Enhanced Catalytic Action of HLA-DM on the Exchange of Peptides Lacking Backbone Hydrogen Bonds between their N-Terminal Region and the MHC Class II α-Chain[1]

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The class II MHC homolog HLA-DM catalyzes exchange of peptides bound to class II MHC proteins, and is an important component of the Ag presentation machinery. The mechanism of HLA-DM-mediated catalysis is largely obscure. HLA-DM catalyzes exchange of peptides of varying sequence, suggesting that a peptide sequence-independent component of the MHC-peptide interaction could be involved in the catalytic process. Twelve conserved hydrogen bonds between the peptide backbone and the MHC are a prominent sequence-independent feature of the MHC-peptide interaction. To evaluate the relative importance of these hydrogen bonds toward HLA-DM action, we prepared peptide variants that lacked the ability to form one or more of the hydrogen bonds as a result of backbone amide *N*-methylation or truncation, and tested their ability to be exchanged by HLA-DM. We found that disruption of hydrogen bonds involving HLA-DR1 residues α51–53, a short extended segment at the N terminus of the α subunit helical region, led to heightened HLA-DM catalytic efficacy. We propose that those bonds are disrupted in the MHC conformation recognized by HLA-DM to allow structural transitions in that area during DM-assisted peptide release. These results suggest that peptides or compounds that bind MHC but cannot form these interactions would be preferentially edited out by HLA-DM. *The Journal of Immunology*, 2004, 172: 1109–1117.

A crucial step of the immune response involves the cell surface presentation of proteolytic fragments of foreign proteins by class II MHC (MHC II)[4] molecules. MHC II molecules bind peptides in specialized endosomal compartments inside the cell and present them on the cell surface for recognition by T lymphocytes (1). Newly synthesized MHC II molecules rapidly associate in the endoplasmic reticulum with the MHC II-associated chaperone invariant chain (2), through an interaction between a flexible loop of the invariant chain and the MHC II peptide-binding site (3, 4). Targeting signals in the invariant chain direct the MHC II complex to endosomal compartments, wherein the invariant chain is sequentially proteolyzed to leave a fragment known as class II-associated invariant chain peptide (CLIP) bound in the MHC II peptide-binding site (5). Endosomal peptides can displace CLIP and bind to MHC II molecules for transport to the cell surface and presentation to T cells. Exchange of MHC II-bound peptides is promoted by the endosomal peptide exchange factor HLA-DM (6), which acts catalytically to promote both peptide binding and release (7, 8). HLA-DM is necessary for efficient peptide exchange in vivo, as demonstrated by the accumulation of MHC II molecules loaded with CLIP on the surface of cells lacking HLA-DM (9, 10).

Intriguingly, HLA-DM is a close structural homolog of its MHC II protein substrate. X-ray crystallographic studies of HLA-DM revealed a similar fold to that of classical MHC II molecules, with the major differences concentrated in the region equivalent to the peptide binding site, which in HLA-DM is collapsed and does not allow space for peptide binding (11, 12). How HLA-DM catalyzes peptide exchange on MHC II molecules is not known, although some information is available regarding the general features of the interaction (13, 14). Biochemical and spectroscopic data suggest the participation of alternate MHC II-peptide conformations differing in the strength of the MHC-peptide interaction (15–18). HLA-DM has been suggested to facilitate peptide exchange by stabilizing a low-affinity state (19) or by promoting conversion between states (20).

Although the cellular substrate for HLA-DM is believed to be predominately the MHC II-CLIP complex, peptides other than CLIP also can be exchanged (21, 22). HLA-DM exhibits different catalytic activity on different MHC-peptide complexes, leading to a potential role for HLA-DM as an intracellular peptide editor (6, 21, 23). Although the determinants of this different susceptibility are not clear, the ability of HLA-DM to catalyze the exchange of a variety of peptides suggests that the catalytic mechanism involves features of the peptide/MHC interaction common to all peptides, such as the network of hydrogen bonds between the MHC II and the backbone of the bound peptide (21). These hydrogen bonds are a general feature of MHC II-peptide interaction that has been observed in all crystal structures determined to date (24). The network is composed of 12–15 hydrogen bonds involving the MHC main chain (α51–53) and conserved MHC residues (α62, α69, α71, β61, β81, and β82). To evaluate the contribution of these hydrogen bonds to the HLA-DM catalytic mechanism, we studied the activity of HLA-DM toward a series of peptide variants, each carrying an *N*-methylated amide bond or a truncation that disrupts

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[4] Abbreviations used in this paper: MHC II, class II MHC; CLIP, class II-associated invariant chain peptide; HA, hemagglutinin; b, biotinylated.

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one or more of the conserved hydrogen bonds. We found that HLA-DM exerted a catalytic effect in all cases, but that the catalytic effect was significantly enhanced when the dissociating peptide lacked the ability to form one or more of the three hydrogen bonds formed between the peptide N-terminal region and the main chain of MHC α51–53. Either no changes or only small reductions in the catalytic efficiency of HLA-DM were observed for peptides lacking the ability to form one or more of the remaining hydrogen bonds studied. Implications of these results for the mechanism of DM-mediated peptide-exchange catalysis and intracellular peptide editing are discussed.

Materials and Methods

Expression and purification of proteins

Recombinant soluble HLA-DR1 (*DRB1*0101*) was expressed by a stably transfected Schneider 2 insect cell line and purified by affinity chromatography as described previously (8, 25, 26). Briefly, insect cells were grown in serum-free medium to $\sim 2 \times 10^7$ cells/ml, and MHC II expression was induced by addition of 1 mM $CuSO_4$. The cell supernatant was collected after 5 days, and MHC II was isolated by immunoaffinity chromatography using the anti-DR monoclonal LB3.1. Purified protein was concentrated by ultrafiltration, dialyzed against 10 mM HEPES and 50 mM NaCl, and either loaded immediately with peptide or frozen in small aliquots at −80°C. Recombinant soluble HLA-DM carrying a FLAG tag (8) was prepared similarly, except that HLA-DM was isolated by anti-flag immunoaffinity with peptide elution, followed by size exclusion chromatography (S200; Pharmacia, Peapack, NJ) and anion exchange chromatography (MonoQ; Pharmacia) (12). The protein was typically stored in aliquots at −80°C, but was found to be stable for several weeks if stored at high concentration (>100 μM) at 4°C. The activity of HLA-DM was found to vary little between different preparations of the protein (<20%; data not shown).

