An Ion Pair in Class II Major Histocompatibility Complex Heterodimers Critical for Surface Expression and Peptide Presentation*

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In this report we demonstrate that the ion pair Arg-80α and Asp-57β, located in the peptide-binding site of nearly all class II major histocompatibility complex (MHC) proteins, is important for surface expression and function of the murine class II heterodimer I-Ad. Charge reversal at either of these two residues by site-directed mutagenesis generated mutant class II molecules that failed to appear at the cell surface. This defect in surface expression was partially reversed when the invariant chain was present or when the mutants were paired with the corresponding charge-reversed variant of the opposite chain. Surprisingly, surface expression was restored when cells expressing the single-site mutants were cultured at reduced temperature. In addition, the substitution of Asp-57β with residues found in alleles of class II molecules associated with diabetes resulted in heterodimers that were inefficiently expressed at the cell surface and presented foreign peptide poorly. Together, these results demonstrate that the formation of a salt-bridge between Arg-80α and Asp-57β is required for efficient surface expression of class II MHC molecules, therefore representing an important step in the assembly and transport of functional class II heterodimers to the cell surface.

T lymphocytes respond to foreign antigens by detecting peptide fragments of those antigens bound to products of the major histocompatibility complex (MHC) and displayed on the surface of antigen presenting cells (reviewed in Ref. 1). The three-dimensional structures of both class I and II MHC molecules complexed with self- or foreign peptide antigens have recently been solved, revealing much about the molecular basis of peptide-binding to MHC molecules (2–8). Structurally, both classes of molecules are similar, as predicted by sequence comparison (9); however, they differ in fine detail, especially in the peptide-binding site (7, 8). These differences partly account for the distinct manner in which each class of molecule binds peptide. In class I molecules, residues forming the "ends" of the peptide-binding site bury the termini of bound peptides (3–6). In contrast, in class II molecules, both ends of the peptide-binding site are "open," allowing the termini of bound peptides to extend out of the site (7, 8). Consequently, peptides bound to class I molecules are predominantly 8–10 residues in length (10–14), whereas peptides bound to class II molecules tend to be 13–25 residues in length (15–17).

In both classes of MHC molecules, the presence of bound peptides influences their tertiary structure and efficiency of surface expression on the cell surface. The binding of self- and foreign peptides to the class I heavy chain promotes the folding, assembly, and surface expression of class I molecules by stabilizing the class I complex (18, 19). Similarly, peptide binding to class II molecules influences the conformation, stability, and surface appearance of class II molecules (20–24). Thus, detailed functional analysis of the peptide-binding sites of both classes of molecules will be necessary to understand the role of peptide binding in determining the tertiary structure, and hence, surface expression of MHC molecules and subsequent display of foreign antigens to the immune system.

We have been interested in the biochemical basis of recognition of peptide antigens bound to MHC molecules by T cells. We have characterized the T cell receptor (TCR) expressed by the murine T cell clone D5 (25), which recognizes arsonate (Ars)-conjugated peptides presented by the class II molecule I-Ad (26). We have engineered single-site substitutions in the putative MHC/peptide antigen-binding site of the D5 TCR and have identified several residues that are important in recognition of the MHC-peptide complex (27, 28). Subsequently, we sought to compensate these single-site TCR mutations with complementary mutations in the I-Ad molecule. In the course of these studies, we identified an ion pair, Asp-57 of the β-chain and Arg-80 of the α-chain of I-Ad, that plays an essential role in surface expression of I-Ad as well as peptide presentation.

