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

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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

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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 βH81A 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 MHC molecules, accelerating the loading process for peptide Ags (2–4) and editing 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 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 pLCX or pLXSN (Clontech). Constructs encoding full-length DM α- 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 pLCX-DR, pLXSN-DRB or MigR1-DMα-2A-DMB (25). T2-DRα-DMβ double positive cell lines were obtained by DrEx 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 FACSVantage 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 BD Cytofix/Cytoperm kit according to the manufacturer’s instructions (BD Biosciences). Stained cells were analyzed on a FACSVerse flow cytometer (BD Biosciences). Data and analysis were performed using 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) and data were analyzed with FlowJo 8.4 software.

**Peptide labeling and sDR1-peptide complex formation**

The peptides HA (PRFVKQNTLRLAT) and CLIP (VSKMRMATPLLMQ) were commercially synthesized with the N terminus acetylated. Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester was used to covalently attach the Alexa Fluor 488 fluorophore to the lysine residue (underlined in the sequences above) in the peptide. Labeled peptides were purified by reverse-phase chromatography and labeling was confirmed by MALDI mass spectrometry. sDR1-peptide 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 on a TSK-GEL G3000SW analytical gel filtration column and concentrated with a TS K-200 plate reader equipped with polarizers and two 485-nm (±20 nm) bandwidth filters for excitation and two 535-nm (±25 nm) bandwidth filters for emission. A total of 25 flashes were used for each reading and the integration time was set to 20 μs. The program i-Control was used to collect data and to calculate the fluorescence polarization, p, which is defined as p = (I// − G I//) / (I// + G I//), where I// and I⊥ are the intensity of the emission at polarizations both parallel and perpendicular to the excitation source, and G is a factor to correct for instrumental differences in detecting emission components. The experiments containing complexes of WT, W61A, or H81A DR1 were monitored for 1000 cycles of 452 s each. For assays containing the s88/HA-N82A complex, the reactions were monitored for 1000 cycles of 9 s each in the absence of sDM and for 600 cycles of 3 s each in the presence of sDM. Rate constants were obtained by fitting the data points to the single exponential decay equation $p = Ae^{−kt} + C$ using Prism 4.0 (GraphPad Software).

**Results and Discussion**

**Susceptibility of mutant HLA-DR molecules to DM-catalyzed CLIP 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 DM. 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
To evaluate the conserved hydrogen bonds formed by the MHC class II \( \beta \)-chain (Fig. 1), alanine substitutions were engineered at each of the three conserved positions to generate \( \beta W61A, \beta H81A, \) and \( \beta N82A \) 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 \( \beta W61A \) or \( \beta H81A \) or \( \beta N82A \) DR1 (Fig. 3). By contrast, CLIP was completely absent from the surface of cells expressing \( \beta N82A \) DR1, suggesting that the bidendate hydrogen bonds formed by \( \beta N82A \) play a disproportionate role in stabilizing CLIP-DR1 binding.

CLIP levels were markedly reduced on cells coexpressing each of the \( \alpha \)-chain mutant DR1 molecules, \( \alpha N62A, \alpha N69A, \) or \( \alpha R76A, \) and this phenotype was reversed in cells coexpressing DM with WT DR1, reflecting normal DM catalytic activity. High CLIP levels were also present on cells expressing each of the \( \alpha \)-chain mutant DR1 molecules, \( \alpha N62A, \alpha N69A, \) or \( \alpha R76A, \) and this phenotype was reversed in cells coexpressing DM with WT DR1, reflecting normal DM catalytic activity. High CLIP levels were also present on cells expressing each of the \( \alpha \)-chain mutant DR1 molecules, \( \alpha N62A, \alpha N69A, \) or \( \alpha R76A, \) and this phenotype was reversed in cells coexpressing DM with WT DR1, reflecting normal DM catalytic activity.

To further evaluate the role of conserved hydrogen bonds involving the MHC class II \( \beta \)-chain in the DM catalytic mechanism, we generated soluble recombinant DR1 and \( \beta W61A, \) on the pH-dependence of DM-catalyzed peptide binding (supplemental Fig. S2).

