or Tyr bound preferentially to DRB1*0405. The third anchor was more promiscuous than either the first or second anchor (Fig. 5 *c*). Thus, although the IC_{50} of the most favored (N and S for DRB1*0405 and 0406, respectively) and least favored (Q and G for DRB1*0405 and 0406, respectively) substitutions differed by as much as several hundredfold, all the substitutions, including basic hydrophilic residues, showed detectable binding to both DR4 complexes. Interestingly, unlike the other anchors, residues such as Q or S revealed significantly higher binding with DRB1*0406 (IC_{50} less than 10% of 0405). When substituting the third anchor to hydrophobic residues, Lys was inserted in the amino terminus of the peptide to increase its solubility. This insertion does not create a new binding motif because lysine in the first anchor had no affinity to the DR4 complexes (Fig. 5 *a*). Hydroxyproline exhibited practically the same levels of affinity as proline at all three anchor positions (not shown).

Binding of Polyalanine-based Peptides Based on Eluted Self-peptide Sequences. The above quantitative structural motif for the DR4 binders allowed us to identify the putative amino acid residues important for binding on the eluted DRB1*0405-

Table 4. *Binding of Insulin-derived Synthetic Peptides to DR4*

Insulin-derived peptide*	IC50 with DRB1*0405	IC50 with DRB1*0406
K-Insαp1-10 (KGIVEQCCTSI)	>500	>500
KK-Insαp8-17 (KKTSICSLYQLE)	180	4.1
K-Insαp12-21 (KSLYQLENYCN)	10.5	12.0
K-Insβp1-18 (KFVNQHLCGSHLVEALYLV)	>500	>500
K-Insβp11-30 (KLVEALYLVCGERGFFYTPKT)	15.0	14.5

* Human insulin-derived synthetic peptides were incubated at various concentrations with DR4 complexes in the presence of ^{125}I-0405BP3 (20 nM) and IC_{50} values (μM) were determined. One to two residues of Lys were inserted at the amino termini to increase the solubility.

binding peptides listed in Table 1. Thus, possible motifs of the DRB1*0405-binding peptides (except 0405BP3), are AAFAAMASAA, AALAAIASAA, AAYAALANAA, and AAYAAVAVAA. These motifs were synthesized and tested by binding inhibition assay. Since the putative motif of AAYAAVAVAA for 0405BP1, MIFp32-51, and PKM2p99-117 was not soluble, lysine was inserted at the NH_2 terminus (KAAYAAVAVAA) to increase the solubility. Indeed, as demonstrated in Table 3, all the polyalanine-based peptides bound to both DR4 complexes whereas AAKAAKAGAA, which was predicted from Fig. 5 to show null binding, had no affinity. This also indicates that there is no detectable binding with the AxxAxA motif. The YxxVxV and YxxLxN motifs did not show very high affinity to the DRB1*0406 compared with DRB1*0405 complex, confirming the results shown in Table 2 and Fig. 5. Thus, Tyr at the first anchor reduces the affinity to DRB1*0406.

*Identification of Human Insulin Fragment That Binds Preferentially to DRB1*0406.* Using the above structural motifs for the DR4 binders, we searched for a human insulin fragment with a higher affinity to the DRB1*0406 than to the DRB1*0405 complex, since such a fragment may be recognized by T cells of patients with IAS. As shown in Fig. 5, such a motif is likely to have Q or S as its third anchor. Interestingly, the α chain does have an IxxLxQ motif which contains a cysteine residue. To confirm allele specificity of the binding, ^{8}TSICSLYQLE^{17} and other insulin fragments were synthesized and tested for binding affinity to the DR4 complexes. Table 4 shows the IC_{50} values for these peptides with DRB1*0405 and 0406. As predicted by the quantitative structural motif, TSICSLYQLE has the highest affinity to the DRB1*0406 complex, with an IC_{50} value 44 times smaller than that for the DRB1*0405. Since this insulin fragment forms a disulfide bond between flanking cysteine residues (^{6}α), reducing agents such as methimazole may linearize this fragment by cleaving the disulfide bond. Insulin βp11-30 and αp12-21 could also bind with lower affinities both to DRB1*0406 and to 0405, but the motif in this location ($^{\beta24}$FxxIxK$^{\beta29}$ and $^{\alpha13}$LxxLxN$^{\alpha18}$, respectively) did not contain cysteine residues (Fig. 6). No other insulin fragments bound to either DR4 complex.