MATERIALS AND METHODS

Cell Lines—The following T hybridomas expressing the TCR of the T cell clone D5 (25) recognize I-Ad/Ars-Ova (36–50) (26) and have been characterized previously: D5h (29), D5sw (27), and D5w (28). The T cell hybridoma 9C127, specific for I-Ad/Prop (323–339) (30), was kindly provided by Dr. P. Marrack (Immunology Corp., Waltham, MA). RT2.3.HC2 (RT2) cells are stable L cell transfectants expressing I-Ad (32) kindly provided by Dr. N. Braunstein (Columbia University, New York, NY). COS cells (33) were used to transiently express I-Ad α- and β-chains, described below. All cells were grown in complete medium containing Dulbecco’s modified Eagle medium (high glucose), 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml β-mercaptoethanol, 2 mM glutamine, and 10% fetal calf serum (HyClone Laboratories, Logan, UT) in 10% CO2 incubators at 37°C unless indicated. CTL-L2 cells (34) used to assay IL-2 were maintained in complete medium supplemented with rat IL-2 (Collabo-
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DNA Constructions—Plasmid constructions were carried out by standard techniques (35). Plasmids containing Aα-DNA (Aα5-Sal-EXV, [36], Aβ cDNA (Aβx-EXV, [37]), and marine invariant chain cDNA (ml-EXV [37]) were kindly provided by Dr. N. Braunstein. Control plasmid encoding chimeric molecule La2-4(β), which consists of the extracellular domains of the 2B4 TCR β-chain fused to the transmembrane and cytoplasmic domains of the TCR γ-chain (γ-chain in pCD-SRα [38]) was kindly provided by Dr. I. Engel (National Institutes of Health, Bethesda, MD). EcoRI fragments containing Aα and Aβ cDNA were subcloned into the polylinker EcoRI site of the expression plasmid pGLP, kindly provided by Dr. P. H. Houghton (Harvard Medical School, Boston, MA), which is derived from the plasmid pCD and contains an SV40 promoter for transient expression of cDNA in COS cells and fd to generate single-stranded templates for mutagenesis (39). Site-directed mutagenesis was carried out on uracil-labeled, single-stranded templates, as described previously (27), using oligonucleotides listed in Table I to generate variants. Variants were initially screened by analyzing restriction enzyme sites introduced or destroyed by the mutagenic oligonucleotides (Table I). Sequences of variants were confirmed by dideoxynucleotide sequencing the entire α- or β-domain of each plasmid using Sequenase (U. S. Biochemical Corp.).

Transfection of COS Cells—COS cells were transfected essentially as described (40). A total of 2 \times 10^5 COS cells was plated in complete medium 24 h prior to transfection. Cells were washed twice with Dulbecco’s modified Eagle’s medium/10 mM HEPES and cultured with a total of 10 μg each of α- and β-chain plasmid DNA in 6 ml Dulbecco’s modified Eagle’s medium, 10 mM HEPES, 100 μM chlothioquine (Sigma), 250 μg/ml DEAE-dextran (Pharmacia) for 4 h at 37 °C. The DNA solution was removed, and cells were left in 6 ml of shock medium containing Dulbecco’s modified Eagle’s medium, 10 mM HEPES, 10% dimethyl sulfoxide for 3 min at room temperature. shock medium was removed and replaced with 20 ml of complete medium. Cells were cultured for 2 days prior to analysis. As a control, in each experiment COS cells were mock-transfected with medium lacking I-Ad plasmids or were transfected with I-Ad-α-chain plasmid alone. When indicated, 20 μg of plasmid DNA encoding the invariant chain or the co-transfection control molecule 284β was co-transfected along with I-Ad plasmid DNA.

Flow Cytometry—Culture supernatants from B cell hybridomas used as control and I-Aα-reactive mAb for flow cytometry are listed in Table II. All B cells hybridomas were purchased from American Type Culture Collection (Rockville, MD), except K24-199 cells, kindly provided by Dr. D. McKean (Mayo Clinic, Rochester, MN). Cell staining was carried out as described (27) using saturating concentrations of mAb supernatants determined by staining I-Ad-α (transfected RT2 L2 cells (data not shown). Cells were analyzed on a FACScan flow cytometer (Becton Dickinson) using the FACScan software program. Dead cells were excluded from analysis by staining with propidium iodide. Results of flow cytometry experiments are displayed as histograms of fluorescence number versus fluorescence intensity or are expressed as “fluorescence units” (40). A total of 5000 cells was analyzed for each sample. Fluorescence units were calculated as the product of the number of “positive” cells exhibiting fluorescence above a reference point and the mean fluorescence of those cells. The reference point was set such that mock-transfected COS cell populations contained fewer than 1% of total cells with fluorescence above this level when the I-Aα test mAb was used (see Fig. 1). In general, fewer than 1% of total COS cells transfected with I-Aα plasmids displayed fluorescence above this reference point when stained with control mAb. The mean fluorescence of the positive cells was calculated from mean channel fluorescence provided by the FACScan program using the formula: Mean fluorescence 100 [mean channel fluorescence + 256].