By contrast to the \( \beta H81 \) mutations, \( \beta W61A \) 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 \( \beta W61 \) was a candidate target in the DM catalytic mechanism. The capacity of DM to catalyze CLIP dissociation from \( \beta N82A \) DR1 could not be evaluated with this approach because of the high rate of spontaneous dissociation, leaving \( \beta N82A \) as an additional candidate.

**Effect of mutations in conserved positions in the DR1 \( \alpha \)-chain on susceptibility to DM-mediated CLIP release.** Expression of DR and CLIP 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 with mAbs L243 (DR) and CerCLIP (CLIP). Total cellular expression of DM was measured in permeabilized cells by flow cytometry using the mAb MaP.DM1 (top panels).

**FIGURE 2.** Effect of mutations in conserved positions in the DR1 \( \alpha \)-chain on susceptibility to DM-mediated CLIP release. Expression of DR and CLIP 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 with mAbs L243 (DR) and CerCLIP (CLIP). Total cellular expression of DM was measured in permeabilized cells by flow cytometry using the mAb MaP.DM1 (top panels).

**Effect of MHC class II \( \beta \)-chain mutations on DM catalytic potency**

To further evaluate the role of conserved hydrogen bonds involving the MHC class II \( \beta \)-chain in the DM catalytic mechanism, we generated soluble recombinant DR1 and \( \beta W61A, \)

**FIGURE 3.** Effect of mutations in conserved positions in the DR1 \( \beta \)-chain on susceptibility to DM-mediated CLIP release. Expression of DR and CLIP 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).

**FIGURE 4.** Effect of mutational disruption of DR1 \( \beta \)-chain hydrogen bonds on DM catalytic potency. a–e, Preformed complexes (50 nM) of Alexa Fluor 488-labeled HA or CLIP peptides bound to mutant sDR1 were incubated with excess unlabeled peptide (20 \( \mu \)M) and various concentrations of soluble DM (pH 5.0) at 37°C. Peptide dissociation rates were measured using a fluorescence polarization assay as described in Materials and Methods. f, Data for mutant sDR1 molecules are compared with WT sDR1 in a log-log plot of peptide dissociation rates vs DM concentrations. g, DM-catalyzed rate enhancements normalized for differences in intrinsic peptide dissociation rates.
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 βH81A 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 selectively 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. 4e) 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 increase 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.

### References


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

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Rates are expressed in units of s⁻¹.


Supplemental Figure Legends

Fig. S1. Asparagine substitution at position 81 in the DR1 beta chain does not affect susceptibility to DM-mediate CLIP release. Expression of DR and CLIP on the surface of T2 cells expressing βH81N DR1 molecules in the absence (middle panel) or presence (right panel) of DM was measured by flow cytometry with mAbs L243 (DR) and CerCLIP (CLIP). A retroviral vector encoding full-length DRβ-H81N was generated using pLXSN-DRb-H81A as a template with primers (DRβ-H81N-F: 5’-GGTGGACACCTACTGCAGAagCAACTACGGGTTGG -3’ and DRβ-H81N-R: 5’-CCAACCCCGTAGTTGgtTCTGCAGTGAGGTGTCCACC -3’), and the product was confirmed by DNA sequencing. T2 cells stably expressing the mutant DR1 heterodimer in the absence or presence of DM were generated as described in Materials and Methods.

Fig. S2. Replacement of the histidine at position 81 in the DR1 beta chain does not change the pH dependence of DM-catalyzed peptide binding. Wild type or mutant purified soluble recombinant DR1 molecules (50 nM) were incubated with 1 μM biotinylated MAT peptide in the present or absent of 1 μM purified soluble DM in a buffer containing 0.2% NP-40 and 40 mM citrate/Na2HPQ4 (pH 3.0-7.0), in a total volume of 30 μl, for 3 hours at 37°C. Following the incubation, samples were pH neutralized and DR-bound peptide was measured using a europium fluoroimmunoassay as previously described (1, 2). No peptide binding was detected under these experimental conditions with βN82A DR1, which forms very unstable peptide complexes.

