A Site for CD4 Binding in the \( \beta_1 \) Domain of the MHC Class II Protein HLA-DR1

Jennifer Brogdon, David D. Eckels, Christopher Davies, Stephen White and Carolyn Doyle

*J Immunol* 1998; 161:5472-5480; [http://www.jimmunol.org/content/161/10/5472](http://www.jimmunol.org/content/161/10/5472)

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

**References**

This article cites 60 articles, 19 of which you can access for free at: [http://www.jimmunol.org/content/161/10/5472.full#ref-list-1](http://www.jimmunol.org/content/161/10/5472.full#ref-list-1)

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**

Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
A Site for CD4 Binding in the $\beta_1$ Domain of the MHC Class II Protein HLA-DR1

Jennifer Brogdon, David D. Eckels, Christopher Davies, Stephen White, and Carolyn Doyle

Using a lymphocyte binding assay, we have previously demonstrated that the CD4 protein can mediate cell adhesion by direct interaction with MHC class II molecules. In this report, we have used this assay to test whether synthetic peptides, corresponding to DR$\beta$ sequences, could inhibit CD4-class II adhesion. A peptide derived from sequences within the $\beta_1$ domain (DR$\beta$1–55), as well as two peptides derived from sequences within the $\beta_2$ domain (DR$\beta$121–135 and DR$\beta$141–155), were shown to inhibit CD4-class II adhesion. Inasmuch as a site for CD4 binding in the $\beta_2$ domain had been previously documented, these studies were designed to investigate the role of the $\beta_1$ domain as an additional site of interaction with CD4. Sixteen site-specific mutations were engineered within the $\beta_1$ domain of DR$\beta$1*0101. Several mutations were shown to disrupt CD4-dependent T cell activation. Based on these results, we propose a model for the molecular interaction of CD4 with MHC class II proteins in which both the $\beta_1$ and $\beta_2$ domains of class II interact with the two amino-terminal Ig-like domains of CD4. The Journal of Immunology, 1998, 161: 5472–5480.

The CD4 glycoprotein is expressed on T helper and CTL cells. It recognizes foreign Ags presented by MHC class II proteins on APCs. However, the TCR-$\alpha$-$\beta$ recognizes polymorphic residues in the peptide binding groove. CD4 interacts with monomorphic determinants of MHC class II molecules. It is likely that Ag receptor recognition requires that CD4 functions as a “coreceptor” that actually contacts the same MHC molecule as the TCR, generating a trimeric complex. In fact, it has been shown that without a productive CD4-class II interaction, the T cell receives a “partial signal,” similar to that seen with “partial agonist” peptides, which rises to altered CD3$\zeta$ and ZAP70 phosphorylation. The colocalization of CD4 and the TCR complex on the effector cell would result in a potent activating signal, thereby requiring a lower Ag density on the APC to activate a T cell (5–8). Similarly, CD8 functions as a coreceptor for T cells recognizing MHC class I molecules on the APC (9) and mediates coreceptor function by prolonging TCR-MHC associations (10). Thus, the coreceptor function of CD4 and CD8 is due to their ability to mediate cell adhesion (11, 12), augment TCR-MHC interactions, and transduce signals through the CD4-associated protein tyrosine kinase, p56$^{1k}$ (13, 14).

Direct evidence for the interaction of CD4 and MHC class II molecules was obtained in our laboratory using a cell binding assay (11). In the initial studies, the human CD4 protein was expressed at high levels in simian CV-1 cells using an SV40-derived viral vector. Human B lymphocytes expressing MHC class II Ag bound to monolayers of CD4-expressing cells, whereas class II-negative B cells did not. Abs directed against CD4 and class II were shown to inhibit cell binding. These experiments provided the first evidence that CD4 and class II proteins can interact to mediate cell adhesion, even in the absence of the TCR. Chinese hamster ovary (CHO) cells expressing the human CD8a protein were used similarly to show that CD8 interacts with MHC class I molecules (12); we have recently established stable cell lines expressing the human CD4 protein in CHO cells for use in the cell-adhesion assay (15). Of note, high-level expression of CD4 (or CD8) was a critical parameter in these experiments.

The CHO-CD8 cell binding assay was used to demonstrate that residues within the membrane proximal $\alpha$-$\beta$ domain of HLA-A2.1 were critical for CD8-class I adhesion (9, 16, 17). The similarity between MHC class I and class II proteins led to the speculation that CD4 might bind to the membrane proximal $\beta_2$ domain of the MHC class II molecule in a manner analogous to CD8-class I. To test this hypothesis, König et al. engineered mutations at conserved sites within the $\beta_2$ domain of the murine I-A$^d$ molecule (18). Murine L cells, cotransfected with A$^d$ and various mutant A$^b$ cDNAs, were tested for the ability to induce activation of an Ag-specific CD4$^+$ T cell hybridomas. Mutations in the region of the $\beta_2$ domain-encompassing residues 137–143 resulted in the most profound defects in T cell stimulation. This region of the class I1 molecule was further implicated by Cammarota and coworkers, using a solid-phase binding assay in which soluble rHLA-DR4 molecules or HLA-DR-derived peptides bound to immobilized soluble rCD4 (19). Peptides encompassing amino acids 134–148 and 138–152, corresponding to sequences in the $\beta_2$ domain, were shown to bind efficiently to soluble human CD4. These results

*Department of Immunology, Duke University Medical Center, Durham, NC 27710; 1Blood Research Institute, The Blood Center, Milwaukee, WI; and 2Department of Structural Biology, St. Jude Children's Research Hospital, Memphis, TN

Received for publication April 24, 1998. Accepted for publication July 14, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† This work was supported by a Cancer Research Foundation Investigator Award in Immunology (C. Doyle), an American Cancer Society Junior Faculty Research Award (C. Doyle), and the National Institutes of Health (GM46391). J.B. was supported by National Institutes of Health Predoctoral Training Grant 5T32CA09058.

