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Immunology

Critical role for the Val/Gly^{86} HLA-DRβ dimorphism in autoantigen presentation to human T cells

(major histocompatibility complex/polymorphism/epitope presentation/acetylcholine receptor/myasthenia gravis)

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ABSTRACT **Helper T lymphocytes recognize fragments of foreign (or self) antigens in the peptide-binding clefts of major histocompatibility complex class II molecules; their activation is a crucial step in the induction of many immune and autoimmune responses. While studying the latter, we raised a T-cell line from the thymus of a myasthenia gravis patient against recombinant α subunit of the human acetylcholine receptor, the target of this autoimmune disease. The line responds to the 144–156 region of the human sequence and not to the same region of the electric fish homolog, which differs by only three residues. These CD4$^+$ T cells recognize this epitope only in the context of HLA-DR4 class II molecules, of which the variants with Gly^{86} are absolutely required. Thus the naturally occurring alternatives Dw14.2 (Gly^{86}) and Dw14.1 (Val^{86})—which differ only at this one position in the entire antigen-binding region—show an all-or-nothing difference in presenting activity. This dimorphism at position 86 is widespread, occurring in subtypes of DR1, DR2, DR3, DR5, and DR6 alleles as well as DR4. Since other DR4 subtypes with substitutions at positions 70–74 also fail to present this peptide, and glycine residues can be uniquely flexible, we suggest that this replacement at position 86 acts locally or at a distance by altering the conformation of the peptide-binding cleft. Such profound functional consequences for T-cell recognition as we report here may explain this example of conserved major histocompatibility complex diversity.**

The striking polymorphism of the major histocompatibility complex (MHC) in most vertebrate species implies strong selection for diversity. As functional and crystallographic studies show (for class I molecules), the role of these MHC heterodimers is to bind peptide fragments of processed antigens in a cleft formed by the folding of their two membrane-distal domains (1, 2) and to present them to T lymphocytes. [For CD4$^+$ helper T cells, the class II molecules on specialized antigen-presenting cells (APCs) fulfill this role.] Specific recognition of the MHC and peptide components of the resulting composite by T lymphocytes underlies the phenomenon of MHC restriction whereby most peptides are recognized in the context of only some of the class II alleles. Thus, the diversity of the MHC molecules probably ensures that the population as a whole maintains responsiveness to a broad repertoire of foreign antigens.

Many autoimmune diseases show associations with particular MHC alleles or haplotypes (reviewed in ref. 3). Myasthenia gravis (MG) is one example, showing strong associations with HLA-A1, -B8, and -DR3 in young-onset Caucasian patients (4). It is clearly mediated by autoantibodies to the acetylcholine receptor (AChR) at the motor endplate (5): this consists of α_2, β, δ, and ϵ or γ subunits varying from 437 to 501 amino acids long, each having at least four transmembrane segments and an N-terminal extracellular domain of about 210 residues (6). The antibodies are heterogeneous high-affinity IgG molecules that are helper T-cell dependent in laboratory animals (7) and almost certainly in humans too. The α subunits (represented twice) are thought to be the main target of autoantibodies and helper T cells (5), and the induction of these autoreactive T cells is widely believed to be a crucial step in autosensitization (8).

To study this process, we have recently been able to raise T-cell lines and clones from MG patients using recombinant *human* AChR α subunit (9): our polypeptide r37-429A (where r indicates recombinant) stimulates human T cells more frequently than either native AChR purified from the electric fish *Torpedo* (T-AChR, ref. 10) or its recombinant 37-437 analog. Others have performed similar studies with recombinant mouse α 36-216 (11). We have concentrated initially on the young-onset patients with their 3:1 female bias, HLA association (4), and infiltration (hyperplasia) in thymic medulla by lymph node-type T-cell areas and germinal centers (5). Furthermore, their hyperplastic thymus is usually particularly rich in AChR-specific T- (12) and B- (5) lineage cells, and their myasthenia tends to ameliorate after thymectomy. We now report a T-cell line raised from the thymus of a typical (HLA-DR3/4) patient that discriminates very sharply between the human AChR and T-AChR sequences and between variants of the restricting HLA-DR4 molecule, depending absolutely on a glycine for valine substitution at β-chain position 86.