Peptide Presentation Assay—A total of 10^5 T cell hybridomas was cultured in triplicate with 2 \times 10^5 transfected COS cells and serial 3-fold dilutions of peptide antigen in 20 μl in 96-microwell plates. After 20 h, supernatants were removed, frozen, and tested for the ability to promote the growth of the IL-2-dependent cell line CTLL-2. Viability of the CTLL-20 cells was measured by their reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Sigma) using a WST-1 microplate reader (Molecular Devices, Menlo Park, CA) by recording OD,750 nm after subtracting OD,560 nm. Arsonate-conjugated Ova peptides spanning residues 33–49 and 33–50, Ars-Ova (33–49) and Ars-Ova (33–50), were prepared as described previously (26). The Ova (33–50) peptide was synthesized by the Tufts University Biopolymer Laboratory (Tufts University, Boston, MA) and purified by high performance liquid chromatography (HPLC). HPLC-purified peptide α d (12–26) was kindly provided by Dr. T. Briner. Relative peptide presentation was defined as the ratio (peptide [WT]-variant)/[peptide [WT]].

| Variant* | Oligonucleotide* | Engineered restriction site |
|----------|-----------------|---------------------------|
| D57Kβ   | GGGGCCGCCGAGAAGGCGGATAC | Introduced Eagl |
| D57Rβ   | GGGGCCGCCGAGAAGGCGGATAC | Introduced Xhol |
| D57Sβ   | GGGGCCGCCGAGAAGGCGGATAC | Introduced SadI |
| D57Tβ   | GGGGCCGCCGAGAAGGCGGATAC | Introduced PstI |
| E59Kβ   | GGCAAGGCCAAATCTGCAGGAC | Introduced Hinfl |
| E66Kβ   | GCGGAGATCTTAAAGGAGCAGGG | Introduced AluII |
| E74Kβ   | CGAACCACCCGCACTGGTGACCA | Introduced Smal |
| D76Kβ   | GCCGAGCTAAGGCCGTCGTC | Destroyed DralI |
| E84Kβ   | CACAACCTAGACCGGAGACCC | Destroyed Apal |
| E87Kβ   | CGAGGGGCCGAAGGCGCA | Destroyed Apal |
| E51Ka   | AGGCTCTAATGTTGCCAAATTT | Destroyed MscI |
| E59Ka   | ATACCTTATTTCAACGAGGTG | Introduced Dral |
| E70Kα   | ATAGCTGACAAATCAACAACA | Destroyed Psrl |
| R80a    | TTGACTAAGGATCAATAATTTC | Destroyed Drdl |
| R80d    | TTGACTAAGGATCAATAATTTC | Destroyed Drdl |

*Variant polyepitides are designated by the amino acid substitution they carry; for example, D57Kβ represents the substitution of Asp-57 in the I-Aα β-chain with Lys, using the single-letter code for amino acid residues and the I-Aα numbering system (9).

Results

We individually substituted Lys for several negatively charged residues in the α-helical regions of the α- and β-domains of the I-Aα heterodimer by site-directed mutagenesis (Table I and Fig. 9). These helices form the “wails” of the peptide-binding site of MHC molecules and, when bound with foreign peptide, are recognized by the TCR. We chose to express these variant I-Aα molecules in COS cells because COS cells can transiently express murine class II molecules even though they fail to express the invariant chain (37). We analyzed expression of these variant I-Aα molecules by flow cytometry using a variety of mAb recognizing different epitopes on I-Ad (Table II). We then tested the ability of the variant class II molecule to present foreign peptides to I-Aα-restricted, antigen-specific T cell hybridomas.

Role of Negatively Charged Residues in Surface Expression of I-Aα and Presentation of Foreign Peptide—Between 10 and 50% of COS cells transiently transfected with wild-type (WT) α- and β-chains of I-Aα displayed significant surface staining with I-Aα-reactive mAb MK-D6 above staining with control mAb (Fig. 1). In contrast, mock-transfected COS cells failed to stain specifically with mAb MK-D6 (Fig. 1). COS cells transfected with I-Aα- or β-chain DNA alone failed to stain with mAb recognizing either I-Aα- or β-chains (see below and data not shown), demonstrating the strict requirement for both α- and β-chains of I-Aα for surface expression of I-Aα heterodimers as previously reported (37).