‡ Current address: Section of Immunobiology, Yale University School of Medicine, New Haven, CT.

‡‡ Address correspondence and reprint requests to Dr. Carolyn Doyle, Department of Immunology, Box 3010, Duke University Medical Center, Durham, NC 27710; E-mail address: doyle004@mc.duke.edu

4 Abbreviations used in this paper: CHO, Chinese hamster ovary; HA, hemagglutinin; IMDM, Iscove’s modified Dulbecco’s medium; MFI, mean fluorescence intensity; CLIP, class II-associated invariant chain peptide.
suggested that CD4 interacts with MHC class II molecules in a manner analogous to the CD8-class I interaction.

However, other studies using hybrid class I-class II MHC molecules suggested that residues within the β1 domain of class II might interact with CD4. For example, Golding et al. engineered a chimeric molecule consisting of the β1 domain of the murine I-Aβ class II molecule linked to the α1 domain of the class I molecule, H-2Dβ (20). The recombinant protein, when expressed at the surface of transfected murine L cells, was recognized by class II allospecific cytotoxic T cells. Interestingly, this response could be inhibited by Abs directed against the L3T4 (CD4) molecule on the effector cell. These data are consistent with the β1 domain serving as the target for CD4 interaction and allore cognition, suggesting that residues contained within both the β1 and β2 domains of the class II protein might interact with CD4. Moreover, mutagenesis studies of the CD4 protein revealed that residues in the two membrane distal domains (D1 and D2) mediate CD4-class II interactions (21–25). These results suggest that a broad surface on the CD4 protein is involved during class II associations, likely via interactions with multiple regions of the class II molecule.

To further investigate the hypothesis that CD4 might interact with multiple sites on the class II molecule, the cell binding assay was performed in the presence of synthetic peptides to localize regions of the DRβ polyprotein that might mediate CD4-class II adhesion. Peptides corresponding to sequences within both the β1 and β2 domains were shown to inhibit CD4-class II adhesion in a dose-dependent manner. Inasmuch as a site for CD4 binding in the β2 domain had been previously documented, site-specific mutations were engineered in the β1 domain of the DRβ1*0101 molecule and cDNAs carrying the mutated sequences were expressed in the class II-negative cell lines, T2 and T2.DM (26, 27). These transfectants, expressing mutant DR1 proteins, were analyzed for the ability to stimulate two CD4-dependent T cell clones. Several mutations within the β1 domain were shown to clearly affect T cell recognition. These results implicate this region of the class II molecule, specifically the region encompassing residues 42–56, as a novel site for CD4-class II interaction. Structural analysis reveals that this region lies on the same face of the class II molecule as the previously identified CD4 binding site in the β2 domain, providing a second docking site for the CD4 protein.

Materials and Methods

Cell adhesion assay

The binding of radiolabeled B cells to adherent CHO-DUKX (CD4-negative) or CHO-CD4 cells was performed as previously described (15, 28). Radioactivity was measured in a beta counter and the number of cells bound in each well was quantified by the following algorithm: cells bound = (number of added cells × experimental value (cpm bound)) / total cpm. All of the results presented are averages of triplicate samples. For peptide inhibition studies, the CHO-CD4 monolayers were preincubated with DRβ or control peptides for 12–16 h. The monolayers were then washed, and radiolabeled Raji (MHC class II+) B cells were added for a 4-h assay. The sequence of the hemagglutinin (HA) peptide used as a control is as follows: IDGASGSAFYDKSFYN.

Cell culture

The dhfr CHO cells (CHO-DUKX) were maintained in MEM-a (without ribonucleosides and deoxyribonucleosides; Life Technologies, Grand Island, NY) supplemented with 10% dialyzed FCS (dFCS; Intergen, Purchase, NY), 15 mM HEPES, penicillin/streptomycin, and 20 μg/ml each of thymidine (Sigma, St. Louis, MO), 2′-deoxyadenosine (Sigma), and (−)-adenosine (Aldrich, Milwaukee, WI). CHO-CD4 cells (28) were cultured in MEM-a (Life Technologies) supplemented with 10% dFCS, 15 mM HEPES, P/S, and 0.80 μM methotrexate (MTX, Sigma). The TxB hybrid cell line, T2 (26), and its derivative, T2.DM, transfected with the HLA-DM genes (27), were generous gifts from Dr. Peter Cresswell (Yale University School of Medicine, New Haven, CT). The cell lines were maintained in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies) with 10% FCS (Life Technologies) and 50 μg/ml gentamicin. The T2.DM cell line was also grown in the presence of 500 ng/ml puromycin (Sigma). The DR1-restricted human T cell clones HA-1.4 (29) and AL14–71 (30) were maintained as previously described. Briefly, 10 × 10^6 T cells were stimulated with 15 × 10^6 irradiated HLA-DR1* PBMCs for 7–10 days in IMDM supplemented with 10% pooled human AB serum (Life Technologies) and 20 U/ml human rIL2 (Genzyme, Boston, MA). For HA-1.4, HA peptide (306–320; Multiple Peptide Systems, San Diego, CA) was included at a final concentration of 2 μM. For proliferation assays, T cells were used at least 7 days following stimulation with PBMCs and Ag.