MATERIALS AND METHODS

Antigens. Native T-AChR or human AChR was solubilized from electric organs or muscle in Triton X-100, purified by affinity chromatography (12), and used at 1–5 μg or 10–20 ng per ml. Recombinant human and *Torpedo* α subunit polypeptides were generated, expressed, and purified (by SDS/PAGE) identically (as described in refs. 13 and 14) and used at about 0.5 μg/ml. Peptides were synthesized (from the sequences in ref. 15) using fluoren-9-ylmethoxycarbonyl-protected amino acid residues on an LKB Biolynx apparatus and used at 10 and 2.5 μg/ml. Phytohemagglutinin (PHA, Difco) was used at a 1:1000 dilution, and purified protein

Abbreviations: AChR, acetylcholine receptor; T-AChR, *Torpedo* AChR; APC, antigen-presenting cell; EBV, Epstein–Barr virus-induced; MG, myasthenia gravis; MHC, major histocompatibility complex; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; PPD, purified protein derivative (of tuberculin); Px, irradiated peripheral blood cells; SI, stimulation index (indices); r, recombinant.

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derivative of tuberculin (PPD, Evans Medical, U.K.) was used at 10 μg/ml.

HLA-Specific Reagents. Monoclonal antibodies L243 (anti-DR), Genox 3.53 (anti-DQ), and B7-21 (anti-DP) were used at 10 μg/ml to block presentation of antigens to T cells (16, 17). To identify HLA-DR4 subtypes, genomic DNA was first amplified (by PCR) using DR4-specific primers; dot blots were then hybridized with ^{32}P-labeled allele-specific oligonucleotides (18–21).

Donor Patient and Cell Culture Methods. A hyperplastic thymus was removed (in 1985) from a 15-year-old patient (PM) 7 months after onset of MG symptoms [severity grade II B (22)]; her serum anti-AChR titer was 180 nmol. Her HLA type is A2, 32; B8, 55 (Bw6); Cw9; DR3,4 (DRw53); DQw7. The thymus cell suspension was cryopreserved (23).

A low-density-enriched fraction was subsequently prepared from thawed thymus cell suspension (12) and was cocultured with fresh 30-Gy ^{137}Cs-irradiated peripheral blood lymphocytes (PBL, Px) and antigen. From 120 Terasaki wells initiated with 10^3 low-density cells (plus 10^4 Px and ≈0.5 μg of r37-429A per ml) per 20-μl well, there was good T-cell growth in three wells; line PM-A was established from one of these by expansion (with interleukin 2) into 0.2-ml and then 2-ml wells (plus further antigen and Px). Of the 60 wells initiated similarly but with 10^4 low-density cells per well, 10 showed good growth; line PM-B established from one of them showed an AChR epitope and MHC restriction pattern almost identical to PM-A (not shown). Both lines were >96% CD3$^+$ and CD4$^+$, <2% CD8$^+$, and 40–70% HLA class II-positive on the two occasions tested.

The lines were maintained by restimulation of 0.5 × 10^6 T cells plus 2–3 × 10^6 autologous Px and 0.5 μg of r37-429A per ml every 2 weeks and expansion in highly purified human interleukin 2 (Biotest Diagnostics, F.R.G.) every 3–4 days afterward. Responses were assayed on the day of restimulation using 2 × 10^4 T cells, 2 × 10^5 autologous Px (or other APCs), and 0.1–0.02 μg of antigens per 0.2-ml round-bottomed well. The wells were pulsed with 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine after 72 hr, harvested 16 hr later, and assayed on a Betaplate flat-bed liquid scintillation counter (Pharmacia-Wallac).