Peptide synthesis and purification

Peptides were synthesized by solid-phase F-moc chemistry, deprotected, and purified by reverse-phase HPLC, with integrity confirmed by electrospray mass spectrometry. *N*-Methyl amides were introduced using the corresponding *N*-methylated F-moc precursors (Chem-Impex International, Wood Dale, IL). For the hemagglutinin (HA)-derived peptides, concentrations were determined using an extinction coefficient (280 nm) of 1215 $M^{-1}cm^{-1}$; for the CLIP peptide, a bicinchoninic acid assay (Pierce, Rockford, IL) was used to estimate the concentration. Biotinylation was performed by reacting the N terminus of the peptide with biotin-X-succinimidyl ester (X = aminocaproyl; Molecular Probes, Eugene, OR) before deprotection and cleavage from the synthesis resin. Biotinylated peptides are designated with a "b", e.g., bHA. The biotinylated (b)$HA4_{Me}$ peptide (see Table I) was synthesized and biotinylated by Multiple Peptide Systems (San Diego, CA) using similar procedures. The b$\Delta_{10,11,12}$HA peptide was obtained in crude form by Anaspec (San Jose, CA) and purified and characterized in the same manner.

Loading of peptides onto HLA-DR1

Peptides were loaded onto the purified HLA-DR1 (10–20 μM) by extended incubation (~72 h) at 37°C using a 50- to 100-fold molar excess of peptide over HLA-DR1, in 10 mM HEPES, 50 mM NaCl, 0.02% NaN_3 (pH 7.0), in the presence of a protease inhibitor mixture consisting of 200 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 17 μM bestatin, 2 μM E-64, 85 μM EDTA, and 20 μM pepstatin A (all from Calbiochem, San Diego, CA). After loading, the mixture was concentrated ~4-fold by centrifugal ultrafiltration (Ultrafree-15; Millipore, Bedford, MA), and MHC-peptide complexes were isolated from free peptide and aggregated free MHC II by gel filtration (S200; Pharmacia) at 4°C. The fractions containing monomeric MHC II-peptide complexes were concentrated to 20–50 μM, aliquoted, and frozen at −80°C until needed. The purity and integrity of the preparation was confirmed by SDS-PAGE.

Characterization of DR1-peptide complexes

The integrity of the constructed DR1-peptide complexes was confirmed by SDS-PAGE analysis without boiling of samples. A total of 5–10 μg of complex was mixed with SDS-loading buffer so that the final SDS concentration was 0.4% and either left on ice or incubated for 5 min at 100°C. The samples were then loaded on a 12% polyacrylamide gel (Bio-Rad, Hercules, CA) that was run at 4°C and at 100 V for 1.5 h. The gel was stained with Coomassie brilliant blue to detect the SDS-stable band at 50 kDa corresponding to the intact DR1-peptide complex. The complexes were further analyzed by differential light scattering using a DynaPro-MS/X instrument and DynamicsV6 software (Proterion, Piscataway, NJ) and size exclusion chromatography using an SEC3000 column (Phenomenex, Torrance, CA) equilibrated with 10 mM HEPES buffer at pH 7.0 with 100 mM NaCl. The complexes were analyzed by differential light scattering at concentrations of 10–20 μM at 20°C and then immediately injected into the SEC3000 column run at the same temperature.

DM-catalyzed peptide exchange kinetics

The kinetics of peptide exchange were measured in a discontinuous manner using native PAGE, by monitoring the loss of peptide from a preformed MHC-peptide complex in the presence of excess bystander peptide. The effect of HLA-DM on the dissociation rate of each peptide was determined at several concentrations of HLA-DM. For peptides $\Delta_{1,2,3}$HA, b$\Delta_{1,2}$HA, and b$HA5_{Me}$, peptide-exchange was measured by a gel-shift assay, taking advantage of the different mobilities of HLA-DR complexes of the test and bystander peptides (HA, or $\Delta_{1,2,3}$HA for b$HA5_{Me}$) in the native PAGE assay. In each case, the shorter peptide carries fewer positive charges than the longer peptide, and the corresponding MHC-peptide complex migrates significantly faster. For the other peptides (b$HA4_{Me}$, b$HA8_{Me}$, b$HA11_{Me,}$ b-HA, b-CLIP, and b$\Delta_{10,11,12}$HA), peptide exchange was measured using a streptavidin shift assay, taking advantage of the reduced mobility of streptavidin/biotin-peptide/DR1 complexes relative to the complexes carrying unlabeled bystander peptide (HA), which do not bind streptavidin.

Table I. *Peptides, dissociation rate constants, and absolute and relative DM catalytic effects for peptides used in this study*[a]

Peptide Name	Sequence[b]	Uncatalyzed Dissociation K_{off} ($\times 10^{-3}$ min^{-1})	DM-Catalyzed Dissociation[c] ($\times 10^3$ $M^{-1}min^{-1}$)	Catalytic Rate Enhancement Factor J[d] ($\times 10^6$ M^{-1})	Affects H-Bond
bHA	b-PKYVKQNTLKLAT	0.06 ± 0.009	0.6 ± 0.07	10 ± 1.2	None
b$\Delta_{1,2}$HA	b-YVKQNTLKLAT	0.08 ± 0.01	5.2 ± 0.5	62 ± 9	1, 2
$\Delta_{1,2,3}$HA	YVKQNTLKLAT	0.54 ± 0.07	21 ± 2	39 ± 6	1, 2, 3
b$HA4_{Me}$	b-PK**Y**VKQNTLKLAT	1.15 ± 0.15	108 ± 4	94 ± 13	4
b$HA5_{Me}$[e]	b-PKY**V**KQNTLKLAT	0.42 ± 0.09	1.9 ± 0.3	4.5 ± 1.2	5
b$HA8_{Me}$	b-PKYVKQN**A**LKLAT	0.047 ± 0.008	0.24 ± 0.02	5.1 ± 1.0	8
b$HA11_{Me}$	b-PKYVKQNTLK**L**AT	0.39 ± 0.03	3.9 ± 0.15	9.9 ± 0.8	11
b$\Delta_{10,11,12}$HA[d]	b-PKYVKQNTL	0.25 ± 0.04	1.26 ± 0.08	5.0 ± 0.8	10, 11, 12
bCLIP17	b-PVSKMRMATPLLMQA	2.0 ± 0.2	15 ± 1.4	7.5 ± 0.9	None