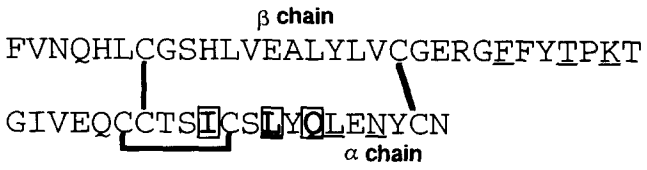

Figure 6. DRB1*0406-binding motifs on human insulin. IxxLxQ motif with the most efficient binding to DRB1*0406 complex is boxed. Other binding motifs (FxxIxK and LxxLxN) are underlined. Lines between cysteine residues indicate disulfide bonds.

Identification of Human Insulin Fragment That Stimulates the Autoreactive T Cell Line Specific to Insulin. To test whether DRB1*0406-bearing individuals carry T cells autoreactive to the insulin peptide with the IxxLxQ motif, we established a short-term T cell line by stimulating PBMC with the recombinant human insulin. With the established T cell line exerting insulin-induced proliferation, we quantitated the T cell reactivity against each synthetic peptide. As shown in Table 5, the T cells showed the strongest reactivity against Insαp8-21 (^{8}TSICSLYQLENYCN^{21}) which contains both IxxLxQ and LxxLxN motifs. The T cells proliferated in response to Insαp8-19 (^{8}TSICSLYQLENY^{19}) but not to Insαp8-17 (^{8}TSICSLYQLE^{17}), which is devoid of the third anchor N of the LxxLxN motif. However, this may not indicate that the T cells are recognizing the DR4–peptide complex through a preferential usage of LxxLxN motif for binding, since the T cells are capable of recognizing amino acid residues lying 8 to 9 residues apart to the carboxy terminus from the first anchor (1). Thus, it is likely that the truncation of ^{18}NYCN^{21} affected the T cell recognition of the DR4–peptide complex formed by using not only the LxxLxN but also the IxxLxQ motif. On the contrary, truncation of four residues of Insαp8-21 at the amino terminus (Insαp12-21; ^{12}SLYQLENYCN^{21}) clearly dissects these two binding motifs, because the first anchor (necessary for binding to DR4 as shown in Fig. 3) Ile of the IxxLxQ motif is removed whereas the LxxLxN motif is kept intact. Stimulation with this truncated peptide decreased the T cell response by 66% as compared with Insαp8-21. These observations indicate that the binding through the IxxLxQ motif rather than

Table 5. *T Cell Reactivity against Human Insulin Fragments*

Synthetic peptide*	Sequence	Percent T cell response
KK-Insαp8-21	KKTSICSLYQLENYCN	100
KK-Insαp8-19	KKTSICSLYQLENY	89.0
KK-Insαp8-17	KKTSICSLYQLE	0.3
K-Insαp12-21	KSLYQLENYCN	33.8
K-Insβp11-30	KLVEALYLVCGERGFFYTPKT	32.1

* A short-term autoreactive T cell line established by stimulating PBMC from a DRB1*0406-bearing individual with reduced human recombinant insulin was incubated with autologous irradiated PBMC in the presence of various soluble synthetic peptides, and subjected to [^{3}H]thymidine incorporation assay. The IxxLxQ motif is underlined. The following formula was used to calculate the percent T cell response using mean values of duplicate cultures. Percent T cell response = 100 × ([cpm with a peptide] − [cpm without peptide])/([cpm with KKInsαp8-21] − [cpm without peptide]). The denominator was 4,514 cpm.