Mutagenesis

A cDNA encoding the full-length DRβ1*0101 chain (1120 bp) was subcloned into M13 mp18, and sdDNA mutagenesis was performed (31). Amino acid residues are numbered according to Tonnelle et al. (32). Mutations were confirmed by sequencing M13 mp18-DRβ sdDNA. Mutant DRβ inserts were excised with BamHI and subcloned into pSrneo (provided by Dr. Hamid Band, Dana-Farber Cancer Institute, Boston, MA). Proper orientation was determined by restriction enzyme digestion with Xhol and ScaI. A cDNA BamHI fragment encoding nucleotides 480–1660 of HLA-DRb was cloned into pSrneo, and proper orientation was determined by restriction enzyme digestion with BseHI and XhoI.

Generation of stable transfectants

Transfection of T2 or T2.DM was performed as described (27) with minor modifications. Briefly, cells in mid-log phase were washed once with room temperature serum-free IMDM and resuspended at 4.0 × 10^6 cells/ml in serum-free medium. Cells (2.0 × 10^5. 0.5 ml) were incubated at room temperature for 10 min with 2 μg pSrneo-DRβ and 20 μg pSrneo-DRα, which had been previously linearized by digestion with the restriction endonuclease BseHI (Life Technologies). Cells were transfected by electroporation at 250 V and 800 μF. Following electroporation, cells were allowed to recover in cuvettes for 10 min at room temperature and then were cultured in IMDM/10% FCS for 24–48 h at 37°C. 5% CO2. Transfectants were pelleted by centrifugation, resuspended in fresh medium plus 1 ng/ml active G418 (Life Technologies), and plated in 96-well flat-bottom plates on irradiated feeder layers (subconfluent HeLa cells, 5400 rad). Approximately 2–3 wk later, clones were expanded and screened by FACS analysis to identify class II-positive clones.

Indirect immunofluorescence and FACS analysis

Cells (0.5–1.0 × 10^6) were incubated with 100 μl primary Ab for 30 min on ice. Normal rabbit serum (10% heat-inactivated; Life Technologies) was included with the primary Ab to block FcR binding. After incubation with primary Ab, cells were washed with cold FACS buffer (PBS plus 2% heat-inactivated newborn calf serum) and then incubated for 10 min at room temperature and then were cultured in IMDM/10% FCS for 24–48 h at 37°C. 5% CO2. Transfectants were pelleted by centrifugation, resuspended in fresh medium plus 1 ng/ml active G418 (Life Technologies), and plated in 96-well flat-bottom plates on irradiated feeder layers (subconfluent HeLa cells, 5400 rad). Approximately 2–3 wk later, clones were expanded and screened by FACS analysis to identify class II-positive clones.

Indirect immunofluorescence and FACS analysis

Cells (0.5–1.0 × 10^6) were incubated with 100 μl primary Ab for 30 min on ice. Normal rabbit serum (10% heat-inactivated; Life Technologies) was included with the primary Ab to block FcR binding. After incubation with primary Ab, cells were washed with cold FACS buffer (PBS plus 2% heat-inactivated newborn calf serum) and then incubated for 30 min on ice with fluorescein-conjugated goat anti-mouse IgG (Cappell, Durham, NC). Cells were washed again and then fixed with 3.7% formaldehyde for flow cytometric analysis using a FACScan (Becton Dickinson, San Jose, CA). The P6/12.2 (American Type Culture Collection, Manassas, VA) anti-V9.1 Fab, as the transfectant parent cell line was used as a negative control for these experiments. For Fig. 2, the following equation was used to assess differences in mAb recognition of a particular mutation (mut) relative to recognition of the wild-type (wt) molecule (MFI, mean fluorescence intensity; neg, negative; percent reactivity = [{mut MFI(mAb) - mut MFI(neg)} / mut MFI(neg)] / [wt MFI(mAb) - wt MFI(neg)] × 100).

Abs and peptides

TSL1/22 (33), L243 (34), L227 (34), and 2.06 (35) were obtained from American Type Culture Collection. The following Abs and FACS analysis to identify class II-positive clones.

Preparation of Fab

The SupT1 #21 hybridoma secretes an anti-CD4 mouse IgG1 mAb and was grown as ascites fluid in nude BALB/c mice. Fab were generated using an ImmunoPure IgG1 Fab Preparation Kit (Pierce, Rockford, IL). The final
Cell Harvester (Cambridge Technology, Cambridge, MA), and quantification by comparison with a known standard was analyzed for purity by SDS-PAGE and Coomassie staining. Quantification of Fab was estimated by comparison with a known standard and judged to be approximately 1 µg/µl.

