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Cutting Edge: HLA-DM Functions through a Mechanism That Does Not Require Specific Conserved Hydrogen Bonds in Class II MHC-Peptide Complexes

Zemin Zhou,2*Kari A. Callaway,2*K Dominique A. Weber,† and Peter E. Jensen3**

HLA-DM catalyzes peptide dissociation and exchange in class II MHC molecules through a mechanism that has been proposed to involve the disruption of specific components of the conserved hydrogen bond network in MHC-peptide complexes. HLA-DR1 molecules with alanine substitutions at each of the six conserved H-bonding positions were expressed in cells, and susceptibility to DM catalytic activity was evaluated by measuring the release of CLIP. The mutants αN62A, αN69A, αR76A, and BH81A DR1 were fully susceptible to DM-mediated CLIP release, and βN82A resulted in spontaneous release of CLIP. Using recombinant soluble DR1 molecules, the amino acid βN82 was observed to contribute disproportionately in stabilizing peptide complexes. Remarkably, the catalytic potency of DM with each β-chain mutant was equal to or greater than that observed with wild-type DR1. Our results support the conclusion that no individual component of the conserved hydrogen bond network plays an essential role in the DM catalytic mechanism. The Journal of Immunology. 2009, 183: 4187–4191.

Class II MHC molecules initially assemble with the chaperone invariant chain (Ii)4 followed by transport to endosomal compartments and proteolytic cleavage of Ii, leaving a fragment, CLIP, largely buried in the peptide-binding groove (1). HLA-DM catalyzes CLIP dissociation and peptide exchange reactions in class II molecules, accelerating the loading process for peptide Ags (2–4) and editing the repertoire of peptides presented to CD4+ T cells. DM is a non-polyorphic MHC class II protein that is structurally similar to other class II molecules (5). However, DM does not have the capacity to bind peptide Ags, and it functions as a chaperone-catalyst, stabilizing peptide-free (“empty”) class II molecules (6) and accelerating CLIP dissociation and peptide exchange through a mechanism that involves transient direct physical interaction with class II-peptide complexes. DM accelerates the rate of dissociation of all peptides (7), not just CLIP, but catalytic potency differs depending on the kinetic stability of the complex (7–10) and other less defined features of the complex (11–13). The capacity of DM to differentially “edit” peptide complexes has important biological implications through skewing of the repertoire of foreign and self-peptide complexes available for activation or tolerance induction in CD4+ T cells.

The structural basis for the DM catalytic mechanism remains poorly understood. The general orientation of the physical interaction between DM and substrate MHC class II molecules (i.e., HLA-DR) has been defined by using mutational and cross-linking approaches (14–16). It is likely that DM preferentially binds to and stabilizes a relatively unpopulated conformational isomer of MHC class II-peptide complexes, characterized by a loss or weakening of noncovalent interactions that stabilize peptide binding (7, 17, 18). Two general sets of interactions are largely responsible for peptide binding: 1) peptide sequence-dependent interactions between peptide side chains (anchors) and subsites or “pockets” in the peptide-binding groove; and 2) a conserved hydrogen bond network formed by nonpolymorphic amino acids in the MHC protein and main chain atoms in bound peptide (19). The anchor-pocket interactions are primarily responsible for determining peptide-binding specificity, whereas the conserved hydrogen bond network provides a basal contribution to stability and constrains the orientation of peptide in the binding site. Destabilization of conserved hydrogen bonds has been hypothesized to be a primary component of the DM catalytic mechanism (5, 7, 20, 21). This is attractive because the hydrogen bond network is a conserved feature, consistent with the universal capacity of DM to accelerate the dissociation of peptide complexes. There is strong evidence that the network contributes greatly to stabilizing peptide complexes (22, 23). In addition, this mechanism would account for the results indicating that catalytic potency is inversely proportional to kinetic stability (7). If one or more
conserved hydrogen bonds were the primary target for disruption in the catalytic mechanism, one might predict that the energy of stabilization would be reduced by an approximately constant factor independent of the sequence of the bound peptide. Indeed, Narayan et al. recently proposed that DM specifically targets the hydrogen bond formed by the conserved histidine at position β81 in MHC class II molecules (21). HLA-DR1 molecules with an asparagine substitution at this position were reported to form highly unstable peptide complexes, and peptide dissociation was not further enhanced by DM, possibly because DM cannot further disrupt a hydrogen bond that does not exist in the mutant molecule.

In the present study, two approaches were used to systematically analyze the effect of conserved hydrogen bond-disrupting mutations on DM catalytic potency. We postulated that mutational disruption of specific hydrogen bonds targeted in the catalytic mechanism would result in reduced catalytic potency, consistent with the results reported by Narayan et al. (21). Instead, our results indicate that the conserved hydrogen bond formed by histidine β81 is not a primary target in the DM catalytic mechanism. Indeed, our findings support the conclusion that none of the conserved hydrogen bonds is a critical target necessary for DM-catalyzed peptide dissociation.

