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Conformational lability in the class II MHC 3–10 helix and adjacent extended strand dictate HLA-DM susceptibility and peptide exchange

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HLA-DM is required for efficient peptide exchange on class II MHC molecules, but its mechanism of action is controversial. We trapped an intermediate state of class II MHC HLA-DR1 by substitution of αF54, resulting in a protein with increased HLA-DM binding affinity, weakened MHC-peptide hydrogen bonding as measured by hydrogen-deuterium exchange mass spectrometry, and increased susceptibility to DM-mediated peptide exchange. Structural analysis revealed a set of concerted conformational alterations at the N-terminal end of the peptide-binding site. These results suggest that interaction with HLA-DM is driven by a conformational change of the MHC II protein in the region of the α-subunit 3–10 helix and adjacent extended strand region, and provide a model for the mechanism of DM-mediated peptide exchange.

Antigen presentation | antigen processing | major histocompatibility proteins | chaperone | protein folding

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The authors declare no conflict of interest.

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Data deposition: Crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID codes 3QXD and 3QXK).

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interaction, but their role in DM-mediated catalysis has been controversial (12, 13, 17, 18), in part because of a lack of methods to examine the strength of these bonds directly. We devised an experimental protocol that uses amide hydrogen-deuterium exchange (HDx) and MS to examine the strengths of the MHC II-peptide H-bonds in native, unlabeled MHC II-peptide complexes. Amide hydrogens exchange with solvent on a time scale of milli-seconds to hours, with the exchange rate depending on the strength of the amide H-bond (28). As outlined in Fig. 1A, we stripped the peptide from the MHC II under conditions that preserve the pattern of amide H/D present in the MHC-peptide complex, and used high-resolution linear trap quadrupole HPLC/MS to evaluate HDx in MHC II-peptide amide bonds by direct examination of the released peptide.

For the free HA peptide in H2O solution, the average mass (1,503.8) and the stable isotope ion distribution pattern (Fig. 1B, Left) correspond with that expected for the HA peptide sequence and the known isotope abundances. For the free HA peptide transferred to 90% D2O solution, the average mass increases to 1,511.9, with changes in the mass spectrum (Fig. 1B, Center), corresponding to addition of 12 D, after accounting for back-exchange during the analysis (SI Appendix, Fig. S1), as expected for the 12 amide bonds in the HA peptide. For the MHC II-HA complex immediately after transfer to 90% D2O, the average mass is 1,504.6 (Fig. 1B, Right). Thus, interaction with the MHC II protein protects ~90% of the peptide amides from exchange.

With increasing time in D2O, additional H/D exchange is observed, with multiphasic kinetics (Fig. 1C, black line). The time-dependent H/D exchange kinetic is fit well by an exponential equation that describes a fast phase (1/2 < 1 min), a fast phase with half-time of a few minutes, and a much longer slow phase with a half-time of ~18 h (SI Appendix, Table S1). The slow phase is still substantially faster than the rate of peptide release (Fig. 1D). Overall, this analysis reveals that the bound peptide’s amide NH bonds exchange at dramatically different rates.

We were able to assign several individual peptide amides to particular kinetic phases using multidimensional electron transfer dissociation (ETD) MS (21). In ETD, peptides are fragmented between NH and CO bonds (Fig. 2A), permitting the detection of c-series and z-series ions (Fig. 2B), analogous to the b-series and y-series of conventional collision-induced dissociation (29). Unlike collision-induced dissociation, however, ETD does not result in H/D scrambling (30), so that the ion fragments retain the pattern of deuterium incorporation present in the eluted peptides. Peptide fragments exhibit different kinetics of deuterium incorporation, depending on the protection of the particular amide NH atoms present in that fragment. For example, the c3 fragment has very little deuterium incorporation, even at 512 min, whereas the c6 fragment is substantially exchanged by 2 min (Fig. 2C).