**Proliferation assays**

Stimulators were irradiated at 24,000 rad with a ^137^Cs gamma irradiator and then treated with mitomycin C (50 µg/ml) and resuspended in culture medium (IMDM, 10% human serum, glutamine, and gentamicin). Cells were plated at 10^4/well in 96-well U-bottom plates. HA peptide (306–320) was included at final concentrations of 0.08–10.0 µM. For the Fab-inhibition experiments, a fixed concentration of 1 µM HA peptide was used. Stimulator/peptide mixtures were preincubated at 37°C for 3–4 h before the addition of responders. Responder cells (HA1.A or AL14.71) were preincubated with #19 Fab for 30–45 min at 37°C, as indicated, and plated at 2 × 10^4/well to obtain a final stimulator/responder ratio of 5:1. Assays were pulsed with 1 µCi/well of ^3H^thymidine during the last 12–18 h of a 72-h incubation. Samples were harvested onto glass fiber filters with a PhD Cell Harvester (Cambridge Technology, Cambridge, MA), and quantification of ^3H^thymidine incorporation was determined by scintillation spectrosopy with a Tri-Carb Liquid Scintillation Analyzer (Packard, Downers Grove, IL).

**Computer modeling**

The coordinates of HLA DR1 (DRα/DRβ1*0101) complexed with the HA (306–318) peptide were provided by Dr. Lawrence Stern (Harvard University, Cambridge, MA). Ribbon diagrams were generated on a Silicon Graphics workstation (Mountain View, CA) with Molscript (41).

**Results**

Peptides derived from the sequences of DRβ inhibit cell adhesion mediated by CD4 and MHC class II molecules

A lymphocyte binding assay was used to screen synthetic peptides for the ability to inhibit CD4-class II-mediated cell adhesion. These peptides, each 15 amino acids in length, were comprised of overlapping sequences from the entire length of the DR2β polypeptide (39, 40). For these experiments, CHO cells expressing high levels of human CD4 (CHO-CD4) were preincubated with peptide for 12 h. The monolayers were washed extensively and radiolabeled Raji (MHC class II+) B cells were added. After 4 h, the monolayers were washed and the number of bound Raji B cells, in the presence or absence of a given peptide, was determined.

In the first series of experiments, a peptide concentration of 200 µg/ml was used (Fig. 1A). A number of peptides appeared to have some inhibitory effects; i.e., the binding of Raji cells was diminished in the presence of peptide. However, only those five peptides (indicated with arrows in Fig. 1A) that caused the greatest inhibitory effects were synthesized in sufficient quantity for more extensive studies. The lymphocyte binding assay was then repeated using a range of peptide concentrations (0–500 µg/ml). In these experiments, one β₁-domain peptide (DRβ41–55; Fig. 1B) and two β₂-domain peptides (DRβ121–135 and DRβ141–155) showed a concentration-dependent inhibitory effect (data not shown). These regions are highlighted in green on a ribbon diagram depicting the structure of HLA-DR1 (see Fig. 6A). Notably, these peptides map to the same face of the class II molecule and are within highly conserved regions of the protein that are localized to solvent-exposed loops (42).

The results obtained with β₁-domain peptides 21–35 and 41–55, as well as an irrelevant control (HA) peptide, are shown in Fig. 1B. The β41–55 peptide displayed the most convincing, dose-dependent inhibition. Moreover, these residues are localized within a solvent-exposed loop underlying the short α helix, juxtaposed to C-terminal residues of the antigenic peptide, and would be highly accessible for intermolecular interactions. Indeed, residues 49–55 are involved in the dimer/dimer interface in the crystallographic “superdimer” (43). By contrast, inhibition by the β21–35 peptide was less efficient. Moreover, inasmuch as these residues constitute part of the peptide binding site, they are less likely to contribute to CD4-class II interactions. Thus, the B41-55 region was targeted for site-specific mutagenesis to investigate our hypothesis that the β₁ domain of class II might serve as a site for CD4 interactions.

**Site-directed mutagenesis and re-expression of HLA-DR1β mutant polypeptides**

The DNA sequences encoding the HLA-DRβ1*0101 were targeted for site-directed mutagenesis. This particular allele was chosen because the DRβ1*0101 protein had been crystallized and its molecular structure determined (43). Mutations engineered in the region encompassing residues 42–56 of the β₁ domain are the focus of this report. Sixteen site-specific mutations were made: SA42, DK43, VL44, GR45, EK46, RQ48, AG49, VA50, TR51, EK52, EM52, LV53, GA54, RQ55, and PT56 (the first letter corresponds to the sequence encoded within DR2β1 domain peptides 21–35 and 41–55, as well as an irrelevant control (HA) peptide, are shown in Fig. 1B). The β41–55 peptide displayed the most convincing, dose-dependent inhibition. Moreover, these residues are localized within a solvent-exposed loop underlying the short α helix, juxtaposed to C-terminal residues of the antigenic peptide, and would be highly accessible for intermolecular interactions. Indeed, residues 49–55 are involved in the dimer/dimer interface in the crystallographic “superdimer” (43). By contrast, inhibition by the β21–35 peptide was less efficient. Moreover, inasmuch as these residues constitute part of the peptide binding site, they are less likely to contribute to CD4-class II interactions. Thus, the B41-55 region was targeted for site-specific mutagenesis to investigate our hypothesis that the β₁ domain of class II might serve as a site for CD4 interactions.