[a] Estimated errors are given as the SD from a nonlinear least square fitted exponential (K_{off}) or linear (slope) dependence.
[b] Residues carrying *N*-Me amide modification are indicated by bold and underline.
[c] Slope of the plot of dissociation rate constant vs DM concentration. This factor indicates the sensitivity of the complex to DM-induced dissociation.
[d] Slope divided by the uncatalyzed dissociation rate constant K_{off}. This factor indicates the catalytic efficiency of DM towards this complex, or the fold rate enhancement per DM.
[e] For the b$HA5_{Me}$ and the b$\Delta_{10,11,12}$HA, values shown represent the slow component only. The fast component also appeared to be somewhat enhanced by HLA-DM (although to a smaller degree), but uncertainty in estimation of fast rates by the native gel assay precluded quantitative analysis.

Purified complexes of HLA-DR1 and test peptides (20 μM) were brought to pH 5.0, mixed with a large excess of free bystander peptide (1 mM), and incubated at 37°C under the same conditions used for peptide loading (see above). At various times, small aliquots of the mixture were removed and immediately mixed with either 1/9 vol of native PAGE loading buffer (1 M Tris (pH 7.5), 30% glycerol, and 0.001% bromophenol blue) for gel-shift assays, or an equal volume of a mixture of loading buffer containing 5 mg/ml streptavidin (Molecular Probes) for streptavidin-shift assays, followed by rapid freezing using crushed dry ice. The zero-time point samples were removed after addition of unlabeled peptide but before the mixture was moved to 37°C. At the end of the experiment, all samples were thawed by brief incubation at 4°C followed by 10% native PAGE analysis (27) run also at 4°C. No substantial peptide loss or exchange occurred during sample processing, as evidenced by the routine observation of 100% starting complex in the zero-time samples and by control experiments where either no competitor peptide was added or the 37°C incubation was bypassed (not shown). Native gels were stained with GelCode blue (Pierce) and scanned by a flatbed scanner to allow densitometric analysis of the bands using the program Image J (http://rsb.info.nih.gov/ij/). Measured intensities were background corrected using the intensity from the corresponding region of a control lane on the gel, and normalized based on the zero time point.

Data analysis

The normalized experimental intensities were fit to a single-exponential decay model (or to a double-exponential decay model in the cases of the bHA5$_{Me}$ and b$\Delta_{10,11,12}$HA peptides) using a nonlinear least squares minimization procedure, to yield for each peptide complex an uncatalyzed dissociation rate constant (K_{uncat}) and a series of catalyzed dissociation rates ($K_{cat,DM}$) obtained at various concentrations of DM. The relative catalytic efficiency of DM was calculated by plotting the observed dissociation rates $K_{cat,DM}$ vs the concentration of DM. This relationship was found to be linear for all complexes and all DM concentrations investigated. The slope of the $K_{cat,DM}$ vs [DM] was calculated by fitting the data to a linear model and restricting the *y*-axis intercept to be the independently measured uncatalyzed rate (K_{uncat}). The catalytic enhancement parameter *J* was calculated as the slope of the linear DM dependence plot divided by K_{uncat}, as previously described (21).

Results

A particular set of hydrogen bonds between MHC II residues and the main chain of bound peptide is a conserved aspect of the MHC-peptide interaction that has been observed virtually unchanged in all crystal structures determined to date. Twelve of these hydrogen bonds involve either the MHC main chain or MHC residues that are highly conserved within the otherwise highly polymorphic peptide binding site, and, as such, are expected to be present in most MHC II-peptide complexes. Fig. 1 shows the location of these main chain hydrogen bonds, which, in this work, we will refer to as H-bonds 1–12. To examine the relative contribution of these hydrogen bonds to the catalytic effectiveness of HLA-DM, we synthesized a series of peptide variants designed to disrupt one or more of these conserved hydrogen bonds, either because of N-terminal truncation (b$\Delta_{1,2}$HA, $\Delta_{1,2,3}$HA), C-terminal truncation (b$\Delta_{10,11,12}$HA), or *N*-methylation (bHA4$_{Me}$, bHA5$_{Me}$, bHA8$_{Me}$, bHA11$_{Me}$) (Table I). These substitutions were introduced into the well-studied antigenic peptide HA$_{306–318}$, derived from influenza virus HA (28), and bound to recombinant soluble extracellular domain of HLA-DR1, a common human MHC II protein.

Each of the peptides except for the C-terminally truncated b$\Delta_{10,11,12}$HA retain all of the side chains that are bound by the five HLA-DR1 specificity pockets (Fig. 1, shaded regions). The b$\Delta_{10,11,12}$HA peptide retains the side chains that are most important for binding onto HLA-DR1 (29). Each peptide except for $\Delta_{1,2,3}$HA carried an N-terminal biotin attached as an amide using a 6-carbon aminocaproyl linker; $\Delta_{1,2,3}$HA has a free N terminus rather than an N-terminal amide. Comparison of b$\Delta_{1,2}$HA and $\Delta_{1,2,3}$HA should allow investigation of the role of H-bond 3, because $\Delta_{1,2,3}$HA lacks the amide carbonyl that normally forms this hydrogen bond by interacting with MHC His$^{\beta 81}$ (Fig. 1).

