



Published in final edited form as:

J Immunol. 2009 October 1; 183(7): 4187–4191. doi:10.4049/jimmunol.0901663.

HLA-DM functions through a mechanism that does not require specific conserved hydrogen bonds in class II MHC-peptide complexes¹

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Abstract

HLA-DM catalyzes peptide dissociation and exchange in class II MHC molecules through a mechanism that has been proposed to involve 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 release of CLIP. The mutants α N62A, α N69A, α R76A, and β H81A DR1 were fully susceptible to DM-mediated CLIP release, and β N82A resulted in spontaneous release of CLIP. Using recombinant soluble DR1 molecules, amino acid β N82 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.

Introduction

Class II MHC molecules initially assemble with the chaperone invariant chain (Ii) 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 antigens (2–4) and editing the repertoire of peptides presented to CD4⁺ T cells. DM is a nonpolymorphic MHC class II protein that is structurally similar to other class

¹This work was supported by National Institutes of Health Research Grants AI30554 and AI33614

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II molecules (5). However, DM does not have the capacity to bind peptide antigens 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 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; peptide sequence-dependent interactions between peptide side chains (anchors) and subsites or “pockets” in the peptide-binding groove, and a conserved hydrogen bond network formed by non-polymorphic 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 results indicating that catalytic potency is inversely proportional to kinetic stability (7). If one or more conserved hydrogen bond was 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 β 81 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 β 81 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 DR1 α and DR1 β (DRA*0101/DRB1*0101) and mutant constructs were cloned into retroviral vectors pLPCX or pLXSN (Clontech). Constructs encoding full-length DM α and β chains were fused with FMDV.2A sequence(24) by PCR. The DMA-2A-DMB

construct was cloned into retroviral vector MigR1, which has a GFP marker driven by an internal ribosomal entry site. T2 and Phoenix cell lines were obtained from American Type Culture Collection. High-titer retroviral supernatants were generated by transfection of Phoenix cells with pLPCX-DRA, pLXSN-DRB or MigR1-DMA-2A-DMB (25). T2-DR α -DR β double positive cell lines were obtained by DR α retroviral infection and puromycin selection, followed by DR β retroviral infection and G418 selection (Invitrogen). To co-express DM, cells expressing wild type or mutant DR1 were infected with DMA-2A-DMB retrovirus and sorted for GFP expression with a FACSVantage Cell Sorter (Becton Dickinson).

Antibodies and flow cytometry

Fluorophore-conjugated mAbs to HLA-DR (L243), CLIP (CerCLIP), 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 Cytotfix/Cytoperm™ kit according to the manufacturer's instructions. Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson) and data were analyzed with FlowJo 8.4 software.

Expression and purification of soluble DR1 (sDR1) and soluble DM (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 media (BD Biosciences) with 1 mM CuSO₄. Cell culture supernatant was collected by centrifugation and sDR1 purified with L243 mAb affinity chromatography and aggregates were removed using a TSK-GEL G3000SW analytical gel filtration column. Soluble DM was purified from supernatants of S2 transfectants as described (4,14).

Peptide labeling and sDR1-peptide complex formation

The peptides HA (PRFVKQNTLR_LAT) and CLIP (VSKMRMATPLL_MQ) 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 (AF488) fluorophore to the lysine residue 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 μ M) with 50 or 250 μ M peptide in 10 mM citrate-phosphate buffer pH 5.3 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 micron centrifugal device.

Fluorescence polarization peptide dissociation assays

To measure peptide dissociation rates, 50 nM sDR1 complexes containing AF488-labeled peptide was incubated with 20 μ M unlabeled peptide in the absence or presence of sDM (0.25–2 μ M). Assays were performed at 37°C in 10 mM citrate-phosphate buffer pH 5.0, 150 mM NaCl, and 0.05 % Tween-20 in a final volume of 75 μ l. All reactions were carried out in Corning 384 well low flange black flat bottom polystyrene NBS microplates. To prevent evaporation during measurements, 10 μ l of mineral oil was layered over the reactions and the plate was covered with a VIEWseal plate seal (Greiner Bio-One). Fluorescence polarization measurements were made using a Tecan Infinite F200 plate reader equipped with polarizers and two 485 nm (\pm 20 nm) bandwidth filters for excitation and two 535 nm (\pm 25 nm) bandwidth filters for emission. A total of 25 flashes were used for each reading and the integration time was set to 20 μ sec. The program i-Control was used to collect data and to calculate the fluorescence polarization, *p*, which is defined as