LxxLxN is a major mechanism to form DR4–Insαp8-21 complex recognized by the T cells. In addition to the minor reactivity against the LxxLxN motif, another weak reactivity was detected against Insβp11-30, which is shown in Table 4 to bind DR4 complexes with an intermediate affinity. Synthetic peptides such as Insαp1-10 that exhibited no detectable binding to DR4 complexes (Table 4) were devoid of T cell–stimulating activity (not shown).

Discussion

In the studies herein, we report that: (*a*) native DRα-DRB1*0405 complex purified from B lymphoblastoid cells have, in their peptide-binding grooves, peptide fragments of CD23, CD20, MIF, pyruvate kinase, and three unidentified peptides; (*b*) three amino acid residues (anchors) on these peptides, such as FxxLxN are important for binding; (*c*) the first anchor, closest to the amino terminus of the binding peptides, must be hydrophobic; (*d*) the second anchor, which is three residues apart from the first one is hydrophobic but some nonbasic hydrophilic residues are also capable of binding; (*e*) the third anchor, lying two residues apart from the second anchor, is promiscuous but the binding affinity was variable; (*f*) although the basic motif for binding to DRB1*0406 is similar to that for DRB1*0405, certain residues reveal a significant difference in binding requirements; (*g*) anchor residues on DR4 binders are predictable; (*h*) among human insulin–derived peptide fragments, the ^{8}TSICSLYQLE^{17} of the human insulin α chain, which is exposed only under reducing conditions, has the highest affinity specific to DRB1*0406 by binding with the IxxLxQ motif; and (*i*) a short-term human insulin-specific T cell line generated from a DRB1*0406-bearing individual recognizes a peptide fragment containing the IxxLxQ motif as a major T cell epitope.

The reasons insulin peptide binding is thought to occur in the grooves include: (*a*) negative binding of 0405BP3 in reducing conditions eliminated nonspecific "stickiness" of the peptide to the DR complexes (not shown), and (*b*) some binders with low IC_{50} (good binders) co-cultured with DRB1*0405- and 0406-restricted T cell clones inhibited the antigen-specific proliferative responses (manuscript in preparation).

Because polymorphic residues of DR alleles are scattered within the peptide-binding grooves, different DR molecules are capable of binding peptides with different structural motifs, and this phenomenon contributes to the HLA-linked polymorphism of immune responses. To date, motifs for binding peptides have been reported in DR1 (DRB1*0101; 5, 8, 10, 11), DR2 (DRB1*1501; 8, 9), DR3 (DRB1*0301; 6, 7, 9), DR4 (DRB1*0401; 9, 12, 19), DR11 (DRB1*1101; 8, 12), DR7 (DRB1*0701; 8, 9, 20), and DR8 (DRB1*0801; 9). The structural motif for DR4 (B1*0405 and 0406) binders clarified in the current study showed significant similarity to a DRB1*0401-binding motif reported by Hammer et al. (12), but not to one reported by Chicz et al. (9). The discrepancy probably arose from the different strategies by which the structural motifs were identified. Although Chicz et al. aligned the sequences without performing DR–peptide binding assay, the DRB1*0401 binders reported in their study all have the basic motif proposed herein. Indeed, solely aligning the sequences of the seven self-peptides shown in Table 1 led to three possible motifs such as hydrophobic-x-x-x-hydrophobic, which failed to show high affinity binding with the DR4 using polyalanine-based peptides (not shown). These data collectively indicate that the DR–peptide binding assay is necessary to identify the structural motifs of DR-binding peptides.