The DNA sequences encoding the HLA-DRβ1*0101 were targeted for site-directed mutagenesis. This particular allele was chosen because the DRβ1*0101 protein had been crystallized and its molecular structure determined (43). Mutations engineered in the region encompassing residues 42–56 of the β₁ domain are the focus of this report. Sixteen site-specific mutations were made: SA42, DK43, VL44, GR45, EK46, RQ48, AG49, VA50, TR51, EK52, EM52, LV53, GA54, RQ55, and PT56 (the first letter corresponds to the sequence encoded within DRβ1*0101, the second letter to the residue after mutagenesis). The strategy was to mutagenize those residues conserved within all human alleles, as well as those conserved between human and murine β polypeptides. Charged residues were also changed, either to the sequence of the corresponding residue in the class I molecule or, in the case of identity, to an alanine residue. Finally, several mutations were introduced that corresponded to rare natural polymorphisms in this
region of the molecule (44). For example, a tyrosine to arginine change was engineered at position 51. Tyrosine is encoded at position 51 in all known DRβ alleles, except in the DRβ3*0201 (0202) alleles where it has been replaced with arginine. Two additional changes that are naturally occurring in the DRβ4*0101 allele, L44 and Q48, were also introduced into the DRβ1*0101 molecule by site-directed mutagenesis.

The mutated HLA-DRβ cDNAs, each containing a single amino acid alteration, were expressed in the T2 (26) and T2.DM cell lines (27) by cotransfection with a plasmid-containing cDNA sequences encoding the nonpolymorphic HLA-DRβ protein and the gene encoding neomycin resistance. Transfectants were generated by transfection of T2 with cDNAs encoding the DM1 protein and the gene expressing neomycin resistance. Transfectants were generated in both T2 and T2.DM cell lines to assess the ability of the mutant molecules to bind peptide and traffic through the normal class II pathway. The T2 mutant cell line, derived by gamma irradiation, carries a homozygous deletion of a large portion of the MHC locus on chromosome 6. All of the class II structural genes (HLA-DR, -DP, -DQ), as well as the genes encoding HLA-DMα and -DMβ, map to the region that is missing in the T2 mutant cell line. HLA-DM expression has been shown to be essential for the release of class II-associated invariant chain peptides (CLIP) and efficient loading of antigenic peptide onto nascent class II molecules in specialized endosomal compartments, termed the MIIC (45, 46). T2.DM was generated by transfection of T2 with cDNAs encoding the DMα and β polypeptides, thereby restoring the ability of newly synthesized class II proteins to acquire antigenic peptides (47).

Following transfection, G418-resistant cells were stained with the DR-specific mAb LB3.1 (37). Successive rounds of cell sorting generated populations that were homogeneous and expressed levels of the mutant class II proteins that were comparable to transfectants expressing the wild-type HLA-DRβ1 protein (see MFI, Fig. 2). To assess the overall integrity of the mutant DR1 molecules, the sorted populations were stained with a panel of class II-specific mAbs. While the specific epitopes recognized by each of the mAbs is unknown, specificity for α- or β-chain, or αβ complex, has been previously analyzed (48). LB3.1, L243, and SG157 recognize αβ complex; L227 recognizes the β-chain of all HLA-DR and -DP proteins, while 2.06 and B8.12.2 see monomorphic determinants on HLA-DR. Finally, IVA12 likely recognizes a determinant conserved among all class II molecules as it binds to all three isotypes of human class II proteins (HLA-DR, -DP, and -DQ). Immunostaining and FACS analysis of transfectants expressing the mutated DR proteins is summarized in Fig. 2. Binding of the DR complex-specific mAb, L243, was not affected by any of the mutations and, thus, was used as a reference mAb for determining the level of reactivity obtained with the other mAbs. In general, most of the mutations had little or no effect on mAb binding, although mutations RQ55 and PT56 slightly diminish the binding of mAb L227. Likewise, GR45 and RQ48 marginally affect recognition by mAbs B8.12.2 and SG157, respectively. Inasmuch as the effects of these four mutations are limited to a single mAb epitope, they likely represent localized effects on mAb binding, rather than major structural alterations in the mutant class II molecules. In contrast, EK46 was the only substitution that dramatically affected Ab binding. Although the loss of three mAb epitopes suggests a substantial effect on the structure of the protein, this residue could simply be part of the contact surface for binding of each of these three Abs. Thus, with the exception of the EK46 mutation, alterations in this region of the β1 domain of HLA-DR had little or no effect on the overall structural integrity of the protein based on Ab recognition and cell surface expression.

β1 mutations do not affect peptide binding or class II trafficking

As the mutations in this region of the β1 domain are proximal to the peptide binding site, it was important to assess the ability of the mutant class II molecules to present peptide(s). A convenient way to measure CLIP dissociation and peptide loading in the MIIC is by CerCLIP.1 mAb staining; the CerCLIP.1 mAb recognizes the αβ-CLIP biosynthetic intermediate (27). In fact, when class II proteins are expressed in T2, virtually all of the surface class II proteins are stained with the CerCLIP.1 mAb, whereas in T2.DM the CerCLIP.1 mAb staining when the mutant molecule was expressed in T2 and a loss of CerCLIP.1 staining when the mutant protein was expressed in T2.DM. Whereas the former would demonstrate efficient invariant chain association with nascent class II molecules (wild-type or mutant), the latter would provide evidence for efficient binding of cognate peptides.