For some experiments we used as APCs the Epstein–Barr virus-induced (EBV) cell lines listed in Table 1 and, for others, mouse L cells transfected with the Mulcos expression vector carrying multiple copies of DRA and DRB1* 0401 full-length cDNA clones and selected for stable high class II expression (24, 25). To prevent autopresentation of peptides by these class II-positive T cells to each other (26), 5 × 10^6 APCs were "pulsed" with 10 μg of peptide per ml for 2 hr at 37°C. Mitomycin C (40 μg/ml) was added for the final 40 min, before washing. We always included Px from a healthy DR4:Dw4$^+$ control for normalizing responses: negative con-

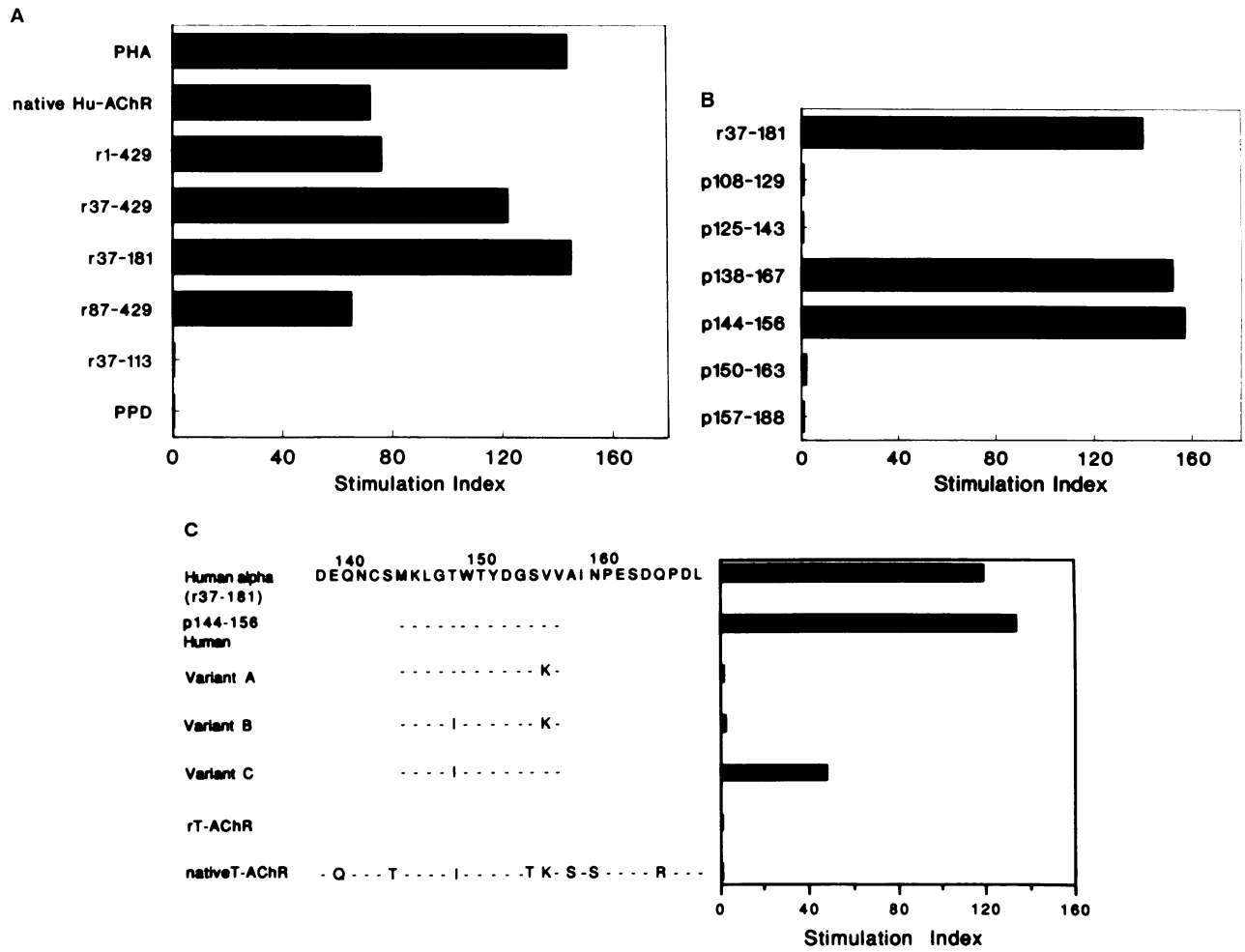

FIG. 1. Identification of the AChR epitope recognized by line PM-A. T-cell proliferative responses to various recombinant (r) human AChR α subunit polypeptides or to synthetic peptides (p) are shown as stimulation indices (SI) or as the increase in cpm above background (Δcpm) after culture with the relevant antigen. SI of 100 correspond to Δcpm of 43,600, 45,600, and 24,300 for *A*, *B*, and *C*, respectively. Standard errors were <5% of the mean unless otherwise stated. PHA was used at 1:1000, PPD was used at 10 μg/ml, and native human (Hu) AChR was used at 10–20 ng/ml. rT-AChR, recombinant *Torpedo* α 37-437, was used at 0.5 μg/ml; native T-AChR was used at 1–5 μg/ml. The sequences are from ref. 15.

trols included DR4:Dw10$^+$ Px and no APCs at all (as explained in *Results*).

RESULTS

AChR Epitope Recognized. The PM-A line was established by stimulation of limiting numbers of thymus cells with the almost full-length recombinant human AChR α subunit r37-429A. After 7–9 weeks, this stimulated as strongly as PHA, whereas PPD (to which this patient also responded) had no effect (Fig. 1). We localized the epitope to the 114–181 region of the AChR α subunit by using two shorter recombinant polypeptides (Fig. 1*A*); the longer of these—r37-181—also stimulated as strongly as PHA. The synthetic peptide p138-167 proved equally potent (Fig. 1*B*), stimulating optimally at 0.3 μM and about 20% maximally even at 