All HA peptide variants tested in this study were able to form stable complexes with DR1 as judged by nondenaturing SDS-PAGE electrophoresis (without boiling of samples before loading), native gel electrophoresis, size exclusion chromatography, and dynamic light scattering analysis. The SDS-stable band was for all variants significantly weaker compared with that of the unmodified HA peptide but of the same magnitude as the CLIP peptide (not shown). All peptide complexes run as monomers with apparent molecular mass of ~50 kDa on a SEC3000 size exclusion column, similar to complexes formed between DR1 and unmodified bHA or bCLIP (not shown). However, the complexes between DR1 and peptides b$\Delta_{10,11,12}$HA and bHA5$_{Me}$ showed small shifts toward

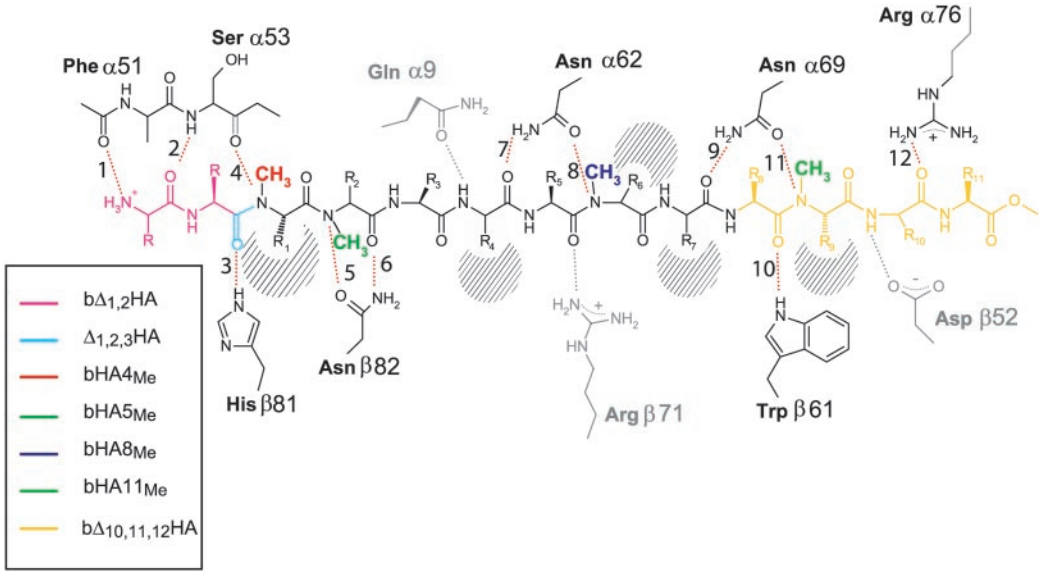

FIGURE 1. Schematic representation of hydrogen bond network between MHC class II and bound peptide backbone. Conserved hydrogen bonds are indicated by a red dashed line and numbered 1–12, counting from the N terminus. Sites of backbone nitrogen methylation or truncation are indicated and colored by variant. Peptide side chains are numbered conventionally based on their position relative to the large hydrophobic residue at position 1. Corresponding side-chain-binding pockets are indicated with crosshatching. Nonconserved but frequently occurring H-bonds are found at positions 4NH, 5CO, and 10NH; these were not investigated in this study. Figure has been modified from Ref. 49.

higher molecular mass similar to non-peptide-incubated insect cell DR1, consistent with fractional peptide loss during the analysis as a result of fast dissociation kinetics (both peptides had a biphasic dissociation behavior with a fast first phase and a slower second phase; see below). Dynamic light scattering analysis for most peptide complexes revealed hydrodynamic radii in the range of 3.26–3.44 Å with polydispersities of 10–13%, consistent with a compact complex of ~50 kDa. Non-peptide-incubated insect cell-derived DR1 showed a slightly increased radius of 3.57 Å with a higher polydispersity of 21% consistent with the notion that it either lacks bound peptide or contains a mixture of weakly bound peptides (42). The DR1/bHA5$_{Me}$ complex presented a more complicated picture and was impossible to analyze in a reliable manner presumably due to rapid peptide dissociation and creation of multiple DR1 oligomerization species (see below). This behavior was not an issue during peptide exchange experiments due to the constant presence of high excess of competitor peptide in the mixture.

Intrinsic dissociation constants for these peptides were measured by a simple exchange assay, in which purified MHC-peptide complexes were incubated at 37°C and pH 5.0 in the presence of a large excess of another peptide, with the rate of peptide exchange determined by native PAGE. Typically, the starting MHC complex carried a biotinylated peptide, and the bystander peptide was unlabelled, with streptavidin used to follow the loss of biotinylated peptide with time (Fig. 2*A*, *top panel*). For the nonbiotinylated peptide $\Delta_{1,2,3}$HA (as well as b$\Delta_{1,2}$HA and bHA5$_{Me}$), differential electrophoretic mobility in the absence of streptavidin was used to follow peptide exchange (Fig. 2*B*, *top panel*). Dissociation rate constants were estimated by single-exponential fits to plots of the amount of complex remaining vs time (Fig. 2, *bottom panels*). Intrinsic dissociation rate constants for bHA (6.0×10^{-5} min^{-1}) and bCLIP (2.0×10^{-3} min^{-1}) were similar to those reported previously (7.3×10^{-5} and 2.1×10^{-3} min^{-1}, respectively) (21), although the dissociation rate for bHA has also been reported to be significantly different (30). The measurements presented in this report are validated by the coincidence of the values of the separately measured uncatalyzed rate and calculated uncatalyzed dissociation rate from the extrapolation of the catalyzed rates to infinite dilution (Fig. 3). Differences in the measurement of such slow rates might be due to the intrinsic difficulties in measuring very slow kinetics that involved incubation times of weeks, where the stability of the protein complex, the excess peptide, and the presence of proteases are complicating factors. Because of this potential problem, the results of the present study, although presented in relation to the bHA peptide control, might be best interpreted in relation to each other without any significant changes in the conclusions.

Several of the peptide substitutions caused large decreases in the MHC-peptide complex lifetime, particularly $\Delta_{1,2,3}$HA, bHA4$_{Me}$, bHA5$_{Me}$, and bHA11$_{Me}$. The importance of H-bonds 3 and 4 has been previously reported (31, 32). bHA5$_{Me}$ exhibited double-exponential behavior, with $K_{off,1}$ of ~0.10 min^{-1} (45%) and $K_{off,2}$ of ~4.1×10^{-4} min^{-1} (55%). Similarly, b$\Delta_{10,11,12}$HA exhibited double-exponential behavior with $K_{off,1}$ of ~0.011 min^{-1} (48%) and $K_{off,2}$ of ~2.5×10^{-4} min^{-1} (52%). Such biphasic behavior has been observed before in the dissociation of native and modified peptides, and has been attributed to conformational isomers (16, 33–37) or to alternative peptide binding registers (38). Alternate binding registers are not expected here because of the strong preference of HLA-DR1 for an aromatic group in the first specificity pocket (39).

