Allele Specificity of Structural Requirement for Peptides Bound to HLA-DRBI*0405 and -DRBI*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-DRBl*0405 complex) were eluted from the purified DR4 complex, fractionated on reverse-phase HPLC, and subjected to NH₂-terminal sequencing. Seven independent sequences were obtained, and all putative peptides synthesized bound to DRBI*0405 as well as DRBI*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 DRBI*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 DRBI*0405 and DRBI*0406. It is noteworthy that all patients with methimazole-induced insulin autoimmune syndrome are positive for DRBI*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 DRBI*0405 and DRBI*0406. Interestingly, the quantitative structural motif identified in this study predicted that 8TSICSLYQLE 17 of human insulin α chain may bind specifically to DRBI*0406 using its 8TSICSLYQLE 17 motif. Indeed, DRBI*0406 complex bound 8TSICSLYQLE 17 with a high affinity, and in striking contrast, DRBI*0405 complex did not. Furthermore, a short-term T cell line specific to human insulin established from a DRBI*0406-bearing individual did show reactivity with a peptide fragment containing the 8TSICSLYQLE 17 motif. Although this fragment probably exists at a very low level under normal physiological conditions due to the disulfide bond between flanking cysteine residues (Cys-1Cys), a reducing compound such as methimazole may cleave the disulfide bond in vivo and allow DRα-DRBI*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 tolerant 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 DRBI*0401 through DRBI*0414 with slight differences in the structure of the peptide-binding groove, thus allowing detailed analyses of peptide-DR interactions. DRBI*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 DRBI*0401 which differs from DRBI*0405 at
only two residues (57th and 71st) on the β chain, and is strongly associated with rheumatoid arthritis (RA) 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 autantigen 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% CO2 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 from EBV-transformed human B lymphoblastoid cell lines EBWa donors used for a generation of an insulin-specific T cell were applied for affinity chromatography with a mAb HU-4 (anti-DR IgG2a; 13, 16), which was previously shown to precipitate DRαβ(DRB1) but not DRαβ(8) 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% non-β-glucoside/PBS. The DR–peptide complex was eluted with 1% non-β-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 (1010 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 0.15 M NaCl 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 0.05% TFA and 5% acetonitrile in water. The fraction was eluted into 0.1 M Tris and 0.15 M NaCl at pH 10.8 (17), and then precipitated by acetonitrile at a final concentration of 80%. Seven cycles of purification with a total of 20 g (1010 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.

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 (102A; 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 [3H]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 L-phenylalanine, 73 μM pepstatin A, 8 mM EDTA, 6 mM N-ethylmaleimide, 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 Seph dex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ) column (4 × 120 mm) equilibrated with 0.5% NP-40, 0.1% NaN3/PBS, and the fractions were assayed for ra-

Abbreviations used in this paper: IAS, insulin autoimmune syndrome; IC50, 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 (IC50) of binding was determined.

**Generation of a Human T Cell Line Specific to Insulin.** PBMC (5 x 10^6/ml) of a DRBl*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 x 10^{-5} M of 2-mercaptoethanol overnight at 37°C in 5% CO2/95% 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 x 10^6/well) in a 96-well flat-bottomed culture plate. 7 d after stimulation, cells were fed with insulin-pulsed, irradiated autologous PBMC (1 x 10^6/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 transferred 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 x 10^4/well) for 72 h with irradiated autologous PBMC (1.0 x 10^6/well) and soluble synthetic peptides (5 µM) in a 96-well plate, in the presence of [3H]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 (DRBl*0405).** Self-peptides eluted from 900 µg of purified DR4 (DRBl*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 [SDPILYRPAVALDTKGPE], 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 DRBl*0405 and DRBl*0406 complexes, and exhibited 10-20% of DR-binding activity at pH 7.0, whereas an irrelevant peptide (NELSGEAKHALKGLY) 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 DRBl*0405 and DRBl*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 DRBl*0405 and DRBl*0406 Complexes.** By using the competitive inhibi-
Table 1. Amino Acid Sequences of Eluted Peptides and Their Homology

| Peak number | Sequence* (upper) and homologous peptide (lower) |
|-------------|--------------------------------------------------|
| 1           | VP I QRAVYIQNVVVNNPXD                            |
| 2           | SPGTGAYYVLLN                                     |
| 3           | GSTVFDNLNPE I GDYYGW                             |
| 4           | XGQLVSI NN                                       |
|             | E----------HSPEEQDFLTKHA (CD23p195-214)           |
| 5           | GPKPLRFRRM                                       |
|             | SXLVGTQSSFF (CD20p26-45)                         |
| 6           | GKKP--------LMAFG                                 |
| 7           | SDP I LYPVAVALD                                  |
|             | ----------TKGPE                                   |
| 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 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) was incubated for 48 h with various concentrations of unlabeled 0405BP3 (GSTVFDNLNPPEIDGDYYGW) 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 (NELSGEAHK-DALKLY) tested as a competitor revealed nearly identical results either with DRB1*0405 or with DRB1*0406 (closed square).
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 (NELSGEAKHDLGKLK)        | 103                                         | 93                                          |
| 0405BP1 (VP1QRAYQNNVNN)                     | 97                                          | 90                                          |
| 0405BP2 (SPGTGAYVYLLN)                      | 90                                          | 90                                          |
| 0405BP3 (GSTVFDNLNPNEIDGDYGYW)              | 90                                          | 59                                          |
| PKM2p99-117 (SDPILLYRVPVAVALDTKGE)          | 90                                          | 90                                          |
| CD20p26-45 (GPKPLFRRMSLGVPTQSSF)            | 59                                          | 63                                          |
| CD23p195-214 (GQQIVSHSPEQDFLTKA)            | 94                                          | 69                                          |
| MIFp32-51 (GKPPQYIAVHVVPQDTMAFG)            | 94                                          | 69                                          |