COS cells expressing WT I-Aα and the 11 variant Lys-substituted heterodimers listed in Table I were analyzed by flow cytometry using several mAb reactive against either the α- or β-domains of I-Aα (Table II). The relative efficiency of surface expression of WT and variant I-Aα molecules was estimated by calculating fluorescence units for each combination of α- and β-chains (Fig. 2). As shown in Fig. 2, of the 11 Lys substitutions, only one, D57Kβ, prevented surface expression of I-Aα,
since neither the α-chain-reactive mAb K24–199 nor any of the
β-chain-reactive mAb recognized COS cells transfected with
this variant. The remaining 10 Lys substitutions did not affect
surface expression of I-Ad since they all reacted with α-chain
mAb K24–199 and at least one β-chain mAb. Of these, seven
(E51K, E59K, E70K, E74K, β, D76K β, E84K β, and E87K β)
had no effect on mAb reactivity: COS cells expressing these
variant heterodimers stained with all mAb tested at intensities
within a factor of three compared to COS cells expressing WT
I-Ad. In contrast, the remaining three substitutions (E59K β,
E66K β, and E69K β) eliminated recognition by one or more of
the β-chain mAb. The E59K β heterodimer failed to stain with
mAb BP107 and N22, the E66K β heterodimer failed to stain
with mAb 34–5–3S, and the E69K β heterodimer failed to stain
with mAb MK-D6, 34–5–3S, BP107, 28–16–8S, and M5/114.
Therefore, Glu-59β, Glu-66β, and Glu-69β are likely to be sol-
vent accessible in I-Ad, in agreement with the orientation of the
corresponding residues in the human class II molecule HLA-
DR1 (7, 8). The loss of reactivity of mAb with these variant I-Ad
molecules agrees with previous reports demonstrating that the
N-terminal half of the β1-domain of I-α molecules contributes to
the epitopes of several mAb (for example, see Refs. 36, 52).
In addition, our results agree with previous epitope mapping
and provide new or more detailed information regarding the
reactivity of several mAb (see Table II).

We determined whether any of the 10 substitutions that
were tolerated with respect to surface expression of I-Ad
affected the presentation of foreign peptides to an I-Ad-restricted
T cell hybridoma. Fig. 3A shows representative experiments
indicating that COS cells expressing each of these variant
heterodimers were able to present Ars-Ova (33–50) to D5h cells.
Fig. 3B summarizes the results of several such experiments.
When the slight differences in surface expression between
the variant and WT I-Ad heterodimers are taken into account
(see Fig. 2), each of these 10 variant heterodimers were within
10-fold as efficient at peptide presentation as WT I-Ad (Fig. 3B).
These results demonstrate that although three of the 10 sub-
stitutions, including E59K β, E66K β, and E69K β, completely
eliminated recognition by at least one mAb, none profoundly
affected peptide presentation.

Mutations at Asp-57 Diminish Surface Expression of T-Ad
and Presentation of Foreign Peptides—As shown above (Fig. 2),
the D57K β substitution impaired surface expression of T-Ad
heterodimers. We further investigated the role of Asp-57 β
in surface expression of T-Ad by substituting Ser, Ala, or Arg at

![Diagram](image_url)
this position (Table I). Ser and Ala were chosen because these residues are found at position β-57 in several natural variants of murine and human class II molecules (9). Fig. 4A shows that variant heterodimers D57Sβ and D57Aβ were expressed on the cell surface but at levels significantly reduced relative to WT I-Adβ. Surface staining of COS cells expressing the variant heterodimer D57Sβ was 21% (± 11%, n = 4) and 23% (± 10%, n = 4) of wild-type levels using mAb K24-199, MK-D6, and M5/114, respectively; for the variant D57Aβ, these values were 18% (± 13%, n = 4) and 21% (± 8%, n = 4), respectively. In addition, the D57Rβ substitution prevented expression of I-Adβ on the surface of COS cells, thus resembling the D57Kβ substitution. Only in experiments in which surface expression of WT I-Adβ was very high, indicative of experiments in which the efficiency of COS cell transfection was high, were we able to detect surface expression of the variant D57Rβ (see below). These results indicate that the identity of the side chain at position 57 in the I-Adβ-β-chain influences surface expression of the I-Adβ heterodimer.