To determine the effect of the peptide substitutions on the ability of HLA-DM to catalyze peptide exchange, dissociation assays were performed for each MHC-peptide complex in the presence of various concentrations of HLA-DM. For all peptides tested, HLA-DM accelerated peptide exchange (Fig. 2). In every case, we observed a linear correlation between the concentration of HLA-DM and the observed rate of peptide dissociation (Fig. 3), as previously observed for detergent-solubilized full-length HLA-DR1 and HLA-DM (21). (Higher concentrations of the soluble extracellular domains as used in this study were required to achieve levels of rate enhancement comparable with those obtained with full-length detergent-solubilized molecules, presumably because of the lack of the local concentration effects provided by the transmembrane domains (40, 41)). The slopes of the linear correlations provide a measure of the catalytic effectiveness of HLA-DM in dissociating each of the variant peptides (Table I). HLA-DM was particularly effective at dissociating peptides that carried modifications at the N-terminal end: peptides $\Delta_{1,2,3}$HA, and bHA4$_{Me}$ were 35- and 180-fold more sensitive to HLA-DM-mediated release than was the unmodified HA-peptide (Table I). Two deletion variants also were more sensitive to HLA-DM-mediated release than was the unmodified peptide, although to lesser degree, b$\Delta_{1,2}$HA (8.5-fold) and b$\Delta_{10,11,12}$ HA (6.5-fold), whereas none of the other peptide variants exhibited changes of >3-fold.

The relative efficiency of HLA-DM-mediated catalysis toward a series of homologous HLA-DR1-peptide substrates previously has been found to correlate with their intrinsic stabilities, such that peptides with fast intrinsic off-rates were released more efficiently by HLA-DM, and peptides with slow intrinsic off-rates less so (21). The catalytic rate enhancement parameter J (equal to the slope of the rate vs [HLA-DM] plot divided by the uncatalyzed dissociation rate) was introduced by Weber et al. (21) to describe this relationship, and found to be essentially invariant for a series of peptides of different sequences related to HA and CLIP (J = 0.8–2.8×10^9 M^{-1}). We found that the J values for bHA (10×10^6) and bCLIP (7.5×10^6) were similar (Table I), indicating that the same correlation applies to the soluble proteins investigated herein. Only small changes in the J value were found for the peptide variants bHA5$_{Me}$ ($J = 4.5 \times 10^6$), bHA8$_{Me}$ ($J = 5.1 \times 10^6$), bHA11$_{Me}$ ($J = 9.9 \times 10^6$), and b$\Delta_{10,11,12}$HA ($J = 5.0 \times 10^6$).

Strikingly, the DM-catalyzed rate enhancements for the three peptides carrying N-terminal modifications were much greater than for the other variants: $\Delta_{1,2,3}$HA ($J = 39 \times 10^6$), b$\Delta_{1,2}$HA ($J = 62 \times 10^6$), and bHA4$_{Me}$ ($J = 94 \times 10^6$) (Fig. 4). Thus, disruption of H-bonds 1, 2, and 4 leads to a peptide that is much more efficiently removed by HLA-DM, whereas disruption of H-bonds 5, 8, 10, 11, and 12 has a minimal effect. H-bond 3 would not appear to play an important role in the mechanism of HLA-DM-mediated peptide release, as inferred indirectly by the similarity of peptides $\Delta_{1,2,3}$HA and b$\Delta_{1,2}$HA, although a small negative effect could be observed, which might however be attributable to unexpected effects of the biotin label present only in b$\Delta_{1,2}$HA. It is noteworthy that H-bonds 1, 2, and 4, which when disrupted yielded strongly enhanced HLA-DM catalysis, involve only the main chain of the extended MHC region α51–53, found at the N-terminal side of the α-chain helical region (Fig. 5).

Discussion

To investigate a possible role in the catalytic mechanism of HLA-DM for the conserved hydrogen bond interactions between the backbone of the bound peptide and the MHC II molecule, we examined the catalytic efficiency of HLA-DM on the dissociation of HA$_{306–318}$ peptide variants that should be unable to form one or more of the conserved hydrogen bonds. The HA$_{306–318}$ peptide was chosen as the backbone for all modifications, because its side-chain composition is almost optimal for binding to the DR1 protein (42). This provided two distinct advantages that make this study