3 nM. The shorter p144-156 only stimulated maximally at higher concentrations (>1 μM), whereas none of the adjacent/overlapping peptides elicited any response at all (Fig. 1*B*). p138-167 and r37-181 so consistently evoked maximal responses that these were used as the 100% standard for normalization in later experiments.

PM-A cells were also consistently stimulated well by picomolar concentrations of affinity-purified native human AChR (Fig. 1*A*). Thus this epitope must be processed very efficiently from the native molecule despite the 127—142 disulfide bridge and the glycosylation at Asn^{141}. However, these T cells totally failed to respond to either purified native T-AChR or recombinant *Torpedo* α37-300 or 37-437 (Fig. 1*C*). Further, the activity of the human peptide 144-156 was reduced by converting its Thr^{148} → Ile and abolished by changing Val^{155} → Lys, both as in T-AChR (Fig. 1*C*). Moreover, preincubation with either of these latter peptides failed to block the submaximal (50%) response to a 7 μM pulse of the human 144–156 peptide (not shown). Interestingly, after substitution of Tyr^{151} → Phe, stimulation was minimal also (not shown).

Restricting HLA Class II Isotype and Haplotype. In initial experiments, only anti-HLA-DR (and not anti-DQ or anti-DP) antibodies blocked presentation by autologous Px (Fig. 2*A*). Use of Px from HLA-sharing donors then mapped the restriction to the DR4- and not the DR3-containing haplotype and excluded presentation by other common alleles (Fig. 2*B*). In further confirmation, transfected mouse fibroblasts expressing human DRα and DR4:Dw4 β chains efficiently presented long and short recombinant polypeptides as well as p138-167 (Fig. 2*C*).

Restricting Class II Allele. Only some DR4$^+$ APC donors were effective (Fig. 2*B*), and it transpired that the differences between the several subtypes of DR4 (summarized in Fig. 3 and Table 1) were crucial. As some of these subtypes are very rare in Caucasians (e.g. Dw15), we also checked six of them using EBV lines: with only one exception (see below) the results were very similar (Fig. 3).