Transfectants, expressing wild-type or mutated DRβ proteins in T2 (Fig. 3A) and T2.DM (Fig. 3B), were stained with the LB3.1 and CerCLIP.1 mAbs; FACs profiles of a representative subset of transfectants are shown. For each T2 transfectant, the LB3.1 and CerCLIP.1 staining profiles were virtually superimposable, demonstrating that the vast majority of class II molecules expressed at the cell surface contain the CLIP peptide and that the mutated class II proteins bind CLIP as efficiently as the wild-type protein. In contrast, T2.DM transfectants are not stained by the CerCLIP.1 mAb (Fig. 3B), demonstrating that the functional expression of HLA-DM results in an equivalent loss of DR-bound CLIP for wild-type and mutant DR molecules. Taken together, these data suggest that these particular mutations in the β1 domain of DR do not affect CLIP or nominal peptide binding. This is consistent with the recent crystallographic analysis of αβ-CLIP complexes. These
Studies showed that αβ heterodimers bind CLIP in the peptide binding groove, in a manner identical to cognate peptide binding (49). Thus, association of the mutant DR molecules with DM and the subsequent exchange of CLIP for cognate peptide is unaffected by these mutations in the β1 domain of HLA-DR.

Mutations in the β1 domain of DR1 disrupt CD4-dependent T cell recognition

It was initially our intention to use the lymphocyte binding assay to assess CD4 binding of mutant β1 class II proteins. However, this was not feasible for two reasons. First, the lymphocyte binding assay requires exceptionally high levels of class II expression; our transfectants expressed 5–10-fold fewer molecules than did the B lymphoblastoid cell line, Raji (data not shown). Second, high levels of nonspecific binding were detected when untransfected parental T2 (or T2.DM) cell lines were incubated with CHO-CD4 monolayers. Therefore, as an alternative approach to studying the functional consequences of the β1 mutations on CD4-class II interactions, T2.DM transfectants were used as APCs for two CD4-dependent, DR1-restricted human T cell clones: HA1.4, which recognizes the HA peptide (HA 306–320) in the context of DR1 (29), and AL14.71, which is alloreactive for DR1 (30).

HA1.4 and AL14.71 were initially characterized for CD4-dependence during T cell activation (see Fig. 5A). Of note, it was necessary to use nearly five times more Fab to inhibit the response of the HA1.4 T cell clone (as described in Materials and Methods). The proliferation of HA1.4 and AL14.71 was inhibited by 75–80% by the anti-CD4 Fab, whereas a half-maximal response for the alloreactive clone, AL14.71, required a ratio of 2.5–4.0:1 (stimulators:responder cells). For each experiment, the panel of mutant β1 transfectants, along with a representative wild-type transfectant, was tested for the ability to stimulate HA1.4 and AL14.71. Each mutant was tested. It was found that the level of class II expression on the APCs (at least within the range seen with this panel of transfectants) did not correlate with the degree of T cell activation as determined with multiple wild-type T2.DM/DR1 subclones expressing varying levels of class II (data not shown). While the overall magnitude of the response varied slightly with each wild-type transfectant, the amount of Ag required to reach the half-maximal proliferative response of the T cell clones was similar; the half-maximal response for HA1.4 required a peptide dose between 0.25–1.0 μM, whereas a half-maximal response for the alloreactive clone, AL14.71, required a ratio of 2.5–4.0:1 (stimulators:responder).
was analyzed with each T cell clone in three to five independent experiments. Strikingly, both the HA1.4 and AL14.71 T cell clones responded similarly to the panel of $\beta_1$ mutations. The results are summarized in Table I. Three patterns of proliferative responses to mutant-bearing APCs were seen: enhanced, impaired, or equivalent to wild-type levels of proliferation (Fig. 5 and Table I). Fig. 5A shows a representative subset of the $\beta_1$ mutants that were able to stimulate HA1.4 (upper panel) and AL14.71 (lower panel) as well as or better than wild-type DR1. Clearly, the alanine to glycine change at residue 49 and the nonconservative mutation of glycine to arginine at position 45 had no effect on the ability of the mutant-bearing APCs to stimulate either clone. Moreover, several substitutions such as valine to alanine at position 50 or glutamic acid to methionine at position 52 appeared to augment CD4 function, particularly at higher Ag doses. However, because these residues are found on a solvent-exposed loop and therefore are of ambiguous orientation, it is difficult to predict how they might enhance or stabilize interactions with the CD4 molecule.

The response patterns of a third class of mutant-bearing APCs, those which poorly stimulated both T cell clones, are depicted in Fig. 5B. Again, the four mutants that fall into this category (EK46, GA54, RQ55 and PT56) exhibited the same phenotype for both HA1.4 clone, the most severe effects were seen with GA54 and PT56, both of which required approximately 50-fold more peptide to reach the half-maximal response of wild-type. Less dramatic, but still significant, were the effects of the EK46 and RQ55 mutations, which required 5–10-fold more peptide to reach the half-maximal response of wild-type. For the AL14.71 alloreactive T cell clone, these same four mutations required 4–10-fold more stimulators to reach the half-maximal response of wild-type transfectants. Although the shift in the dose-response curve for AL14.71 is not as pronounced as for HA1.4, this may simply reflect a difference in

**FIGURE 5.** Mutations in the $\beta_1$ domain of DR1 disrupt CD4-dependent T cell recognition. A and B, upper panel, HA peptide (306–320) was titrated against T2.DM/DR1 wild-type and mutant transfectants. Lower panel, T2.DM/DR1 wild-type and mutant transfectants were titrated against a fixed number of AL14.71 responder cells ($2 \times 10^4$/well). Activation of both T cell clones was assessed by standard proliferation assays as described in Materials and Methods. Open squares, stimulation with T2.DM/DR1 wild-type. Closed squares, stimulation with the indicated T2.DM/DR1 mutant transfectant.