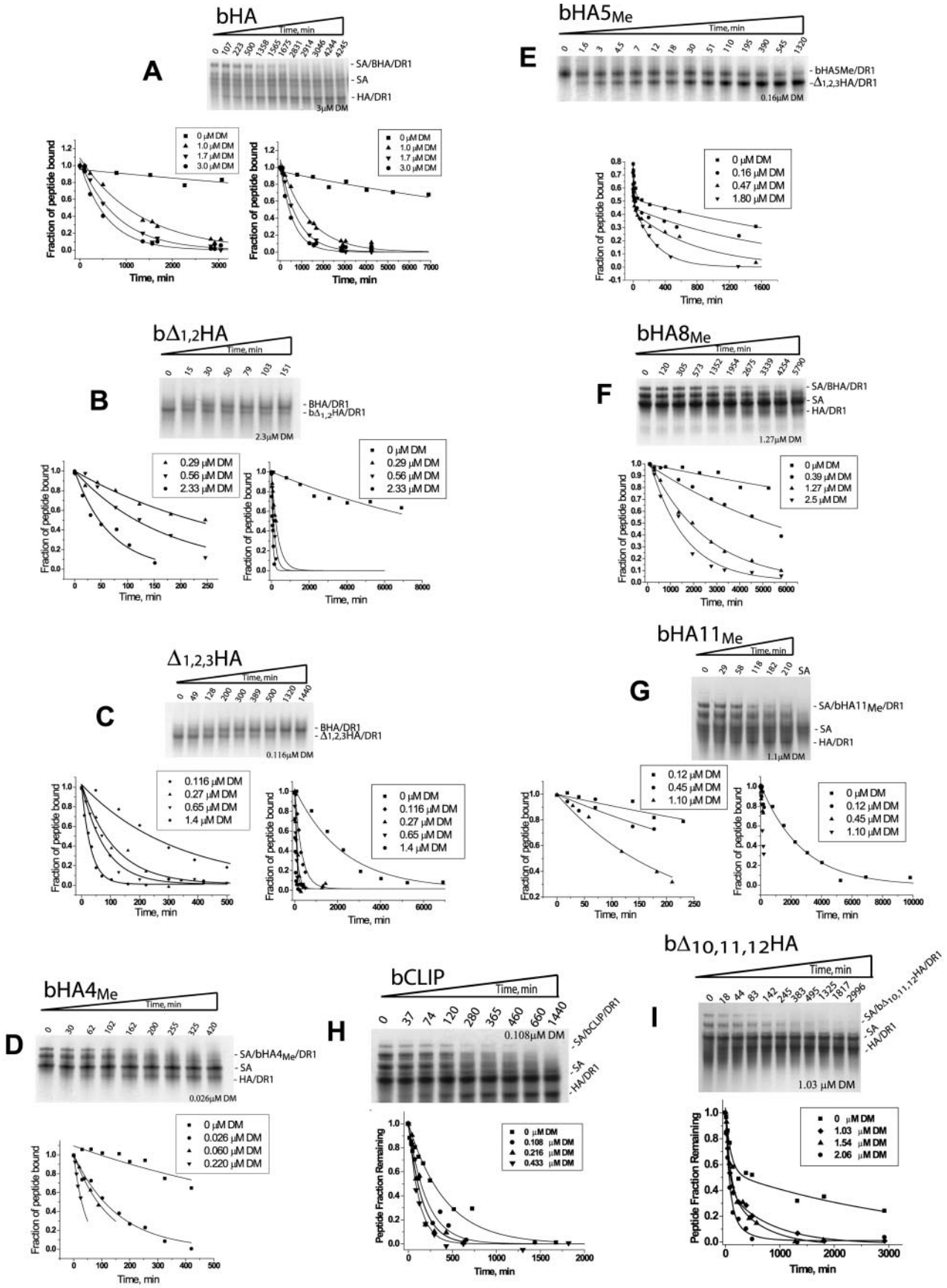

FIGURE 2. Kinetics of peptide dissociation from HLA-DR1 in the absence or the presence of HLA-DM. *Top subpanels*, Native PAGE analysis for a representative DM concentration. *Bottom subpanels*, Graphs of the time course of the dissociation of the respective peptide. For each peptide, a representative native gel that was used to follow the kinetics is shown above (see *Materials and Methods*). In each case, except *E* and *I*, the data were fit to a single-exponential decay model. The data in *E* and *I* were fit to a double-exponential decay model. Two decay plots at different scales are given for *A*, *B*, *C*, and *G*, to allow better visibility of the fits at fast time scales.

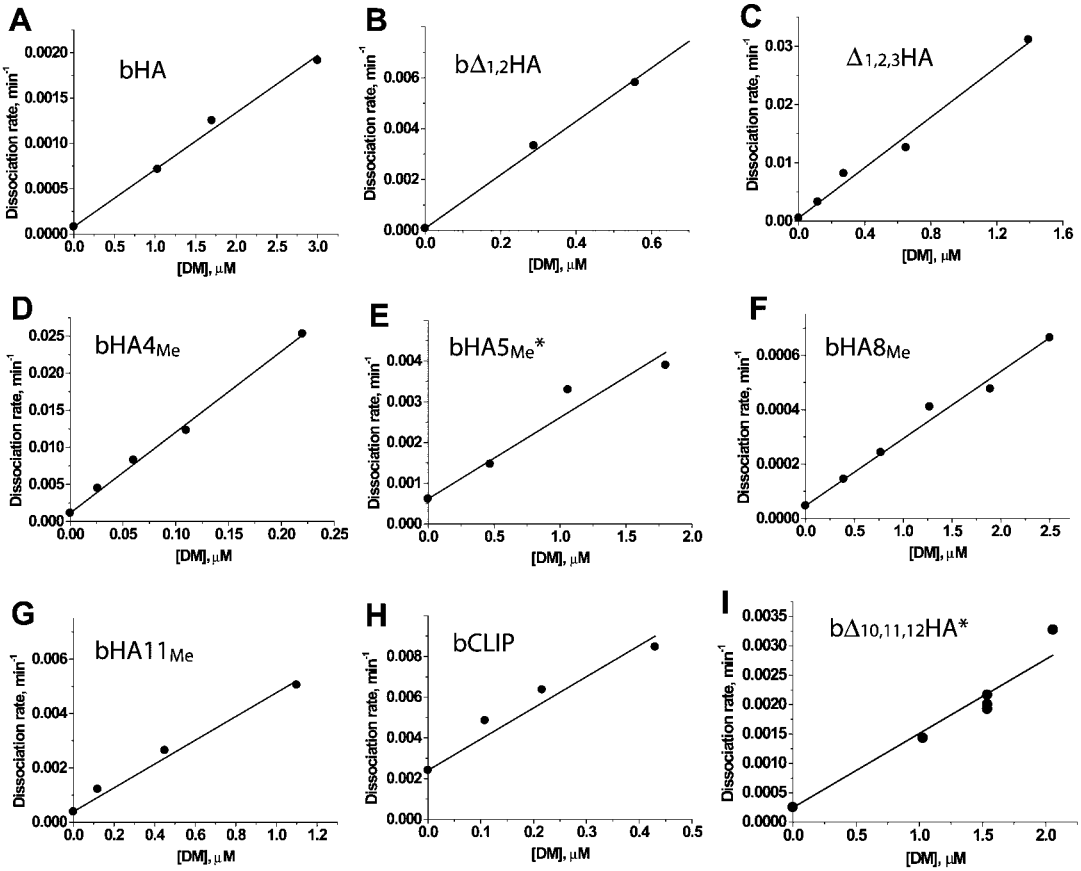

FIGURE 3. Effect of HLA-DM on the observed dissociation rate of the peptides used in this study. In each case, the data were fit to a linear model with the *y*-axis intercept set to the independently calculated point at zero concentration DM. The catalytic rate enhancement factor *J* is the catalytic efficiency (slope of the linear dependence) divided by the uncatalyzed dissociation rate constant (*y*-intercept of the linear dependence). ∗, Only the slow component of the dissociation curve for peptides $bHA5_{Me}$ and $b\Delta_{10,11,12}HA$ was analyzed.