Since the invariant chain has been demonstrated to regulate expression of class II molecules in vivo (53) and enhance surface expression of certain class II molecules containing haplo-type-mismatched α- and β-chains (40), we tested whether co-expression of the invariant chain could enhance expression of WT or variant I-Adβ molecules on the surface of transfected COS cells. In agreement with previous reports (40, 54), co-transfection with the invariant chain had little effect on surface staining of WT I-Adβ by mAb MK-D6 and M5/114 but reproducibly enhanced staining of WT I-Adβ by mAb K24-199 by 2.8-fold (± 0.7-fold, n = 4) (Fig. 4B). Co-transfection of the invariant chain reproducibly enhanced surface staining of variant heterodimers D57Sβ and D57Aβ by mAb K24-199, MK-D6, and M5/114. Enhancement of staining by mAb K24-199 was 14-fold (± 7-fold, n = 4) for D57Sβ and 13-fold (± 5-fold, n = 4) for D57Aβ; by MK-D6 was 3.2-fold (± 0.7-fold, n = 4) for D57Sβ and 2.3-fold (± 0.5-fold, n = 3) for D57Aβ; and by M5/114 was 3.6-fold (± 1.0-fold, n = 4) for D57Sβ and 3.8-fold (± 1.1-fold, n = 4) for D57Aβ. In fact, when the invariant chain was co-transfected, surface staining of these variant heterodimers was nearly as intense as observed with WT I-Adβ (Fig. 4B); for D57Sβ and D57Aβ staining was 89% (± 15%, n = 4) and 78% (± 26%, n = 4), respectively, of wild-type levels using mAb M5/114. Co-expression of the invariant chain reproducibly enhanced expression of variants D57Kβ and D57Rβ to levels slightly above background (Fig. 4B); however, this effect was not observed in experiments in which the efficiency of COS cell transfection was low. Co-transfection with plasmid DNA encoding the chimeric cell surface molecule ZB4β-ζ (38) in place of invariant chain DNA did not restore surface expression of I-Adβ variants substituted at Asp-57β (see below).

The relative efficiency of transfection of WT or variant DNA in these experiments was monitored by co-transfection of DNA encoding ZB4β-ζ. Surface expression of the reporter molecule ZB4β-ζ was approximately equivalent when it was co-transfected with either WT or variant I-Adβ DNA (Fig. 4C), demonstrating that the decreased expression of these variants was not due to nonspecific effects such as toxicity or inhibition of the expression of cell surface molecules.

We tested whether the D57Sβ or D57Aβ substitution had any effect on the presentation of foreign peptides to I-Adβ-restricted T hybridomas in experiments in which surface expression of these variants was made equivalent to WT I-Adβ by co-transfecting the invariant chain. Fig. 5 shows that COS cells expressing variant heterodimers D57Sβ or D57Aβ were at least 25-fold less efficient at presentation of Ars-Ova (33-50) peptide to the D5h T hybridoma compared to WT I-Adβ. These substitutions also diminished presentation of Ova (323-339) peptide to the D5h T hybridoma compared to WT I-Adβ. These results demonstrate that the identity of the side chain at position 57 in the I-Adβ-β-chain not only plays a role in surface expression but also influences peptide presentation.

Charge Reversal at Arg-80α Decreases Surface Expression of I-Adβ and Presentation of Foreign Peptide but Compromises Charge Reversal at Asp-57β—In HLA-DR1, Asp-57β forms an interchain salt-bridge with Arg-76α (8) the residue of human class II molecules corresponding to Arg-80α of murine class II molecules (9). Therefore, peptide binding to Arg-80α does not affect surface expression of I-Adβ by substituting Asp or Glu for Arg-80α. Fig. 6 shows that the variant heterodimer R80Dα was not expressed at levels above background and that the variant R80Eα was expressed at
levels slightly above background; in experiments in which COS cell transfection was more efficient, greater surface expression of the variant R80E was observed. When the invariant chain was co-transfected with I-A\(^d\)- and 2B4\(\beta\)-DNA to detect surface 2B4\(\beta\)-molecules; in this experiment, cells co-transfected with WT \(\alpha\)-chain and the invariant chain serve as control cells staining with mAb H57-597. These results are representative of several independent experiments.