**Table I. Summary of CD4-dependent T cell responses to mutant DR$\beta_1$ stimulators**

<table>
<thead>
<tr>
<th>DR$\beta$ Mutation</th>
<th>HA1.4$^a$</th>
<th>AL14.71$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT DR1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SA42</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>DK43</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>VL44</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>GR45</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>EK46</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>RQ48</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>AG49</td>
<td>+ ++</td>
<td>+ +</td>
</tr>
<tr>
<td>VA50</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>T151</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>TR51</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>EM52</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>LV53</td>
<td>++ ++</td>
<td>+</td>
</tr>
<tr>
<td>GA54</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>RQ55</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PT56</td>
<td>+/−</td>
<td>+</td>
</tr>
</tbody>
</table>

$^a$ +/−, Mutant stimulators required 10-50-fold more peptide to reach the half-maximal response of wild-type stimulators; +++, 5-10-fold more peptide than wild-type; ++, equivalent to wild-type; ++++, 2-5-fold less peptide than wild-type.

$^b$ 1/2, AL14.71 required 4-10-fold more mutant stimulators to reach the half-maximal response of wild-type; ++, equivalent to wild-type; ++++, at least twofold fewer stimulators than wild-type.
the overall Ag density necessary for T cell activation. Neverthe-
less, the significant reduction in CD4-dependent T cell acti-
vation from a localized group of mutations (GA54, RQ55 and PT56) im-
plies this region in the $\beta_1$ domain of DR1 as a site for CD4

Discussion

Previous studies have shown that CD4 interacts with residues 137
and 142 in the $\beta_2$ domain of the murine class II molecule, I-A$^d$
(18), and with the same region (amino acid residues 134–152) in
the $\beta_2$ domain of HLA-DR4 (19). In addition, König et al. iden-
tified residues in the $\alpha_2$ domain of the I-A$^d$ molecule that contrib-
ute to CD4 coreceptor function (51). Our studies provide further
evidence that a region in the $\beta_2$ domain of class II, namely residues
134–155 of HLA-DR, mediates CD4-class II associations. In ad-
inition, we have identified a novel site for CD4 binding in the $\beta_1$
domain (residues 41–56). Both sites within the $\beta_1$ and $\beta_2$
domains are solvent exposed and highly conserved between human and mu-

line class II molecules, consistent with the idea that CD4 associ-
ates with monomorphic determinants of class II. The inhibitory
effects of the $\beta_2$-domain peptides are not entirely surprising in that
this region of the protein is analogous to the region of class I that
interacts with CD8 (9). Moreover, this region clearly mediates
CD4 function, as previously described (18, 19).

The results of the $\beta_1$ peptide (DRB41–55) inhibition studies and
subsequent functional analysis of site-directed mutants of this re-


gion were particularly intriguing. These studies revealed that four
residues in the $\beta_1$ region of HLA-DR1 (E46, G54, R55, and P56)
were critical for CD4 coreceptor function (Fig. 6B). Mutation of
glycine to alanine at position 54 or proline to threonine at position
56 greatly diminished CD4-dependent T cell activation by both the
alloreactive (AL14.71) and peptide-specific (HA1.4) T cell clones.

The location of these residues at the beginning of the $\beta$-chain $\alpha$

helix (Fig. 6B), as well as their biophysical character (i.e., the

flexibility of glycine and the propensity of proline to form kinks),
suggests a role in determining secondary structure. As such, mu-
tation of either residue may affect the position(s) of neighboring
residues on the $\alpha$ helix that point up toward the TCR or into the
groove. Nevertheless, any disruption in overall structure must be
subtle inasmuch as staining with a panel of mAbs revealed no
discernible differences. Moreover, both mutants bound CLIP when
expressed in T2. The observation that the DR1/CLIP association is
of high affinity (52) and that CLIP binds DR1 in a manner that is
identical to nominal peptide binding to DR1 (49) suggests that
peptide binding by GA54 and PT56 is not severely compromised.

Whereas structural effects on the nearby peptide-binding pockets
or on TCR recognition are possible, our data suggest that mutation
of G54 or P56 disrupts a site on the HLA-DR1 molecule recog-
nized by CD4.

Mutation of glutamic acid at position 46 also abrogated T cell
recognition by both T cell clones and completely disrupted three of
seven Ab epitopes. Interestingly, this mutation did not affect rec-
ognition by conformation-specific mAbs, such as L243, implying
that $\alpha\beta$ polypeptide interactions are intact. Furthermore, like
GA54 and PT56, the EK46 mutant protein binds CLIP when ex-
pressed in T2, implying that peptide binding is not affected. As
such, the inhibition of T cell proliferation seen with APCs express-
ing the EK46 mutant may simply be a consequence of disrupting
its association with CD4. Previous studies by two other groups
found that peptides corresponding to residues 35–46 within HLA-
DR-blocked CD4/class II adhesion and T cell proliferation (53–
55). Both our peptide inhibition studies and the EK46 mutant data
are consistent with these findings. As with the three $\beta_1$ mutants
discussed above, recognition by both AL14.71 and HA1.4 is dis-
rupted by the arginine to glutamine mutation at position 55. Based
on the protein structure of HLA-DR1, R55 is highly accessible for
intermolecular associations and does not appear to contribute to the
localized secondary structure (Fig. 6B). Indeed, crystallo-

graphic analysis of HLA-DR1 reveals that R55 is capable of such
interactions as it forms an intermolecular salt bridge with E52 of
the neighboring class II molecule.