We were able to measure deuterium incorporation rates for six c-series ions (c2, c3, c5, c6, c8, and c9) and seven z-series ions (z4, z5, z7, z9, z10, z11, and z12), although with weaker signals for the z-series (complete analysis in SI Appendix, Table S2). By subtracting the mass differences (ΔMW) between natural (c) and deuterated (ΔΔMW) ions, the contributions of particular peptide NH atoms (or pairs of NH atoms) can be obtained. Fig. 2D shows such a ΔΔMW analysis, with the extracted H/D exchange of each peptide NH atom (or pairs of NH atoms) at various times shown in open bars and the fully exchanged mass difference shown in black bars. Corresponding z-series data are shown in Fig. S2A, with an overall summary of both c-series and z-series data in SI Appendix, Fig. S3. It is readily apparent that the V4 position is the most strongly protected of all of the peptide NH, indicating that this residue participates in the strongest H-bonding interaction. The next strongest H-bonds emanate from the Y3 and N7 positions. The V4 and Y3 positions flank the α1 side-chain binding pocket. Thus, it would be highly unlikely for the α1 side chain to spontaneously escape the P1 pocket, as recently proposed (9), and so we sought other explanations for the key role of this region in DM-mediated peptide exchange catalysis.

Substitution of α54 Dramatically Increases Susceptibility of MHC II to DM-Catalyzed Peptide Dissociation. To determine the relevance of the region proximal to the P1 pocket for DM catalysis, we engineered mutations in the extended strand region of the α-subunit at residues α51, α53, and α54, and surrounding the P1 pocket, at residues αL45, αF48, ββ89, and βW153 (Fig. 3A, and SI Appendix, Fig. S4 and Table S3). As a control, we also mutated αQ57, located on the α-subunit helix one turn beyond the extended strand region. We monitored the intrinsic rate of peptide dissociation from the mutant proteins using Alexa488-labeled CLIP peptide in a fluorescence polarization assay (Fig. 3B–G, blue triangles, and SI Appendix, Fig. S4 B–E). Dissociation time courses fit well to single exponential decays, with τβ2 and kα1 values shown in SI Appendix, Table S1, ranging from a 9.5-fold increase in dissociation rate for αF54C to a 1.9-fold decrease for αS33A.

We tested the susceptibility of the MHC II mutant proteins to DM in a functional assay (Fig. 3B–G and SI Appendix, Fig. S4 B–E). In the range tested, peptide dissociation rate constants increased linearly with increasing DM concentration for each of the mutant proteins (Fig. 3H and SI Appendix, Fig. S5), with the slopes reflecting the susceptibility of each mutant protein to DM-mediated peptide dissociation. As previously observed in the context of another MHC II protein (11), substitution of αF51 resulted in a protein that was resistant to DM (Fig. 3E). The DM-susceptibility of the control mutant αQ57C and P1 pocket mutants αL45A, αF48A, ββ89A, and βW153A were similar to WT, whereas αS33A was approximately sevenfold more sensitive to DM than WT (Fig. 3B–G and SI Appendix, Fig. S4 B–E). In contrast, αF54C and
αF54A were exquisitely sensitive to DM, with concentrations as low as 30 nM DM, inducing large increases in peptide dissociation rates (Fig. 3D and G). Values for the slopes of the DM-dependent rate profiles (i.e., the DM susceptibility) were 302- and 378-times greater for αF54C and αF54A, respectively, compared with WT (Fig. 3H and SI Appendix, Table S3).

Previous studies have used the specific rate enhancement (i.e., the slope of the DM susceptibility curve divided by the intrinsic dissociation rate constant) as a measure of DM’s catalytic efficacy toward a particular substrate (6, 15). Even after this normalization the αF54 mutations still are extreme outliers, with specific rate enhancements over 400 μM⁻¹ for αF54A, almost 90-times greater than for WT (Fig. 3I and SI Appendix, Table S3). The extreme sensitivity of αF54C to DM was not restricted to the CLIP peptide, as it was also observed for the HA peptide (Fig. 3J, open bars, and J, and SI Appendix, Fig. S6).