* Purified DR4 complexes were incubated for 48 h with 20 nM of 125I-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 alleles 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-
Table 3. Binding of Polyalanine-based Peptides to DR4

| Synthetic peptide as a competitor* | DRB1*0405 | DRB1*0406 |
|-----------------------------------|-----------|-----------|
| AAFAAMASAA                        | 3.6       | 1.1       |
| AALAAIASAA                        | 11.0      | 8.8       |
| AAYAALANAA                        | 1.4       | 5.0       |
| KAAYAAAVAA                        | 5.1       | 12.2      |
| AAKAAGAA                          | >500      | >500      |

* Polyalanine-based peptides were incubated at various concentrations with DR4 complexes in the presence of 125I-0405BP3 (20 nM) and IC50 values were determined. KAAYAAAVAA 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 IC50 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 (IC50 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).

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

| Insulin-derived peptide*         | IC50 with |
|----------------------------------|-----------|
|                                  | DRB1*0405 | DRB1*0406 |
| K-Insop1-10 (KGIVESQCTS1)        | >500      | >500      |
| KK-Insop8-17 (KKTSICSGLYQLE)     | 180       | 4.1       |
| K-Insop12-21 (KSLYFLENVCYN)      | 10.5      | 12.0      |
| K-Insβp1-18 (KFWNQHLCGLYVEALMAC) | >500      | >500      |
| K-Insβp11-30 (KLWAYSILVCGERQFFYPKT) | 15.0   | 14.5      |

* Human insulin-derived synthetic peptides were incubated at various concentrations with DR4 complexes in the presence of 125I-0405BP3 (20 nM) and IC50 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 DRBl*0405-binding peptides (except 0405BP3), are AAAFLAMASAA, AALAAIASAA, AAYALANAA, and AAYAAVAVAA. These motifs were synthesized and tested by binding inhibition assay. Since the putative motif of AAYAAVAVAA for 0405BP1, MIFp32-51, and PRK2p99-117 was not soluble, lysine was inserted at the NH2 terminus (KAAYAVAVAA) to increase the solubility. Indeed, as demonstrated in Table 3, all the polyanaline-based peptides bound to both DR4 complexes whereas AAAYAAKAGAA, which was predicted from Fig. 5 to show null binding, had no affinity. This also indicates that there is no detectable binding with the AxxAXxA motif. The YxxVxxV and YxxLxN motifs did not show very high affinity to the DRBl*0406 complex, confirming the results shown in Table 2 and Fig. 5. Thus, Tyr at the first anchor reduces the affinity to DRBl*0406.

Identification of Human Insulin Fragment That Binds Preferentially to DRBl*0406. Using the above structural motifs for the DR4 binders, we searched for a human insulin fragment with a higher affinity to the DRBl*0406 than to the DRBl*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, TSICSLYQLE17 and other insulin fragments were synthesized and tested for binding affinity to the DR4 complexes. Table 4 shows the IC50 values for these peptides with DRBl*0405 and 0406. As predicted by the quantitative structural motif, TSICSLYQLE has the highest affinity to the DRBl*0406 complex, with an IC50 value 44 times smaller than that for the DRBl*0405. Since this insulin fragment forms a disulfide bond between flanking cysteine residues (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 higher affinities both to DRBl*0406 and to 0405, but the motif in this location (β2SLYQLE19 and αLxxLxN18, respectively) did not contain cysteine residues (Fig. 6). No other insulin fragments bound to either DR4 complex.

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

| Synthetic peptide        | Sequence                  | Percent T cell response |
|--------------------------|---------------------------|-------------------------|
| KK-Insap8-21             | KKTSICSLYQLENACN          | 100                     |
| KK-Insap8-19             | KKTSICSLYQLEN             | 89.0                    |
| KK-Insap8-17             | KKTSICSLYQLE              | 0.3                     |
| K-Insap12-21             | KLSLYQLENAC               | 33.8                    |
| K-Insap11-30             | KLVEALYLCGERGFFTPKT       | 32.1                    |

* A short-term autoreactive T cell line established by stimulating PBMC from a DRBl*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 [3H]thymidine incorporation assay. The IxxLxX 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 x ([cpm with a peptide] - [cpm without peptide]) / ([cpm with KKInsap8-21] - [cpm without peptide]). The denominator was 4,514 cpm.