Analysis of three different crystal forms of HLA-DR1 depicted
dimers of the class II $\alpha\beta$ heterodimer, often referred to as “super-
dimers,” that interfaced at two major contact regions (43). One
area spans residues 49–55 in the $\beta_1$ domain and contacts the same
region of the second $\alpha\beta$ heterodimer. The other dimer interface
occurs between residues in the $\beta_2$ and $\alpha_2$ domains of opposing class II $\alpha\beta$ heterodimers. It remains unclear whether the class II superdimers revealed in these studies represent physiologic structures or whether the dimer structure is merely an artifact of the crystallization process. However, one might speculate that superdimers are important for T cell activation by allowing for more efficient cross-linking of TCRs and, subsequently, enhanced intracellular signaling. The region of the $\beta_2$ domain that is thought to comprise the dimer interface (49–55) has been extensively mutagenized in our studies.

Brown et al. postulate that residues 49–51 form a stable hydrophobic interface while E52 and R55 are involved in reciprocal intermolecular salt bridges (43). Disruption of a key residue that participates in formation of the salt bridge (i.e., mutation of either E52 or R55) might destabilize the molecular superdimer and, as a consequence, diminish T cell activation. If this were true, then mutation of either E52 or R55 should affect T cell recognition. Indeed, mutation of E52, either to lysine or methionine, has little or no effect on CD4-dependent T cell recognition, whereas mutation of R55 clearly inhibits CD4-dependent T cell activation from both clones. Thus, the decreased recognition of RQ55 is very likely due to impaired CD4 recognition, as opposed to disruption of intermolecular dimers. Moreover, results from other mutations in the dimer interface also argue against the “superdimer” model in that mutations can be made (e.g., AG49, VA50, TI51) that do not appear to affect CD4-class II interactions. While these data do not formally exclude the possibility that intermolecular dimers are important for CD4 binding, it is difficult to imagine how potentially monomeric DR molecules (e.g., EK52) would be stronger ligands for the TCR than dimeric DR molecules. Rather, the simplest interpretation is that this region forms part of the site of physical interaction with the CD4 molecule.

Both mutational analyses (56) and the recent crystallization of the CD8$\alpha$-HLA-A2/peptide complex (57) demonstrated that electrostatic interactions are involved in the association of the HLA-A2 $\alpha_2$ and CD8 $\alpha_2$ domains. Moreover, the crystal structure revealed that CD8$\alpha$ homodimers contact both $\alpha_2$ and $\alpha_3$ domains of the class I heavy chain. Our data suggests that both the $\beta_1$ and $\beta_2$ domains of the class II molecule are similarly involved in its interaction with CD4, although cocystalization of CD4 and MHC class II molecules will be necessary to confirm this at a molecular level. Interestingly, the crystal structure of class I and CD8$\alpha$ revealed that interactions were mainly electrostatic in nature and limited to side-chain contacts. This confirmed mutational studies that had described three charged/polar $\alpha_2$-domain residues (Q115, D122, and E128) that affect class I-CD8$\alpha$ interactions (56). Likewise, our data suggests that polar amino acids, E46 and R55 in the $\beta_2$ domain of DR1, influence the association of MHC class II with CD4.

Finally, from the structure of HLA-DR1 it is apparent that affected residues within the $\beta_1$ and $\beta_3$ domain lie on the same face of the molecule and are solvent-accessible. Moreover, several of these residues are capable of electrostatic interactions with residues on the CD4 protein that would stabilize the protein-protein interactions. Similar studies predict a crucial role for hydrophilic interactions between coreceptors and their ligands to stabilize intermolecular associations (9, 57–59). In our model for CD4-class II interactions, both the membrane-distal $\beta_2$ as well as the membrane-proximal $\beta_3$ domains of class II would interact with the two amino-terminal Ig-like domains of CD4. Mutagenesis studies of the D1 and D2 domains of CD4 are consistent with this hypothesis in that many residues encompassing a broad surface on the CD4 protein appear to be involved in its interaction with class II proteins (21–25). While the physiologic significance of MHC class II “superdimers” remains to be conclusively demonstrated, our data argues they are not important for the activation of two human CD4-dependent T cell clones. Likewise, the recently described crystal structure of the murine class II molecule, I-E^K, reveals molecular dimers that are quite distinct from the DR1 superdimers (60). While the DR dimer can be anchored in the membrane in an upright position with sites for TCR and CD4 binding exposed, the structure of the I-E^K dimer would dictate that these molecules would lie nearly flat on the membrane surface. These studies of the I-E^K molecule bring into question the biologic relevance of the superdimers described in the original DR1 crystal structure.

Acknowledgments

We thank Mike Cook and Alan Fisher for flow cytometry, Drs. Lisa Denzin and Peter Cresswell (Section of Immunobiology, Yale University School of Medicine) for cell lines and thoughtful discussion, and Dr. Jack L. Strominger (Harvard University) for his support at the initial stages of this work.

References