**Substitution of αF54 Weakens MHC-Peptide H-Bonding Interactions.** We determined the effect of the αF54C mutation on the overall H-bond network by HDx MS/MS. The amplitudes of the unresolved initial HDx phases are the same for both WT and αF54C (Fig. 1C). The fast-phase amplitudes also are similar for WT and αF54C, as are the respective half-lives for this phase (SI Appendix, Table S1). However, the slow phase of HDx is significantly faster for αF54C than for WT, with half-lives of 1.8 and 18.1 h, respectively (Fig. 1C and SI Appendix, Table S1). The pattern of deuterium incorporation observed by ETD/MS/MS is similar for αF54C as for the WT-HA complex; however, the Y3, V4, and N7 NH exchange is faster than for WT (Fig. 1I, open bars, and I, and SI Appendix, Figs. S2, S3, and S7). For the WT-HA complex, these H-bonds represent the most stable of the interactions, and would be expected to correspond to those most important in determining the overall peptide-MHC lifetime. Our results suggest that the αF54C mutation disrupts these key H-bonds in the N-terminal half of the peptide-binding groove.

**Substitution of αF54 Dramatically Increases Binding to DM.** To determine whether the increased DM susceptibility observed upon substitution of αF54 was associated with increased binding affinity for DM, we used a surface plasmon resonance binding assay. Specific, saturable, dose-dependent binding was detected for both αF54 variants but not for WT, αS53A, αF51A, or any of the other mutants (Fig. 4A–F and SI Appendix, Fig. S4 F–I). Equilibrium binding analysis revealed K_d values of 0.5 μM and 0.7 μM for αF54A and αF54C, respectively (Fig. 4G). We performed several experiments to verify the specificity of the tight binding observed for αF54A and αF54C to DM. First, we observed substantially reduced binding of DM to immobilized αF54C at pH 7.0 relative to pH 5.6 (Fig. 4H), consistent with the known pH dependence of the interaction (31). Second, binding was abrogated by preincubation with a monoclonal antibody (LB3.1) specific for a conformational epitope on MHC II (32) near the presumptive DM binding interface (SI Appendix, Fig. S8 A–D). Finally, DM binding was observed also for CLIP peptide complexes of αF54C but not WT (SI Appendix, Fig. S8 B–D). These results indicate that αF54 substitutions, which increase sensitivity to DM-mediated peptide release, also increase the binding affinity for DM.
Crystal Structure of αF54C-CLIP Shows a Conformational Change in the Vicinity of a Critical DM Contact Residue. To investigate the structural basis for the altered H-bond strengths, increased DM susceptibility, and increased DM binding activity of αF54C, we determined its 2.3 Å crystal structure in complex with the CLIP peptide, and for comparison the 2.7 Å crystal structure of WT bound to CLIP, in the same unit cell (SI Appendix, Table S4). Both crystal structures have two molecules in the asymmetric unit. For WT-CLIP structure, the molecules (blue and green in Fig. 5 and SI Appendix, Fig. S9 A–E) overlay with no major deviations (except for an exposed loop near βP108 that shows large rmsd between most MHC II structures solved to date). The structure of αF54C (purple and red in Fig. 5 and SI Appendix, Fig. S9 A–E) overall was very similar to WT except for a region proximal to the αF54C mutation (indicated by carets in SI Appendix, Fig. S9 B and F), for which large differences were observed specifically in one of the two molecules in the asymmetric unit (SI Appendix, Fig. S9 F–I).