Identification of Human Insulin Fragment That Stimulates the Autoreactive T Cell Line Specific to Insulin. To test whether DRBl*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 Insap8-21 (TSICSLYQLENACN) which contains both IxxLxQ and LxxLxN motifs. The T cells proliferated in response to Insap8-19 (TSICSLYQLEN) but not to Insap8-17 (TSICSLYQLE), 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 LxxLxX 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 aNYCN21 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 Insap8-21 at the amino terminus (Insap8-21; STSICSLYQLENACN) clearly dissects these two binding motifs, because the first anchor (necessary for binding to DR4 as shown in Fig. 3) Ile of the LxxLxX 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 Insap8-21. These observations indicate that the binding through the IxxLxQ motif rather than...
LxxLxN is a major mechanism to form DR4–Insop8-21 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 Insop1-10 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 “TSICSLYQLE” 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 LxxLxQ 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 LxxLxQ 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 IC0 (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 IC50 cannot be used to directly assess DR–peptide affinity among different studies because of the different concentrations of 125I-labeled peptide applied. However, regardless of the relatively high IC50, the Kd 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 (Lxx1xS) 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. 5a were strictly hydrophobic, which is consistent 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 αDRβ is polymorphic among the DR4 subtypes. In a comparative study with DRB1*1101 and 1104, Demetz et al. observed that glycine on αDRβ is capable of accommodating more species of hydrophobic residues than valine on αDRβ.
The same results were obtained in the present study. Thus the DRBI*0405 (glycine on 9βDr), compared with the DRBI*0406 (valine on 9βDr), 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 DRBI*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 74Dr, which is Ala in DRBI*0405 and Glu in DRBI*0406 (23). However, the allele specificity of the second anchor cannot be fully explained solely by the interaction with 9βDr, because none of the basic residues (R, K, or H) exhibited high affinity binding with the DRBI*0406 (Glu for 9βDr). 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) 9βDr 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 DRBI*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 DRBI*0405—peptide interactions; and/or (b) there is a significant difference between DRBI*0401 and DRBI*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 (1SPGTVYAYVLLN) showed the importance of this position. Thus, substitution of 7th Y and 10th L (SPGTGAYVLLN) to Ser residues completely eliminated the binding to DRBI*0405, whereas the same substitutions at 8th Y and 11th L (SPGTGAYVLLN) 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 YAAALAAAL peptide bound to DRBI*0101, 0401, and 1101. Moreover, substitution of 6Ala 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 DRBI*0401 complex. In light of the structural similarity between DRBI*0401 and 0405, this observation corroborates the results of the present study.

Recent crystallographic analysis of a DR1—peptide (206PKYVKQNTKLKAT) complex by Stern et al. (23) clearly supports the current observations. Thus, the side chains of 30Y, 31Q, 31T, and 31L, which correspond exactly to 9F, 8L, 10N, and 11P on GSTVFDNLPNPE, 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 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 DRBI*0401 according to Sette et al. (19), although three DRBI*0405-binding peptides (PKM2p99-117, CD23p195-214, and MIP3p32-51) analyzed in our current study have acidic residues in the position. Among the polymorphic residues, 9βDr, which forms a banklike side wall of a pocket accommodating 316L of 206PKYVKQNTKLKAT (23), is acidic (Asp) in DRBI*0401 (thus causing repulsive interaction with acidic residues which enter the groove), whereas it is neutral (Ser) in DRBI*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 AAALAAAL 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 Boelcke 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 AAALAAAL revealed significantly higher affinity than A or S in all the three positions, which allowed us to identify these anchors by observing decreased binding. Negative differences in affinity among AAALAAAL, 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 DRBI*0406 did not reveal any other anchor positions for the binding with DRBI*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 sulphhydryl compounds such as methimazole, mercaptopropionyl glycine, or glutathione. The direct association of DRBI*0406 with IAS (relative risk = 281) was successfully explained in this study by quantitative comparison of DRBI*0406- and 0405-binding peptide motifs. Thus, three human insulin-derived synthetic peptides showed detectable binding to DRBI*0406 complex, among which the most efficient binder αp8-17, has the LxxLxQ motif that preferentially binds to the DRBI*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 αCys residue. Therefore, in view of the clinical observations, it is conceivable that insulin αp8-17 is a reduction-induced DRBI*0406-specific cryptic self.

In this respect however, a recent study by Ito et al. (26) showed that even T cells of DRBI*0406-bearing healthy individuals can proliferate in response to human insulin presented by the DRBI*0406 complex, whereas T cells of DRBI*0405-bearing healthy individuals cannot. This observation is supported by another report that APC have a reducing capacity bearing healthy individuals cannot. This observation is supported by quantitative comparison of DRBI*0406- and 0405-binding peptide motifs. Thus, three human insulin-derived synthetic peptides showed detectable binding to DRBI*0406 complex, among which the most efficient binder αp8-17, has the LxxLxQ motif that preferentially binds to the DRBI*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 αCys residue. Therefore, in view of the clinical observations, it is conceivable that insulin αp8-17 is a reduction-induced DRBI*0406-specific cryptic self.

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