Structural motifs reported previously confirmed that serologically distinguishable DR alleles such as DR1, DR3, and DR4 have distinct binding motifs (6, 12). However, quite a few immune responses of humans or susceptibility to immunity-related diseases have been shown to be allele specific, even among alleles that can only be distinguished at the DNA level. For instance, DRB1*0405 confers increased susceptibility to RA but not to IAS, whereas serologically identical DRB1*0406 conversely confers susceptibility to IAS but not to RA (3, 4) among Japanese. This type of discrepancy was explained based on peptide–HLA interactions in the current study; the basic structural motif is similar between DRB1*0405 and 0406, but the allele specificity of peptide binding is determined by differences in affinity to certain amino acid residues at the important anchor positions. In view of the reports that anti-HLA alloantisera can recognize not only the structure of the peptide-binding grooves but also bound peptides existing within the grooves (21), it is likely that serologically indistinguishable alleles (e.g., DR4 subtypes) physiologically bind analogous sets of self-peptides. Indeed, although native DRB1*0406-binding peptides were not sequenced in this study, all the peptides eluted from the DRB1*0405 could bind to the DRB1*0406 complex with variable affinity.

The IC_{50} cannot be used to directly assess DR–peptide affinity among different studies because of the different concentrations of ^{125}I-labeled peptide applied. However, regardless of the relatively high IC_{50}, the K_d value for 0405BP3–DRB1*0405 interaction calculated from the data shown in Fig. 2 was 70 nM, approximately the same level of DR–peptide affinity reported by others (18). Of the seven self-peptides eluted from the DRB1*0405 shown in Table 1, a motif for the CD23 fragment (LxxIxS) did not show very strong binding, as predicted by the quantitative structural motif. However, because of the abundant expression of CD23 on EBV-transformed B lymphoblastoid cells, it is conceivable that this fragment existed within the groove at a detectable level.

The first anchors on binding peptides shown in Fig. 5 *a* were strictly hydrophobic, which is concordant with other reports (12). The hydrophobic pocket formed by the α helix of the DRα and DRβ chains is probably responsible for the hydrophobic interaction (1). Among amino acid residues on DR complexes presumably involved in this interaction, only ^{86}DRβ is polymorphic among the DR4 subtypes. In a comparative study with DRB1*1101 and 1104, Demotz et al. observed that glycine on ^{86}DRβ is capable of accommodating more species of hydrophobic residues than valine on ^{86}DRβ

(22). The same results were obtained in the present study. Thus the DRB1*0405 (glycine on ^{86}DRβ), compared with the DRB1*0406 (valine on ^{86}DRβ), exhibited higher affinity with some of the first anchors on binding peptides such as W, C, or Y, whereas none of residues bound preferentially to DRB1*0406.

Some of the second anchor residues also exhibited allele specificity in binding, especially with F, W, or Y. The closest polymorphic site within the groove should be ^{74}DRβ, which is Ala in DRB1*0405 and Glu in DRB1*0406 (23). However, the allele specificity of the second anchor cannot be fully explained solely by the interaction with ^{74}DRβ, because none of the basic residues (R, K, or H) exhibited high affinity binding with the DRB1*0406 (Glu for ^{74}DRβ). Possible explanations for this discrepancy include: (*a*) the side chains of basic amino acid residues in this position of the peptides are too large to fit into the groove; and (*b*) ^{74}Drβ does not directly interact with the second anchor but rather affects the three-dimensional and physicochemical characteristics of the groove, where the second anchor of DR4-binding peptides interacts with HLA (20). The DRB1*0401-binding motif reported recently by Sette et al. (19) did not contain the second anchor in our current study possibly because: (*a*) their data is based on substitutions of Q to E, A, H, L, T, N, and K which are not favored residues in this position for DRB1*0405–peptide interactions; and/or (*b*) there is a significant difference between DRB1*0401 and DRB1*0405 in this position. However, their observation that K in this position is the least favored substitution agrees with our results.

Unlike the first and second anchors, none of the substitutions in the third anchor completely eliminated the binding. However, this position conceivably plays an important role in high affinity binding because single amino acid substitution analysis using another DR4 binder 0405BP2 (^{1}SPGTGAYYVLLN^{12}) showed the importance of this position. Thus, substitution of 7th Y and 10th L (SPGTGA*YYVLL*N) to Ser residues completely eliminated the binding to DRB1*0405, whereas the same substitutions at 8th Y and 11th L (SPGTGAY*YVLL*N) did not affect the binding, presumably by losing the putative third anchor N. Substitution of the 12th N to A also affected the binding significantly (not shown). These data indicate that the third anchor is necessary for binding to DR4. In this regard, Hammer et al. (12) reported that polyalanine-based ^{1}YAALAAAAL^{9} peptide bound to DRB1*0101, 0401, and 1101. Moreover, substitution of ^{6}Ala to Thr or Arg, which exactly corresponds to the third anchor in the current study, clearly resulted in allele-specific binding, with Thr having the highest affinity to the DRB1*0401 complex. In light of the structural similarity between DRB1*0401 and 0405, this observation corroborates the results of the present study.