The conformational changes, which involve a loop between two strands of the β-sheet platform, the short 3₁₀ helix encompassing residues αL45 to αF50 at the edge of the binding site, and the extended strand region αF51 to αF54, can be seen in Fig. 5. The conformational change results in a ~20° reorientation of the short 3₁₀ helix, together with a partial unwinding toward a more canonical α-helical pitch (compare Fig. 5 B and C). These changes are accompanied by a concerted set of rotamer changes in residues that surround the P1 pocket. Large changes are observed for αF48, which positions its side chain on the side of the P1 pocket, displacing βF89, which also undergoes a rotamer change, and for αL45, which moves to contact βW153, replacing the contact normally made by the αF48 side chain. The rearrangements of αL45, αF48, and βF89 resulted in changes in the shape and volume of the P1 pocket (SI Appendix, Fig. S10).

The conformational changes in the 3₁₀ helix and adjacent strand region appear to be induced by contact at the αF51 position in molecule 2 of the asymmetric unit with another molecule in the crystal (SI Appendix, Fig. S11). The contact is observed for both WT and αF54C, but only in the mutant is the conformational change induced. These changes result in lowered B-factors at the contact site relative to the remainder of the αβ1 peptide-binding domain, but increased B-factors for the bound peptide (SI Appendix, Fig. S12). The αF51 position is known to be a critical determinant for DM binding and activity (11), and contact with another molecule at the αF51 position may simulate the effect of a contact at this position with DM.

Discussion

The mechanism of DM-mediated catalysis of peptide exchange on MHC II proteins and the nature of the DM-MHC interaction has been the subject of intensive investigation (6, 9–18, 24, 27). In this work, we evaluated the contribution of MHC II structural elements at the N-terminal side of the peptide binding site, using HDx MS to measure MHC-peptide H-bonding strengths, site-specific mutagenesis to identify MHC II side chains with important contributions to DM binding and facilitated peptide exchange, and crystal structure analysis of the HLA-DR1 mutant αF54C. Substitution of αF54 in the MHC II extended strand region of the peptide binding site results in a protein with greatly increased susceptibility to DM-mediated peptide release, increased DM-binding affinity, and increased MHC II-peptide dynamics, particularly in the MHC II-peptide H-bonding interactions that surround the P1 pocket. The crystal structure of αF54C reveals conformational alterations near the N terminus of the bound peptide involving the reorientation of the 3₁₀ helical region (α45–

Fig. 3. Substitution of αF54 results in a dramatic increase in the rate of DM catalyzed peptide exchange. (A) Location of mutated residues in MHC peptide-binding groove (additional mutations shown in SI Appendix, Fig S4). (B–G) Peptide release kinetics in the presence of various concentrations of DM. (H) The K_{app} values plotted against the concentration of DM. (Inset) Shows WT, αQ57C, and αS53A with expanded y axis. (I) DM-mediated rate enhancements were calculated for each mutant by dividing the slope of the K_{app} vs. [DM] plots by the intrinsic dissociation rates. Closed bars, MHC II-CLIP. Open bars, MHC II-HA. (J) HA K_{app} values plotted against the concentration of DM.
Surface plasmon resonance experiments were conducted on a Biacore 3000 instrument using CM5 chips and 10 mM sodium citrate pH 5.5, 150 mM NaCl, 3 mM EDTA, and 0.05% (vol/vol) surfactant P20 at 30 μL/min. Protein was immobilized using amine coupling using ethyl (dimethylaminopropyl) carbodiimide and N-Hydroxysuccinimide. In the standard ligand coupling procedure, excess avidated dextran carbohydrate layers are capped with ethanolamine. For surfaces capped this way we observed significant nonspecific binding of DM at pH 5.5, presumably because of interactions with the carboxydrxen matrix (pK_a ~ 5). Instead we copped with a different reagent, 2-amino-ethanol, which maintains a negative charge at low pH, and observed low nonspecific binding at pH 5.5. Regeneration of the DM-coupled surface was carried out using 0.1 M glycine pH 11.5 for 30 s until a stable baseline was reached. Binding interactions were fit to a heterologous binding model using BIAeval software.