$p = \frac{I_V - GI_H}{I_V + GI_H}$, where I_V and I_H 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 were monitored for 1000 cycles of 432 sec. For assays containing the $^{488}\text{HA-N82A}$ complex, the reactions were monitored for 1000 cycles of 8 sec in the absence of sDM and for 600 cycles of 3 sec 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 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 wild type (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 co-expressing 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 co-expressing DM (Fig.2). Thus, none of the five conserved hydrogen bonds formed by amino acids αN62 , αN69 , and αR76 is critical for 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 co-expressing 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 gated for

identical expression of DR and DM were analyzed. Thus, the hydrogen bond formed by β W61 was a candidate target in the DM catalytic mechanism. The capacity of DM to catalyze CLIP dissociation from β N82A DR1 could not be evaluated with this approach because of the high rate of spontaneous dissociation, leaving β N82 as an additional candidate.

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 β chain in the DM catalytic mechanism, we generated soluble recombinant DR1 and β W61A, β H81A, and β N82A mutant proteins. A fluorescence polarization assay was employed 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 β H81A mutation had essentially no effect on the intrinsic stability of the DR1-HA complex (Table 1) and the mutation did not reduce the potency of DM in catalyzing peptide dissociation (Fig.4c,f,g). Indeed, catalytic potency was somewhat greater for β H81A compared to WT DR1. Thus, the hydrogen bond formed by β H81 does not play a major role in stabilizing DR1-peptide complexes and it is not an essential target in the DM catalytic mechanism.

The β W61A mutation reduced peptide complex stability, with ~5-fold increase in the intrinsic HA dissociation rate (Table 1). However, DM catalytic potency was not reduced, even after normalization for the effect on the intrinsic peptide dissociation rate (Fig.4d,f,g). These results contrasted with the finding that CLIP release from β W61A 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 β W61A DR1. However, DM was observed to catalyze the dissociation of a variant CLIP peptide β W61A DR1 (Fig.4e) with potency similar to that observed for HA (Fig.4f). Full-length β W61A DR1-CLIP complexes were also highly susceptible DM (data not shown). While we did not observe any gross alteration in endosomal co-localization of β W61A 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 co-localization in membrane subdomains or through an effect on trafficking kinetics (26).

Strikingly, the β N82A mutation was observed to increase the intrinsic HA dissociation rate by >3000-fold (Table 1). 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 β N82 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 β N82A DR1, DM further accelerated peptide dissociation (Fig.4b,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 β N82 appear to be critical for stabilizing DR1-peptide complexes, yet they are not essential targets in the DM catalytic mechanism.

Concluding Remarks

In addition to the nine hydrogen bonds that we analyzed by mutational analysis in the current study, three conserved hydrogen bonds that interact with main chain atoms in the amino terminus of bound peptide are contributed by main chain atoms of amino acids at positions α 51 and α 53 in DR1 (Fig.1) (19). The potential role of these hydrogen bonds in the DM catalytic mechanism cannot be analyzed by mutation. However, Stratikos, et al., used peptide truncation and amide *N*-methylation to prevent the formation of these hydrogen bonds in DR1-HA peptide complexes (20), demonstrating that DM catalytic

potency was enhanced ~6–9-fold with peptide complexes lacking one or more of these hydrogen bonds. They observed only minor effects on catalytic potency with *N*-methylated peptide analogs designed to disrupt each of four additional conserved hydrogen bonds (20). It is possible that disruption of hydrogen bonds mediated by α 51, α 53, or β 81 increases DM binding affinity by increasing DR structural flexibility, resulting in small increases in DM catalytic potency.