Patient PM proved to have the uncommon DR4:Dw14.2 allele, and this and the very similar Dw4 consistently presented optimally, whereas there was no response with DR4:Dw10 (Figs. 2*B* and 3). Still more strikingly, there was also no detectable presentation by DR4:Dw14.1$^+$ cells—whether they were fresh Px or any of four different EBV cell lines (Fig. 3). This all-or-nothing difference in activity between Dw14.1 and Dw14.2 correlates with the Gly/Val dimorphism at position 86—which is the only difference between these two subtypes in the entire antigen-binding region (18–20). This correlation explains almost all subsequent findings. Thus the Val^{86} forms were uniformly inactive, and the only effective APCs of any DR4 subtype had Gly^{86}—including Dw4 and Dw14.2. However, with the rare Dw15 subtype, binding affinities may have been more limiting, since two EBV lines presented well but variably in seven tests, and fresh Px were almost inactive in one experiment.

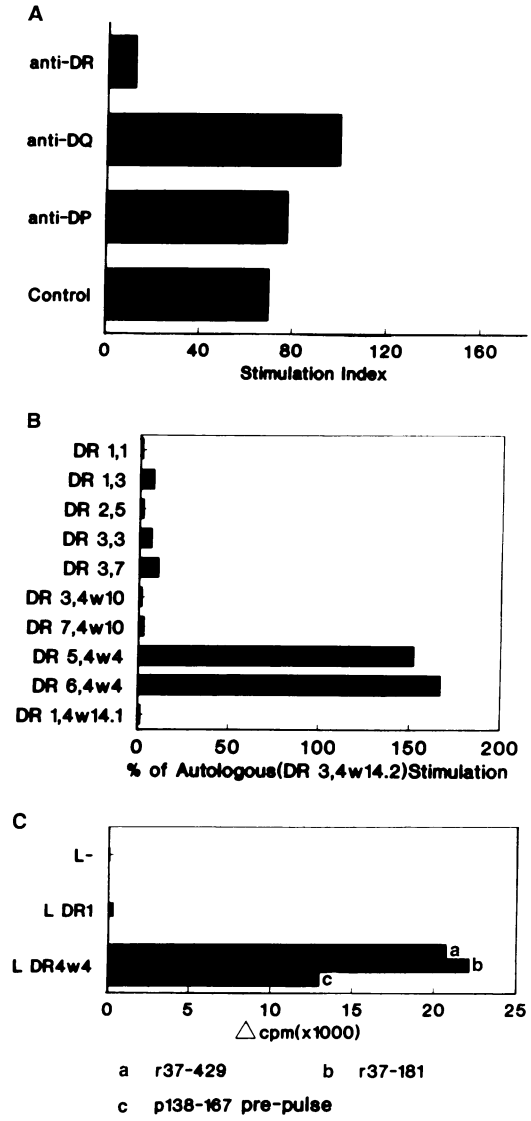

FIG. 2. Identification of the MHC isotype and haplotypes that restrict the presentation of antigens to line PM-A. (*A*) Blocking of the response to r37-429A by culturing with monoclonal antibodies to DR, DQ, or DP isotypes (16, 17). With one-fifth the antigen dose, the smaller response was blocked completely by anti-DR but unaffected by anti-DQ and -DP. Data are representative of two experiments using autologous irradiated PBL (Px). (*B*) Screening of fresh Px from healthy Caucasians with the common HLA-DR alleles and -DR4 subtypes for ability to present r37-181. Data derive from four different experiments and are normalized as the mean % of the Δcpm given by autologous positive control Px in each; this ranged from 40,000 to 160,000 cpm. (*C*) Presentation by mouse L cells transfected with DR α plus DR4:Dw4 β DNA. Line PM-A cells were cocultured with mouse L cells (5×10^4 per well) expressing the indicated class II antigens. A second experiment gave almost identical results: in both, fresh autologous Px presented considerably better than the Dw4$^+$ L cells, presumably because of additional adhesion molecules and/or cytokines such as interleukin 1.

Finally, the additional Ala^{74} → Glu^{74} substitution in Dw13.2 greatly reduced presenting activity, despite the glycine at position 86.