**Fig. 4.** Surface expression of wild-type or variant I-A\(^d\) containing substitutions at Asp-57\(\beta\) in COS cells. COS cells transfected with plasmids encoding the indicated WT \(\alpha\)-chain and WT or variant \(\beta\)-chains were analyzed by flow cytometry using control (14–4-4S) or I-A\(^d\)-reactive mAb. In B and C, cells were co-transfected with control (C) 2B4\(\beta\)- or invariant chain (ii) DNA. In C, COS cells co-transfected with I-A\(^d\) and 2B4\(\beta\)-DNA were stained with TCR \(\beta\)-chain-specific mAb H57-597 to detect surface 2B4\(\beta\)-molecules; in this experiment, cells co-transfected with WT \(\alpha\)-chain and the invariant chain serve as control cells for staining with mAb H57-597. These results are representative of several independent experiments.

**Fig. 5.** Response of D5 T cell hybridoma to antigenic peptide presented by COS cells expressing wild-type or variant I-A\(^d\) containing substitutions at Asp-57\(\beta\). In A, COS cells co-transfected with WT or variant I-A\(^d\) D57S\(\beta\) and D57A\(\beta\) heterodimers and the invariant chain were tested for the ability to present Ars-Ova(33–49) to D5h cells. Relative levels of surface expression of WT and variant heterodimers determined by flow cytometry are shown in the inset. In B, the relative ability of these variants to present antigenic peptides to D5h in independent experiments is summarized as in Fig. 3B.
with the WT \(\beta\)-chain were 50–100-fold less efficient at presentation of Ars-Ova(33–49) peptide to D5h cells than WT I-\(\alpha^d\) when expressed on the cell surface in COS cells co-transfected with the invariant chain. In addition, when matched for surface expression with WT I-\(\alpha^d\), the doubly substituted variant R80E/\(\alpha/D57R\beta\) was also 50–100-fold less efficient at peptide presentation than WT I-\(\alpha^d\) (Fig. 7). These single- and double-site substitutions also decreased presentation of Ova(323–339) peptide to 3DO-54.8 cells (data not shown). Therefore, as do substitutions at Asp-57\(\beta\), substitutions at Arg-80\(\alpha\) affect surface expression of I-\(\alpha^d\) and its ability to present foreign peptides.

Surface Expression of Mutant I-\(\alpha^d\) Heterodimers Is Restored at Reduced Temperature—Thus far we have identified two residues in the peptide-binding site of I-\(\alpha^d\), the substitution of which results in poor surface expression of class II heterodimers. We suspected that the single- and double-site variants we had constructed were poorly expressed on the cell surface because they were defective in an important step in the transport of class II heterodimers to the cell surface. Because exogenously added foreign antigen has been demonstrated to enhance surface expression of normal class II molecules (23), we attempted, with limited success, to enhance the surface expression of these class II variants by culturing transfected COS cells in the presence of high concentrations of Ova(323–339) peptide antigen (data not shown). Subsequently, we tested whether culturing transfected COS cells at reduced temperature could enhance surface expression of variant heterodimers, since normal class I molecules (55) and class II heterodimers (22) that are apparently devoid of endogenous peptides can be expressed on the surface of insect cells, which are grown at 25°C. Also, at reduced temperature class I molecules appear on the surface of RMA-S cells (56), which do not supply foreign peptides to newly synthesized class I molecules and consequently do not express class I molecules on the cell surface at 37°C (18).