The central conclusion of the current study is that no individual component of the conserved hydrogen bond network plays an essential role in the DM catalytic mechanism. DM catalytic activity is preserved or even enhanced with artificial disruption of any component of the conserved network. Given the universal capacity of DM to catalyze peptide dissociation, the conserved network has been an attractive potential target in the catalytic mechanism. If this is the case, however, individual components of the network must play a redundant role. It has become increasingly clear that MHC class II-peptide binding involves cooperative interactions throughout the peptide-binding groove and the hydrogen bond network appears to be relatively isolated energetically from the anchor-pocket interactions (27–29). It is possible, for example, that DM might initially promote the destabilization of *N*-terminal hydrogen bonds leading to a global change in conformation that destabilizes multiple elements of the hydrogen bond network. Removing any subset or single H-bond does not prevent DM from having its catalytic effect, but instead, it might even enhance the catalytic impact. Alternatively, DM might reduce the stability of peptide complexes by impacting other global features such as the dynamics or general structural flexibility of the peptide-binding groove, possibly by altering the interaction of the groove domain with the supporting Ig domains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. Elizabeth Mellins for providing critical reagents.

Abbreviations

Ii	invariant chain
WT	wild type

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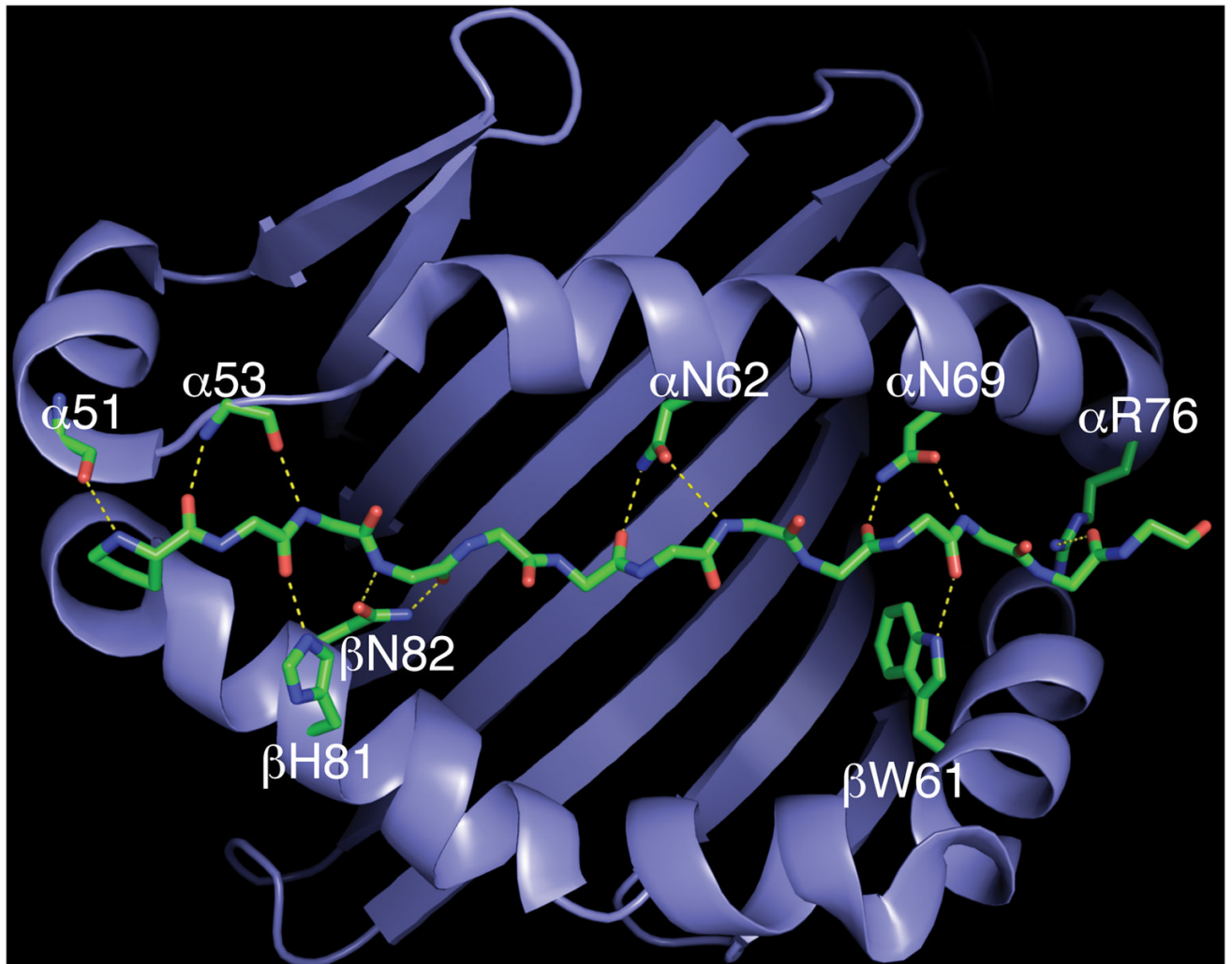