Recent crystallographic analysis of a DR1–peptide (^{306}PKYVKQNTLKLAT^{318}) complex by Stern et al. (23) clearly supports the current observations. Thus, the side chains of ^{308}Y, ^{311}Q, ^{313}T, and ^{314}L, which correspond exactly to ^{5}F, ^{8}L, ^{10}N, and ^{11}P on ^{1}GSTVFDNLPNPE^{12}, respectively, are buried in the pockets formed by residues containing many polymorphic sites. Some previous studies on DR-binding motifs suggested the existence of another important hydrophobic anchor lying eight residues from the first anchor (12, 19, 20), which is also buried in a small pocket that was revealed by crystallography (23). However, the importance of this anchor was not confirmed in the present study because: (*a*) truncated 0405BP3 that lacks this position had the same affinity as the original 0405BP3; and (*b*) of the seven DR4 binders analyzed in this study, two did not even have this position, and those which did have it did not show any definite residue selectivity at this position. It might be that the degree of the involvement of this possible anchor in DR–peptide interactions may depend on the DR alleles and binding peptides, as demonstrated by Hammer et al. (12). It is especially important to note that acidic residues (Asp or Glu) in this position prevent interaction with DRB1*0401 according to Sette et al. (19), although three DRB1*0405-binding peptides (PKM2p99-117, CD23p195-214, and MIFp32-51) analyzed in our current study have acidic residues in the position. Among the polymorphic residues, ^{57}DRβ, which forms a banklike side wall of a pocket accomodating ^{316}L of ^{306}PKYVKQNTLKLAT^{318} (23), is acidic (Asp) in DRB1*0401 (thus causing repulsive interaction with acidic residues which enter the groove), whereas it is neutral (Ser) in DRB1*0405. The small neutral side chain of Ser at this position should accommodate a greater variety of amino acid residues since steric hindrance and repulsive ionic interactions are smaller. Thus it is likely that this polymorphism plays a major role in the existence or variability of peptide binding at a residue lying eight residues apart from the first anchor.

Some polyalanine-based peptides, such as AALAAIASAA had slightly higher affinity to DR4 than the original peptides. It may be that the native sequence contains amino acid residue(s), which inhibit binding as reported by Boehncke et al. (24), and substitution to alanine removes this inhibition. Single amino acid substitution to alanine on antigenic peptides is frequently applied in studies on MHC–peptide and TCR–MHC/peptide interactions; however, this strategy might occasionally lead to incorrect results because, as shown in Fig. 5, alanine clearly plays a role in the interaction between HLA and peptide fragments. In this sense, the strategy for the identification of anchor positions shown in Fig. 3 (detection of decreased binding by substitution to A or S) can only be applied to peptides that exhibit very high-affinity binding with HLA. Fortunately, F, L, and N of AAFAALANAA revealed significantly higher affinity than A or S in all the three positions, which allowed us to identify these anchors by observing decreased binding. Negligible differences in affinity among AAFAALANAA, GSTVFDNLPNPE, and the original 0405BP3 also ruled out possible oversight of important anchor residues. However, if this strategy is used for a low-affinity peptide, extensive substitutions in all the positions (19, 20) might be necessary to avoid missing critical anchors. Indeed, our present study on extensive substitutions of streptococcal M12 peptide recognized by a T cell clone in the context of DRB1*0406 did not reveal any other anchor positions for the binding with DRB1*0406 complex (manuscript in preparation).