Materials and Methods
Expression of mutant DR1 molecules in T2 cells
Full-length DR1α and DR1β (DRA*0101/DRB1*0101) and mutant constructs were cloned into the retroviral vectors pLPCX or pLXSN (Clontech). Constructs encoding full-length DMα- and β-chains were fused with the FMDV-2A sequence (24) by PCR. The DMA-2A-DMB construct was cloned into the retroviral vector MigR1, which has a GFP marker driven by an internal ribosomal entry site. The T2 and Phoenix cell lines were obtained from the American Type Culture Collection. High-titer retroviral supernatants were generated by transfection of Phoenix cells with pLPCX-DMα, pLXSN-DMβ or MigR1-DMα-2A-DMB (25). T2-DR1α-β double positive cell lines were obtained by DRα retroviral infection and puromycin selection, followed by DRβ retroviral infection and G418 selection (Invitrogen). To coexpress DM, cells expressing wild-type (WT) or mutant DR1 were infected with DMA-2A-DMB retrovirus and sorted for GFP expression with a FACS Vantage cell sorter (BD Biosciences).

Abs and flow cytometry
Fluorophore-conjugated mAbs to HLA-DR (L243), CLIP (CerCLIP), and HLA-DM (MaP.DM1) and isotype-matched negative control mAbs were purchased from BD PharMingen. Cell surface staining was performed with a combination of mAbs according to the standard procedures. Intracellular staining was performed using FITC/Cytoperm kit according to the manufacturer’s instructions (BD Biosciences). Stained cells were analyzed on a FACS Scan flow cytometer (BD Biosciences) and data were analyzed with FlowJo 8.4 software.

Expression and purification of soluble (s) DR1 (sDR1) and sDM
Stable S2 transfectants expressing sDR1A (residues 1–192) and sDR1B (residues 1–198) were induced for 7 days in BD BaculoGold Max-XP serum-free medium (BD Biosciences). Stained cells were analyzed on a FACScan flow cytometer (BD Biosciences). sDR1 complexes were formed by incubating sDR1 (5–10 μM) with 50 or 250 μM peptide in 10 mM citrate-phosphate buffer (pH 5.0) and 150 mM NaCl. After an overnight incubation at 37°C, complexes were purified by DRα peptide dissociation in cells
We mutated the amino acids in MHC class II molecules that participate in conserved hydrogen bonds to evaluate the effect on DM catalytic activity. As an initial approach, mutant HLA-DR1 molecules were expressed in T2 lymphoid cells in the presence or absence of sDM. T2 cells express endogenous Ii but not DR or DM. Retroviral expression constructs were generated encoding DR1 molecules with alanine substitutions for each of the three conserved MHC class II α-chain amino acids that form hydrogen bonds, αN62A, αN69A, and αR76A (Fig. 1), thus preventing the
formation of specific hydrogen bonds. Cell lines were generated expressing comparable levels of total and cell surface DR1. High levels of CLIP were present on the surface of cells expressing WT DR1 in the absence of DM (Fig. 2). Spontaneous dissociation of CLIP from DR1 is inefficient, and DM is required to catalyze CLIP dissociation and replacement with other peptides. CLIP expression was markedly reduced on cells coexpressing DM with WT DR1, reflecting normal DM catalytic activity. High CLIP levels were also present on cells expressing each of the α-chain mutant DR1 molecules, αN62A, αN69A, or αR76A, and this phenotype was reversed in cells coexpressing DM (Fig. 2). Thus, none of the five conserved hydrogen bonds formed by the amino acids αN62, αN69, and αR76 is critical for the stable association of CLIP with DR1, and each mutant is fully susceptible to DM-catalyzed CLIP dissociation.

To evaluate the conserved hydrogen bonds formed by the MHC class II β-chain (Fig. 1), alanine substitutions were engineered at each of the three conserved positions to generate βW61A, βH81A, and βN82A DR1 molecules. The mutants were observed to assemble efficiently with Ii (data not shown). In the absence of DM, high levels of CLIP were present on cells expressing βW61A or βH81A DR1 (Fig. 3). By contrast, CLIP was completely absent from the surface of cells expressing βN82A DR1, suggesting that the bidentate hydrogen bonds formed by βN82 play a disproportionate role in stabilizing CLIP-DR1 binding. CLIP levels were markedly reduced on cells coexpressing DM and βH81A DR1 (Fig. 3), demonstrating that DM can efficiently catalyze CLIP dissociation from DR1 complexes lacking the βH81 hydrogen bond, a result that was further confirmed with the asparagine substitution mutant βH81N (supplemental Fig. S1), directly contradicting the conclusions of Narayan et al. (21). βH81A DR1 molecules in DM-expressing cells were stable in SDS detergent, evidence that CLIP is exchanged for high affinity peptides in these cells (data not shown). We were also interested in determining whether the histidine at β81 influences the pH-dependence of DM-catalyzed peptide binding, which is optimal at acidic endosomal pH (2–4). The βH81A and βH81N mutations had little or no effect on the pH-dependence of DM-catalyzed peptide binding (supplemental Fig. S2).