Figure 1.

The conserved hydrogen bond network in class II MHC-peptide complexes. The binding site of DR1 is represented as a ribbon diagram and the backbone of bound peptide is shown as a stick representation. Hydrogen bonds with main chain atoms of bound peptide are shown as yellow dashed lines. Main chain atoms of amino acids at DR1 positions $\alpha 51$ and $\alpha 53$ mediate hydrogen bonds, whereas conserved side chains form hydrogen bonds at other positions in the MHC molecule. The figure was generated with PyMOL software (<http://www.pymol.org>; PyMOL Molecular Graphics System; DeLano Scientific, San Carlos, CA) using Brookhaven Protein Data Bank coordinate file 1DLH (19).

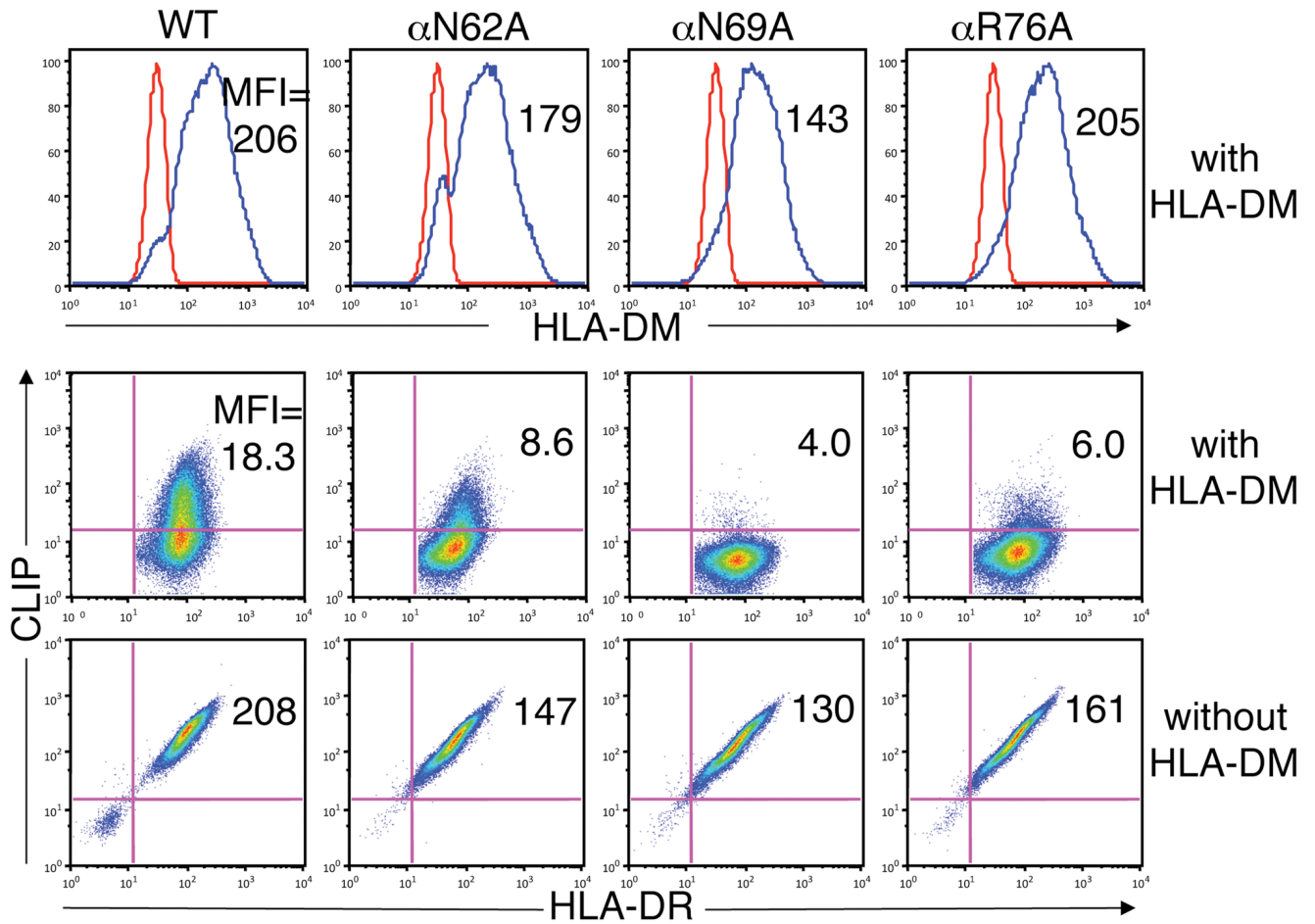


Figure 2.

Effect of mutations in conserved positions in the DR1 alpha chain on susceptibility to DM-mediate 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 mAb MaP.DM1 (top panels).

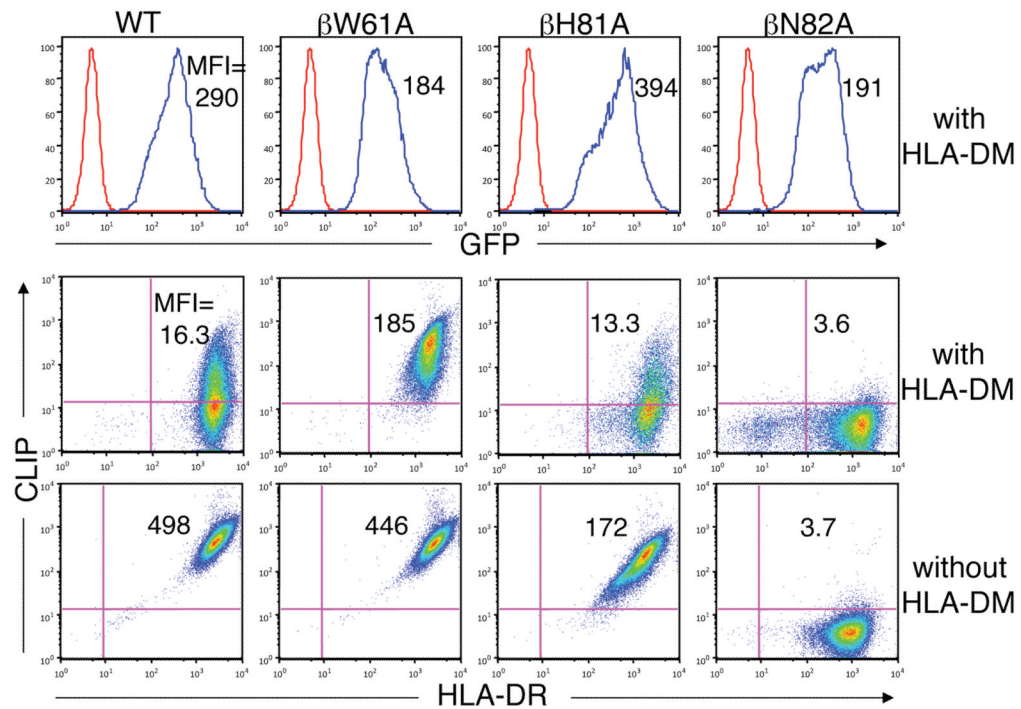


Figure 3.