DISCUSSION

Potential Significance for Autoimmune Responses. The DR4 subtype-dependent recognition pattern was shown by lines PM-A and PM-B; their origins from low-density (probably activated) thymus cells and the response to native human

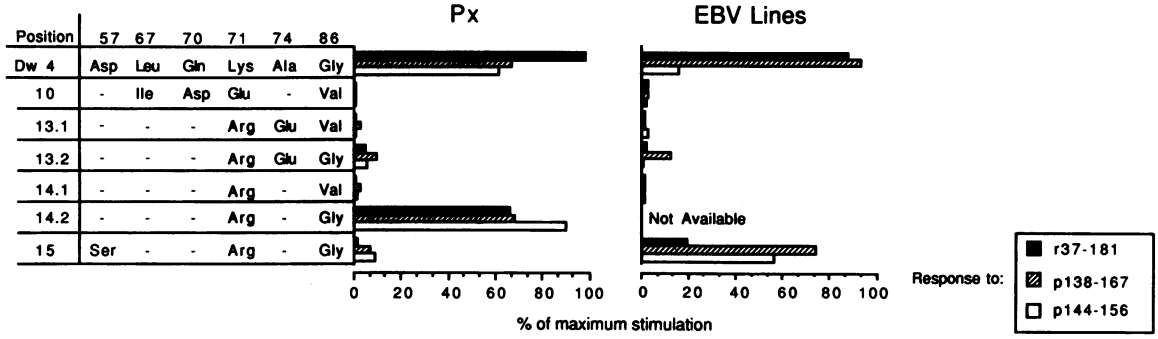

FIG. 3. Presentation by the different subtypes of DR4 and correlation with their sequences (27, 28). A dash indicates identity with Dw4. See Table 1 for details of cells used.

AChR (Fig. 1*A*) suggest that it was disease-related in this patient. Moreover, even 4 years later, her fresh PBL still responded almost as strongly to p138-167 as to r37-429A (SI 4–5)—in parallel with persisting disease activity. Nevertheless, since DR4 with Gly^{86} occurs in <10% of our 155 young-onset myasthenics, this line's recognition pattern cannot predominate in the whole patient population. Interestingly, however, although 65% of these cases are $DR3^+$, we have seen no obvious bias toward DR3-restricted responses to date (9, 10, 12), as illustrated now with this patient—in spite of previous suggestions that class II susceptibility alleles might monopolize presentation of autoantigens. Finally, 27 of these 155 cases were $DR4^+$; they showed a modestly increased proportion of Dw14.1 at the expense of Dw4, compared with either healthy or rheumatoid arthritis subjects (29), but no reduction in Dw10 or Dw13 as in the latter.

Role of Position 86 in Peptide Presentation. The DR4 restrictions of most T-cell clones studied have correlated with the classical T-cell-defined subtypes of DR4 (30). Although a contribution from position 86 has been suggested by results from some other T-cell clones (31, 32), it may have been overlooked with others. It has proved even more clear-cut with the present PM-A line, which detects an all-or-nothing difference in presentation by Dw14.2 (Gly^{86}) and Dw14.1 (Val^{86}). Indeed, the presence of Gly^{86} also largely overrides any potential effects of the $Asp^{57} \rightarrow Ser^{57}$ substitution in Dw15, though not of the $Ala^{74} \rightarrow Glu^{74}$ replacement in Dw13.2. Other residues are clearly crucial too. For example, DR1 β has an identical sequence to Dw14.2 from residues 40–230 but is totally inactive (Fig. 2*B*), presumably because of the major differences in its 9–13 and 26–33 regions.

Variations between MHC alleles show two distinct patterns. In class II molecules, for example, the most striking differences are the clusters of substitutions in the "allelic hypervariable regions" at positions 9–14, 26–38, 57–60, and 67–74 of the β chain, which are thus entirely confined to its first domain (27, 28). By analogy with class I (2), the first two regions (and thus the DR1/DR4 differences) probably contribute to the β strands in the floor of the peptide binding cleft and the others to its α-helical sides. Other variants, by contrast, differ in the first domain only by single amino acid changes—e.g., at position 57 of the DQ β chain (3). Such point substitutions may affect recognition by some T cells equally dramatically (33), which may, in turn, explain the correlation of these dimorphisms with susceptibility to several autoimmune diseases (3), their stabilization in the population, and their maintenance through several primate species (34).