feasible. First, the resulting tight complex with DR1 can accommodate disrupting mutations without severe loss in binding affinity that would make analysis of the kinetics with the techniques used in this study impossible. Second, because recent work points to potential side-chain-pocket effects on DM catalysis (22, 30), the HA peptide represents an useful extreme case where the side-chain-pocket interactions are very strong and constant among almost all mutant peptides tested (with the exception of $b\Delta_{10,11,12}HA$) allowing us to isolate the effect of backbone hydrogen bonds as a whole and in relation to each other (in the absence of complicating factors by side-chain-pocket effects) and therefore evaluate their role in the catalytic mechanism of DM.

We find that the dissociation of peptides lacking the ability to form hydrogen bonds 1, 2, and 4 is preferentially accelerated by HLA-DM. Peptides lacking the ability to form other hydrogen bonds, including those important in determining the intrinsic (uncatalyzed) dissociation rate, do not have large effects on the efficiency of HLA-DM-mediated catalysis. In earlier work, Weber et al. (21) observed that the catalytic rate enhancement factor *J* was not sensitive to side-chain substitutions within a series of homologous peptides, and suggested that a different feature of the MHC-peptide interaction such as the conserved main-chain hydrogen bonds would be implicated in the HLA-DM mechanism. In this study, we show that disruption of hydrogen bonds 1, 2, and 4 strongly potentiates HLA-DM action, suggesting that these particular interactions are involved in HLA-DM-mediated catalysis.

Hydrogen bonds 1, 2, and 4 are all formed by interactions between the peptide main chain near its N terminus and MHC residues α51–53, an extended region at the N-terminal side of the α-chain helix that forms a parallel β strand arrangement with the peptide (Figs. 1 and 5). This result is consistent with two recent studies implicating the same general region of HLA-DR in interactions with HLA-DM. Tethered HLA-DR-HLA-DM complexes were observed to be functional when cysteine β46 of HLA-DM was linked closely to the peptide N terminus but not the C terminus, implicating the HLA-DR region near peptide N terminus in interactions with HLA-DM (14). A study of HLA-DR substitutions that alter HLA-DM interaction also implicated the lateral face of HLA-DR that includes the N-terminal end of the peptide-binding side as the major locus of HLA-DM interaction (13). That study identified phenylalanine α51, included in the α51–53 region identified here, as a particularly important HLA-DM-HLA-DR contact (13).

The effect of peptide truncation and *N*-methylation on HLA-DM-mediated catalysis suggest that disruption of interactions between the MHC and the peptide backbone is an important aspect of HLA-DM's catalysis of peptide dissociation. Other factors including effects of the peptide sequence could also help to promote DM catalysis (22, 30). One possible mechanism for HLA-DM-mediated catalysis could involve transient binding and stabilization of a conformational state of HLA-DR1 in which some of the backbone hydrogen bonds have been disrupted. Our observation of enhanced HLA-DM catalytic activity toward peptides that cannot form hydrogen bonds 1, 2, and 4 could suggest that these interactions are disrupted in the state recognized by HLA-DM; the lack of these hydrogen bonds in peptides $\Delta_{1,2,3}HA$, $\Delta_{1,2}HA$, and $bHA4_{Me}$

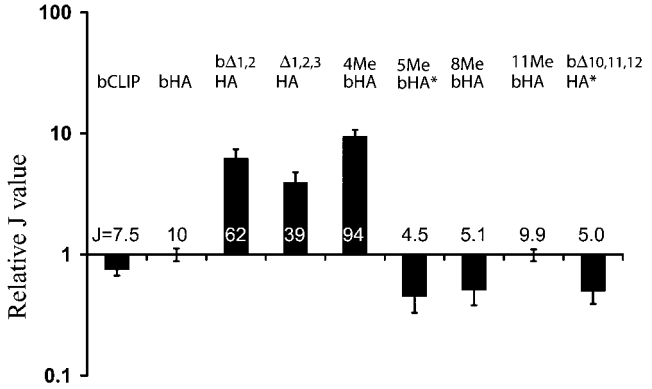

FIGURE 4. Effect of disruption of peptide backbone hydrogen bonds on the catalytic activity of DM. Relative fold change of the catalytic rate enhancement factor J = slope/K_{uncat} (21). J values of the peptide variants were compared with those of the parent HA peptide. Absolute J values are indicated at the base of each bar. The y-axis is in logarithmic scale.