Effect of mutations in conserved positions in the DR1 beta chain on susceptibility to DM-mediate 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).

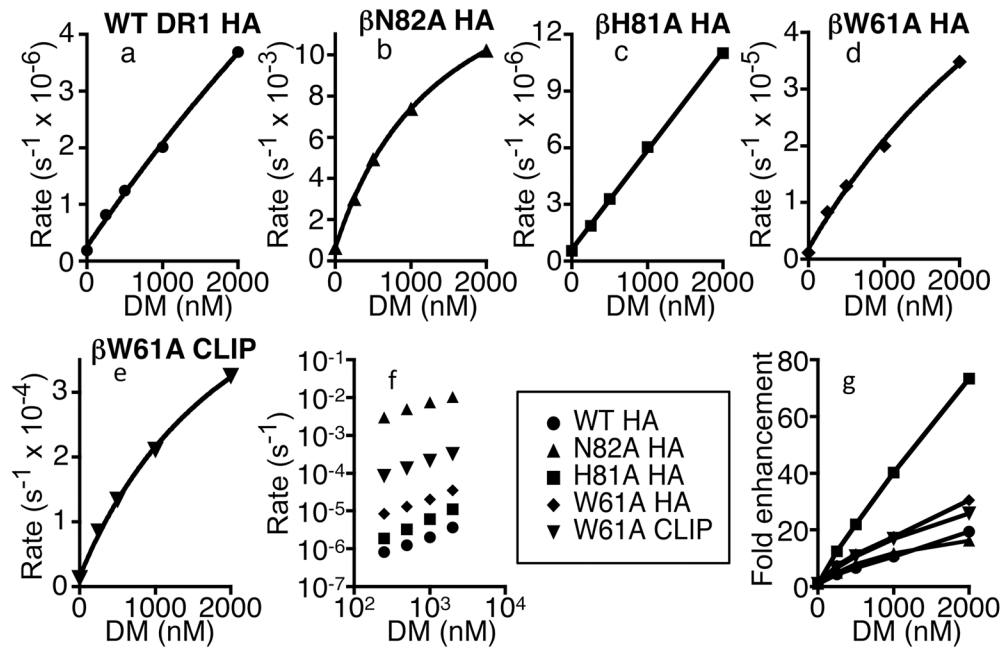


Figure 4. Effect of mutational disruption of DR1 beta chain hydrogen bonds on DM catalytic potency. (a-e) Preformed complexes (50 nM) of Alexa Fluor 488 labeled HA or CLIP peptides bound to mutant sDR1 were incubated with excess unlabeled peptide (20 μM) and various concentrations of soluble DM at pH 5.0, 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 to wild type sDR1 (WT) in a log-log plot of peptide dissociation rates versus DM concentrations. (g) DM-catalyzed rate enhancements normalized for differences in intrinsic peptide dissociation rates.

Table 1

Peptide dissociation rates for mutant DR1 molecules^a

	DM (nM)					n
	0	250	500	1000	2000	
DR1 (HA)	1.9×10^{-7}	8.2×10^{-7}	1.2×10^{-6}	2.0×10^{-6}	3.7×10^{-6}	3
N82A (HA)	6.3×10^{-4}	3.0×10^{-3}	4.9×10^{-3}	7.4×10^{-3}	1.0×10^{-2}	3
H81A (HA)	1.5×10^{-7}	1.9×10^{-6}	3.3×10^{-6}	6.0×10^{-6}	1.1×10^{-5}	2
W61A (HA)	1.1×10^{-6}	8.3×10^{-6}	1.3×10^{-5}	2.0×10^{-5}	3.5×10^{-5}	2
W61A (CLIP)	1.3×10^{-5}	8.5×10^{-5}	1.3×10^{-4}	2.1×10^{-4}	3.3×10^{-4}	1

^aRates are expressed in units of s⁻¹.