Fig. 8 shows that singly substituted variant class II heterodimers containing charge reversal at Asp-57\(\beta\) or Arg-80\(\alpha\), which were expressed poorly at 37°C in the absence of the invariant chain, appeared on the surface of COS cells cultured at room temperature. After room temperature incubation, the level of surface expression of singly substituted charge-reversed variants R80E\(\alpha\) or D57R\(\beta\) was identical to expression of the doubly substituted charge-swapped variant R80E\(\alpha/D57R\beta\) and WT I-\(\alpha^d\) heterodimers, as determined by staining with mAb MK-D6 (Fig. 8A) and \(\alpha\) and \(\beta\)-chain reactive mAb (Fig. 8B). The increase in fluorescence of singly substituted charge-reversed variants at room temperature was due to an increase in both the number of cells expressing I-\(\alpha^d\) as well as the intensity of those cells (Fig. 8A). On the other hand, the decrease in fluorescence of R80E\(\alpha/D57R\beta\) and WT heterodimers (Fig. 8B) was primarily due to the reduction of cells expressing I-\(\alpha^d\) at room temperature, rather than a decrease in the intensity of staining (Fig. 8A). It is important to note that the pattern of staining of heterodimers containing WT or variant \(\alpha\) and \(\beta\)-chains expressed in COS cells at room temperature and at 37°C was similar when tested with the \(\alpha\) and \(\beta\)-chain-specific mAb (Fig. 8B), demonstrating that the differences in temperature did not cause selective changes in the epitopes or conformations of I-\(\alpha^d\) molecules detectable by these mAb. These results demonstrate that surface expression of mutant class II molecules expressed poorly at 37°C in the absence of the invariant chain can be enhanced at reduced temperature.

**DISCUSSION**

We have conducted extensive mutational analysis of negatively charged residues in the peptide/TCR-binding domain of the class II molecule I-\(\alpha^d\), which has been modeled onto the structure of HLA-DR1 (Fig. 9). We have demonstrated the importance of the ion pair Arg-80\(\alpha\) and Asp-57\(\beta\) in both surface expression of and peptide presentation by class II heterodimers. Our results suggest that interactions in the peptide-binding site, including the formation of the salt-bridge between Arg-80\(\alpha\) and Asp-57\(\beta\) and possibly peptide binding, are important for folding and surface expression of class II molecules at 37°C. These results agree with previous studies demonstrating that residues pointing into the peptide-binding site within the \(\alpha\)- and \(\beta\)-domains influence surface expression of class II molecules (32, 36, 57, 58). The mutant class II molecules described here resemble haplotype-mismatched class II \(\alpha\)- and \(\beta\)-chains, which are partially blocked in egress to the medial Golgi compartment (40), and mutant heterodimers that are blocked in a relatively late stage of transport to the cell surface (57). We hypothesize that an important step in the transport of
class II molecules to the cell surface can be blocked by disrupting the salt-bridge between Arg-80 in murine class II α-chains, is present in nearly all class II molecules (9) and is likely to represent an important universal structural feature of class II molecules (8). Based on the structure of HLA-DR1 (8), the formation of this salt-bridge replaces the hydrogen-bonding network found at the end of the peptide-binding site of class I molecules in which the C termini of bound peptides are buried. Asp-57β, present in the β-chain of most class II molecules, occupies a position analogous to Thr-143 conserved in all class I α2-domains; similarly, Arg-80α, conserved in all α-chains of class II molecules, occupies a position analogous to Tyr-84, conserved throughout all class I α1-domains (9). Together Tyr-84 and Thr-143 in class I heavy chains form hydrogen bonds with C termini of bound peptides. The interchain salt-bridge formed between Asp-57β and Arg-80α and the hydrogen bonds they form with the backbone of peptides bound to class II heterodimers might contribute a great deal to the stability of the class II heterodimer and its ability to bind peptides. The reversal of charge in singly substituted mutants D57R, D57K, R80E, and R80D would bring together in close proximity similarly charged residues in the peptide-binding domains of assembling class II heterodimers, potentially destabilizing a portion of the interface between the α- and β-chains and preventing these mutant class II heterodimers from adopting a conformation such that they can bind peptides efficiently. Similarly, the substitution of Ser or Ala for Asp-57β disrupts the salt-bridge, but unlike charge reversal at Asp-57β, this substitution might be less disruptive because only one charged residue, Arg-80α, remains unpaired. However, since the remaining unmutated charged residue is still available to hydrogen bond to the main chain of a bound peptide, the singly substituted mutant class II molecules might be stabilized to some degree when provided with peptide. The pairing of charge-swapped α-chains R80Eα or R80Dα with β-chains D57Rβ or D57Kβ restores a salt-bridge between α- and β-chains; however, these variants might not bind peptides efficiently because hydrogen bonding between residues at these positions and carbonyl oxygen and amide hydrogen atoms of bound peptides is disrupted. Alternatively, the new charge-swapped salt-bridge might not be as strong as the original WT salt-bridge because the I-Aβ microenvironment that stabilizes the WT salt-bridge might destabilize the charge-swapped salt-bridge, as predicted on theoretical grounds (59).