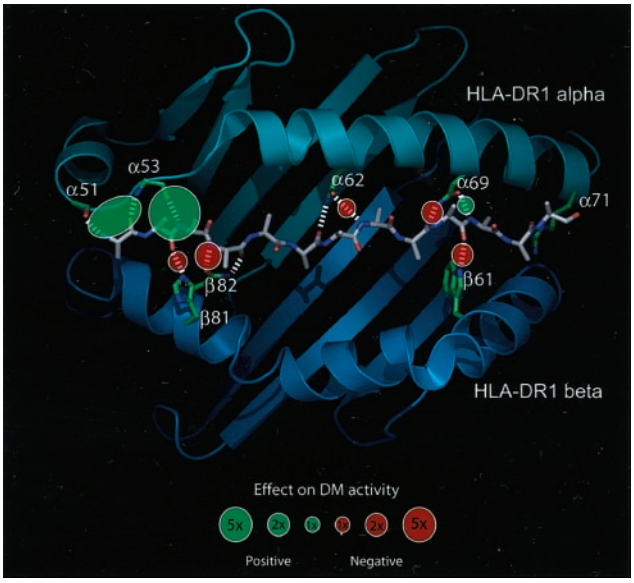

FIGURE 5. Schematic representation of the conserved peptide backbone-MHC interactions and the effect of their perturbation on DM catalytic efficiency reveals the importance of the N-terminal region interactions. The binding site of DR1 is shown as a ribbon diagram with the α-chain colored cyan and the β-chain in blue. The bound HA peptide is shown as a stick representation with the conserved hydrogen bonds indicated as white dashed lines. The effect of specific hydrogen bond disruption on the catalytic efficiency of HLA-DM is illustrated as semitransparent circles around each bond tested. Green color indicates an enhanced effect, and red indicates a reduced effect. The area of each circle indicates the magnitude of the effect. Notice that the largest positive effects on DM activity are seen when the three hydrogen bonds between the α-chain of DR1 and the backbone of the N terminus of the peptide are disrupted. Figure was made with PyMOL (http://www.pymol.org; PyMOL Molecular Graphics System; DeLano Scientific, San Carlos, CA) using Brookhaven Protein Data Bank coordinate file 1DLH.

would facilitate HLA-DM binding and, as a result, enhance catalysis. Previous work has suggested such a model and implicated the P1 pocket as the major area involved in such a conformational change (19); the spatial proximity of these hydrogen bonds to the P1 pocket is almost certainly not coincidental. Furthermore, both the P1 pocket and H-bonds 1, 2, and 4 make interactions with the N-terminal region of the bound peptide, near the lateral side of DR1 that has been suggested that interacts with DM (13, 14). The α51–53 region forms an extended strand within an otherwise helical region of the MHC and might easily adopt another conformation with increased affinity for HLA-DM. Such a conformation could in turn lead to other subtle conformational changes in the region leading to a more open DR1/peptide conformation, such as the fast-exchange form detected by kinetic experiments (15, 16, 29). However, formation of an open state with increased dissociation does not necessarily lead to enhanced DM efficiency (e.g., $bHA_{11}Me$), and peptide variants with enhanced DM catalytic efficiency do not necessarily exhibit enhanced intrinsic dissociation (e.g., $\Delta_{1,2}HA$), suggesting mechanistic differences in the DM-promoted and uncatalyzed pathways of peptide dissociation. A possible model of DM action that could account for the observations seen in this study would involve a two-step pathway of DM-catalyzed peptide dissociation. In the first step, DM would stabilize a high-energy and/or sparsely populated conformation of DR1 where MHC-peptide interactions in the region around the N-terminal end of the peptide are disrupted. The opening of the DR1 region near the N terminus of the peptide could presumably lead to secondary and more global conformational changes in DR1, leading to the creation of the DR1 open state mentioned above, which allows rapid peptide dissociation. Peptide variants that disrupt MHC-peptide interactions outside the N-terminal region could directly lead to enhanced dissociation without a corresponding effect on DM catalytic efficiency.

Overall, although the truncations or the presence of backbone methyl groups affected the kinetics of peptide release considerably, they did not seem to affect the integrity of the peptide-DR1 complex. We found no correlation between the results of the biophysical characterization of the DR1/peptide complexes and the efficiency of DM action onto them, indicating that the effects found in this study are not related to misfolding of the DR1 binding site due to the peptide mutations. Characteristically, we found virtually identical results in our analysis by SDS-PAGE, light scattering, uncatalyzed dissociation kinetics, and size exclusion chromatography for the complex between DR1 and CLIP as well as with the complex between DR1 and $bHA4_{Me}$, although they presented a >12-fold difference in DM catalytic efficiency.

Although we have interpreted the effects of peptide truncation and *N*-methylation primarily in terms of disruption of hydrogen bonds, it should be noted that the peptide variants used in this study introduce additional changes besides deletion of hydrogen bonds. This is especially true for the truncated peptides, which lack main-chain and a few side-chain interactions relative to the full-length peptides, and could potentially be a factor also for the *N*-methylated peptides, because of the larger size of the introduced methyl group compared with the amido hydrogen. Although in principle this could complicate our interpretation of the results of this study, the observation that most of the peptides tested in this study (which contain changes in the primary sequence, N- and C-terminal truncations, and *N*-methylations) exhibited DM efficiencies similar to the control HA and CLIP peptides suggests that the introduced substitutions generally were tolerated well. Even in the absence of the control HA and CLIP peptides, a comparison of the relative DM efficiencies for the mutants still reveals an enhanced DM effect for peptides that lack H-bonds 1, 2, and 4 compared with the other positions, essentially operating as an internal control for the validity of the approach. Finally, the only peptides that showed significantly altered catalysis by DM all clustered in the same region, and were observed in both the *N*-methylated and deletions series. Regardless whether hydrogen bonds are the sole determinants of the effects observed in this study or other interactions also play a role, it seems clear that the region including the

N-terminal end of the bound peptide and α51–53 of the MHCII is a major participant in the DM-induced peptide dissociation pathway.

The results from this study have implications for our understanding of the editing capabilities of HLA-DM. Previously, HLA-DM was thought to affect the repertoire of peptides that reach the cell surface by accelerating peptide exchange in a sequence-dependent manner. We suggest that additional editing occurs in the context of N-terminally truncated peptides, specifically that peptides unable to form hydrogen bonds with the α51–53 region would be preferentially edited out by HLA-DM. An example of that can be found in the comparison of the CLIP peptide with the $b\Delta_{1,2}HA$ or the $bHA4_{Me}$ peptide. Although the intrinsic rate of dissociation of CLIP is actually faster than that of the other two peptides and thus CLIP would be expected to be edited out, the enhanced action of HLA-DM on the $b\Delta_{1,2}HA$ and $bHA4_{Me}$ peptides would preferentially dissociate them, leaving CLIP bound. This effect might explain why sequenced pools of naturally presented peptides generally include only sequences starting several residues N-terminal to the P1 position (43–45). Preferential HLA-DM editing of short peptides can also help explain the poor correlation between binding affinity and inhibitory potency for synthetic peptide-based inhibitors of Ag presentation, which lack some of the N-terminal H-bonds investigated in this study (46). Finally, these results have consequences for peptidomimetic inhibitors that include backbone substitutions and N-terminal truncations in efforts to increase serum stability and bioavailability (47, 48), but which could result in an undesired enhancement of editing by HLA-DM.

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