By contrast to the βH81 mutations, βW61A DR1 molecules appeared to be partially resistant to DM-mediated CLIP dissociation. This was true even when subpopulations that gated for identical expression of DR and DM were analyzed. Thus, the hydrogen bond formed by βW61 was a candidate target in the DM catalytic mechanism. The capacity of DM to catalyze CLIP dissociation from βN82A DR1 could not be evaluated with this approach because of the high rate of spontaneous dissociation, leaving βN82 as an additional candidate.

**Effect of MHC class II β-chain mutations on DM catalytic potency**

To further evaluate the role of conserved hydrogen bonds involving the MHC class II β-chain in the DM catalytic mechanism, we generated soluble recombinant DR1 and βW61A, on the surface of T2 cells expressing WT or mutant DR1 molecules in the absence (bottom panels) or presence (middle panels) of DM was measured by flow cytometry. GFP fluorescence was determined by flow cytometry as a surrogate measure of DM expression (top panels).
βH81A, and βN82A mutant proteins. A fluorescence polarization assay was used to quantify the impact of DM on the kinetics of peptide dissociation. A DM concentration-dependent acceleration of the rate of peptide dissociation was observed with WT DR1 complexes containing the high affinity peptide HA (Fig. 4a). The βH81A mutation had essentially no effect on the intrinsic stability of the DR1-HA complex (Table I), and the mutation did not reduce the potency of DM in catalyzing peptide dissociation (Fig. 4, c, f, and g). Indeed, catalytic potency was somewhat greater for βH81A compared with WT DR1. Thus, the hydrogen bond formed by βH81 does not play a major role in stabilizing DR1-peptide complexes and is not an essential target in the DM catalytic mechanism.

The βW61A mutation reduced peptide complex stability with an ∼5-fold increase in the intrinsic HA dissociation rate (Table I). However, DM catalytic potency was not reduced, even after normalization for the effect on the intrinsic peptide dissociation rate (Fig. 4, d, f, and g). These results contrasted with the finding that CLIP release from βW61A DR1 was relatively resistant to DM in cells (Fig. 3). We considered the possibility that DM might selectivity catalyze the dissociation of HA but not CLIP from βW61A DR1. However, DM was observed to catalyze the dissociation of a variant CLIP peptide from βW61A DR1 (Fig. 4c) with potency similar to that observed for HA (Fig. 4f). Full-length βW61A DR1-CLIP complexes were also highly susceptible to DM (data not shown). Although we did not observe any gross alteration in endosomal colocalization of βW61A DR1 with DM (data not shown), it seems likely that this mutation affects the capacity of DR1-CLIP complexes to interact optimally with DM in T2 cells, either by impacting colocalization in membrane subdomains or through an effect on trafficking kinetics (26).

Strikingly, the βN82A mutation was observed to increased the intrinsic HA dissociation rate by >3000-fold (Table I). This is consistent with the spontaneous release of CLIP observed with this mutant expressed in cells (Fig. 3). Previous studies with mouse class II molecules support the idea that βN82 may in general have a dominant role in stabilizing MHC class II-peptide complexes (23, 27). Remarkably, despite the very rapid spontaneous dissociation of HA from βN82A DR1, DM further accelerated peptide dissociation (Fig. 4, b and f). The catalytic potency was similar to that observed with WT DR1, even with data normalized for intrinsic rates (Fig. 4g). Thus, the two hydrogen bonds formed by βN82 appear to be critical for stabilizing DR1-peptide complexes, yet they are not essential targets in the DM catalytic mechanism.

### Table I. Peptide dissociation rates for mutant DR1 molecules

<table>
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<tr>
<th>DM (nM)</th>
<th>0</th>
<th>250</th>
<th>500</th>
<th>1000</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR1 (HA)</td>
<td>1.9 × 10^-7</td>
<td>8.2 × 10^-7</td>
<td>1.2 × 10^-6</td>
<td>2.0 × 10^-6</td>
<td>3.7 × 10^-6</td>
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<tr>
<td>N82A (HA)</td>
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<td>3.0 × 10^-5</td>
<td>4.9 × 10^-6</td>
<td>7.4 × 10^-6</td>
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<td>H81A (HA)</td>
<td>1.5 × 10^-7</td>
<td>1.9 × 10^-6</td>
<td>3.3 × 10^-6</td>
<td>6.0 × 10^-6</td>
<td>1.1 × 10^-5</td>
</tr>
<tr>
<td>W61A (HA)</td>
<td>1.1 × 10^-6</td>
<td>8.5 × 10^-6</td>
<td>1.3 × 10^-5</td>
<td>2.0 × 10^-5</td>
<td>3.5 × 10^-5</td>
</tr>
<tr>
<td>W61A (CLIP)</td>
<td>1.3 × 10^-5</td>
<td>8.5 × 10^-5</td>
<td>1.3 × 10^-4</td>
<td>2.1 × 10^-4</td>
<td>3.3 × 10^-4</td>
</tr>
</tbody>
</table>

* Rates are expressed in units of s^-1.

### References