Because the substitutions for Arg-80α and Asp-57β affect
both surface expression of I-A<sup>d</sup> and presentation of peptides to the TCR, we propose that the defect in efficient surface expression of the variant heterodimers results from intracellular editing of the heterodimers as incorrectly folded proteins. Class II α- and β-chain DNA of I-A<sup>d</sup> were synthesized initially in the endoplasmic reticulum as heterodimers complexed with the invariant chain and devoid of tightly bound peptides (21). The invariant chain forms multimers with class II heterodimers (60), precludes the binding of endogenous peptides (61, 62) and may, like low affinity peptide binding, prevent the aggregation and denaturation of peptide-free class II heterodimers at physiological temperature (24). The invariant chain directs the intracellular transport of class II heterodimers (63), which includes passage through the Golgi complex and subsequent entry into the endocytic pathway. In specialized endocytic compartments (64–66), the invariant chain is proteolytically processed and dissociates from class II heterodimers at physiological temperature (24). The invariant chain directs the intracellular transport of class II heterodimers (63), which includes passage through the Golgi complex and subsequent entry into the endocytic pathway. In specialized endocytic compartments (64–66), the invariant chain is proteolytically processed and dissociates from class II heterodimers, resulting in the stable binding of peptides to class II molecules and their transport to the cell surface. Class II heterodimers that fail to acquire peptide undergo aggregation and denaturation, are presumably recognized as misfolded proteins, and are blocked from transport to the cell surface (23). The class II mutants described in this report that are defective in surface expression might be inactivated by aggregation, denaturation, or degradation more rapidly than WT I-A<sup>d</sup> heterodimers. In the presence of the invariant chain, surface expression of these variants is partly restored, perhaps because the invariant chain stabilizes the association of the nascent polypeptides, provides a peptide to the peptide-binding site, or enhances the transport of the variant heterodimers to a subcellular compartment where peptides can be supplied.

In addition, the mutant class II molecules described here resemble class I molecules that fail to appear on the surface of RMA-S cells at physiological temperature but reappear at the cell surface at reduced temperature (56). It is likely that at reduced temperature, both class I and II MHC molecules do not require bound peptide for surface expression because, when expressed in insect cells cultured at reduced temperature, they appear on the cell surface apparently devoid of bound peptide (22, 55). Perhaps at reduced temperature, rates of inactivation of peptide-deficient MHC molecules and the mutant class II molecules described here are decreased. Our results support the hypothesis that both class I and II molecules might be best suited for surface expression at physiological temperature only when bound with peptide.

Finally, the importance of Arg-80<sup>a</sup> and Asp-57<sup>b</sup> in surface expression and the function of I-A<sup>d</sup> might explain why these residues are conserved throughout nearly all class II molecules. Several naturally occurring class II molecules have been identified that contain substitutions at position 57<sup>b</sup>, which are analogous to the variants D57S<sup>b</sup> and D57A<sup>b</sup> described in this report. It is noteworthy that these alleles have been implicated in autoimmune disease. Deviations from Asp-57 in human class II molecule β-chains have been observed in alleles associated with insulin-dependent diabetes mellitus, including DQw3.2, DQw2, DQw1.1, and DQw1.A2H, in which Ser, Val, or Ala has been substituted for Asp-57<sup>b</sup> (67). In the I-A β-chain of non-obese diabetic mice, Ser is substituted for Asp-57<sup>b</sup> (68). Although such alleles have other substitutions in addition to the change for Asp-57<sup>b</sup> that might account for the association...
of these alleles with diabetes, the identity of the side chain at β-chain position 57 appears to determine susceptibility and resistance to diabetes: the presence of Asp appears to be protective against diabetes (67). Expression of these variant forms of class II molecules might result in the presentation of an abnormal array of self- or foreign peptides or an altered conformation of class II molecule on the cell surface. Indeed, self-peptides eluted from IA^7 molecules of non-obese diabetic mice are reportedly distinct from other peptides that bind class II molecules in that they possess acidic C-terminal residues (69). Thus, antagonists directed at the peptide-binding site of the variant class II molecule might be designed that selectively inhibit presentation of these abnormal polypeptides.

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