IAS is characterized by a large amount of total serum-

immunoreactive insulin, the presence of anti-insulin autoantibodies, and fasting hypoglycemia, and is reported to be the third leading cause of hypoglycemia among Japanese (25). Another interesting feature of IAS is that one-quarter of the patients had been taking medication prior to the onset, 94% of which were sulphydryl compounds such as methimazole, mercaptopropionyl glycine, or glutathione. The direct association of DRB1*0406 with IAS (relative risk = 281) was successfully explained in this study by quantitative comparison of DRB1*0406- and 0405-binding peptide motifs. Thus, three human insulin-derived synthetic peptides showed detectable binding to DRB1*0406 complex, among which the most efficient binder αp8-17, has the IxxLxQ motif that preferentially binds to the DRB1*0406 complex due to the presence of Gln at the third anchor. Moreover, this sequence has a cysteine residue in the middle of the binding motif (ICSLYQ), which forms a disulfide bond with the flanking α^{6}Cys residue. Therefore, in view of the clinical observations, it is conceivable that insulin αp8-17 is a reduction-induced DRB1*0406-specific cryptic self.

In this respect however, a recent study by Ito et al. (26) showed that even T cells of DRB1*0406-bearing healthy individuals can proliferate in response to human insulin presented by the DRB1*0406 complex, whereas T cells of DRB1*0405-bearing healthy individuals cannot. This observation is supported by another report that APC have a reducing capacity to some extent (27, 28). Thus, the insulin fragment with disulfide bond(s) may not be "cryptic" in a strict sense, but rather the cleaved insulin fragment may exist within APC at a low level in physiological conditions, against which self-tolerance is not completely acquired. Forced reduction of insulin probably augments this immune response and transmits intense "helper" signals to B cells for the production of autoantibody to self-insulin. Other fragments, βp11-30 and αp12-21, could also bind with lower affinities to both DRB1*0406 and 0405 nearly equally, presumably with their $^{\beta24}$FxxTxK$^{\beta29}$ and $^{\alpha13}$LxxLxN$^{\alpha18}$ motifs, respectively. It may be that these possible T cell epitopes exist physiologically at a level sufficient to acquire partial self tolerance, because the $^{\beta24}$FxxTxK$^{\beta29}$ and $^{\alpha13}$LxxLxN$^{\alpha18}$ motifs, as well as flanking residues, contain no disulfide bonds, and can be recognized by T cells in the context of HLA molecules without forced reduction. Indeed, the unexpected minor T cell reactivity observed against these fragments might be explained by the development of anti-insulin autoantibody through T cell recognition of αp8-19 (Table 5), by which B cells can be involved more efficiently in the presentation of insulin molecules to T cells. In light of the equal affinity of the fragments containing the $^{\beta24}$FxxTxK$^{\beta29}$ and $^{\alpha13}$LxxLxN$^{\alpha18}$ motifs to DRB1*0405 and DRB1*0406 (Table 4), the observation that T cells from DRB1*0405-bearing individuals do not proliferate at all in response to insulin (26) may support this hypothesis.

Another possible factor for DR–peptide binding is the substrate specificity of lysozomal proteases for antigen processing. The extent, if any, of polymorphism in this regard remains obscure. However, insulin α chain after S-S cleavage may be short enough to be presented without the involvement of proteases (9, 29).

The quantitative identification of structural motifs shown in this study not only facilitate the understanding of allele-specific binding of self- or non-self-peptides to HLA molecules but also help in the prediction of the hierarchy of binding affinity of multiple T cell epitope sites on natural protein antigens. In this regard, human collagen type II, one of the most likely autoantigens to be recognized by T cells in RA (30), has 11 possible sequences (not counting proteolytic sites) for binding to the DRB1*0405 complex. Studies are underway to: (*a*) determine T cell precursor frequency by stimulating T cells of DRB1*0406-bearing patients with IAS and healthy subjects using synthetic human insulin fragments; and (*b*) establish T cell clones from RA patients recognizing possible DRB1*0405-binding fragments of collagen type II.

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