Position 86 is highly conserved in HLA-DRβ, always being either valine or glycine (27, 28). Furthermore, this Gly/Val dimorphism is very widespread, recurring within the DR1, DR2, DR3, DR4, DR5, DRw6, and DRw52 groups, in many of them as the only difference between subtypes (28). Moreover, in every case the same two bases are changed to generate the rarest (15%) of the four glycine codons, changes that have probably recurred through homologous recombination rather than coincident point mutations (35), again implying persistent selection for diversity.

Structural models of class II are largely hypothetical, and the exact location of residue 86 is not known. Its apparent class I equivalent, Tyr^{171}, forms a critical part of the peptide side-chain-binding "A pocket." This is near the end of the α-helix and thus relatively far from these hypervariable regions and from the other influential substitutions at 70–74 and 9–33. We suggest that the unique rotational flexibility or compactness of glycine may permit a conformational state forbidden by valine—or any other residue—that directly or indirectly affects peptide binding and/or T-cell recognition. A particularly dramatic example of indirect conformational consequences of a single Gly → Val substitution has been studied crystallographically in Ha-ras p21. The $Gly^{12} \rightarrow Val^{12}$ mutation by itself is oncogenic, because it affects GTP binding by altering the fine structure of the L4 loop at positions 59–65 (36).

In current models, position 86 could also affect α/β chain pairing—an analogy with Asp^{57} of DQβ that may form a salt bridge with Arg^{79} of DQα at the opposite end of the α-helices (3). A further analogy is that both of these dimorphisms (i.e., at 57 and 86) by themselves define distinct class II alleles/subtypes. We therefore suggest that, whereas clustered hypervariability mainly occurs in regions that directly interact with peptide or T-cell receptor, the single residue dimorphisms predominantly affect conformation and/or chain pairing and so tend to be located at pivotal sites.

Hence, we propose that Gly^{86} permits conformational—and thus peptide binding—options that are foreclosed by other residues. Thus, for example, some peptides might only bind to DR4 molecules that have glycine at position 86, others to those with Val^{86}, and yet others impartially to either: competition would be much more likely within than between these peptide families. Indeed, very recently, Busch and colleagues (37) have found that this dimorphism by itself profoundly affects binding of certain peptides (dependent in one case on Val^{86}) regardless of other variations between DR alleles. However we cannot exclude the alternatives that this substitution primarily affects the conformation of the peptide in the cleft or the recognition of the resulting composite by the T-cell receptor.

Recent reports show that position 86 can play an important role in allorecognition (38, 39). Our present finding that it determines the presentation of a defined epitope opens more opportunities for direct analysis of peptide binding and T-cell stimulation. The widespread occurrence of this dimorphism and its quantifiable effects on antigen presentation demand further functional and crystallographic studies to elucidate

Table 1. Total number of tests in Fig. 3

DR4 subtype	Px No. of donors	Px No. of tests*	EBV cell lines No. of tests*	EBV cell lines Line
Dw4	5	10	3	Boleth
Dw10	3	6	3	YAR
Dw13.1	1	1	1	SSTO
Dw13.2	2	2	4	JHAF, DF, KW
Dw14.1	3	6	7	PE 117, MTF, LS40, BIN 40
Dw14.2	2	8	None available	
Dw15	1	1	7†	HAS 15, HINND

APCs were fresh irradiated PBL (Px) or mitomycin C-treated EBV cell lines. The results have been normalized as for Fig. 2*B*. See Fig. 3.
*Total number of tests.
†With the two Dw15$^+$ EBV lines, the SEMs were 5.4 for r37-181, 16.2 for p138-167, and 21.2 for p144-156.

the relative contributions of the allelic clusters and of these conserved single codon substitutions.

Note Added in Proof. Four sublines of PM-A have all proved to share a single functional VDJβ and VJα combination with the parent line.

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