Protein crystalllography. Crystals of αF54C CLIP complexes were grown at 4 °C in hanging drops over 0.7% precipitant, 100 mM sodium citrate (pH 5.5), 150 mM NaCl, and 30% PEG 4000. Protein was transferred to solution with 25% ethylene glycol before flash freezing in liquid nitrogen. Diffraction data collected under cryo conditions at the SADBE beamline (λ = 1.1 Å) from single crystals. Crystals of the αF54C-CLIP complex reported here describe a unique characterization of conformational changes involved in peptide exchange by DM, and provides a structural model for the DM-receptive conformation of MHC II that is intermediate between a stable MHC II-peptide complex and the proposed peptide-free conformation.

Materials and Methods

Protein Production. The extracellular domains of wild-type and mutant HLA-DR1 α- and β-chains were individually expressed in Escherichia coli and folded in vitro as previously described (37). Soluble DM molecules secreted from stably transfected Drosophila S2 cells were isolated by immunoaffinity chromatography. HLA-DR1 and DM were further purified by size exclusion chromatography before use (Superdex 200).

Polarization Assays. N-termally acetylated HA (Ac-PKFRQNLTLRAT-OH) and CLIP (Ac-VSKMRMTAPTLIMQ-OH) peptides (21st Century Biochemicals) were labeled with Alexa-488 tetrafluorophenyl ester (Molecular Probes) at position K2 (HA) or K3 (CLIP). Peptide complexes were prepared by incubation of WT or mutant MHC II (150 nM) with 25-nM labeled peptide for 3 d at 37 °C in 96-well plates coated with avidated dextran. Association with DM was followed by fluorescence polarization (488-nm excitation, 520-nm emission) after addition of 100-fold excess unlabeled HA peptide. Dissociation rate constants were determined by fitting to a single phase exponential decay. The rate enhancement by DM was calculated as the slope of the linear rate vs. DM concentration plot, and the DM-enhancement factor calculated as the slope divided by the intrinsic (i.e., no DM) rate constant (6). Similar values were obtained regardless of whether intrinsic dissociation rates were determined by experiments performed in the absence of DM or by extrapolation of DM-dependent rate profiles.

Surface Plasmon Resonance. Surface plasmon resonance experiments were carried out on a Biacore 3000 instrument using CMS chips and 10 mM sodium citrate pH 5.5, 150 mM NaCl, 3 mM EDTA, and 0.05% (vol/vol) surfactant P20 at 30 μL/min. Protein was immobilized using amine coupling using ethyl (dimethylaminopropyl) carbodiimide and N-Hydroxysuccinimide. In the standard ligand coupling procedure, excess avidated dextran carbohydrate layers are capped with ethanolamine. For surfaces capped this way we observed significant nonspecific binding of DM at pH 5.5, presumably because of interactions with the carboxydrxen matrix (pK_a ~ 5). Instead we copped with a different reagent, 2-amino-ethanol, which maintains a negative charge at low pH, and observed low nonspecific binding at pH 5.5. Regeneration of the DM-coupled surface was carried out using 0.1 M glycine pH 11.5 for 30 s until a stable baseline was reached. Binding interactions were fit to a heterologous binding model using BIAeval software.

Protein crystalllography. Crystals of αF54C CLIP complexes were grown at 4 °C in hanging drops over 12% PEG 4000, 100 mM sodium acetate (pH 5.6), 100 mM histidine, 5 mM DTT. WT crystals were obtained under the same conditions, except by streak seeding. Crystals were transferred to well buffer with 25% ethylene glycol before flash freezing in liquid nitrogen. Diffraction data collected under cryo conditions at the National Synchrotron Light Source on the x29 beamline (λ = 1.1 Å) from single cryo cryo conditions by streak seeding crushed F54C CLIP crystals. Crystals were obtained regardless of whether intrinsic dissociation rates were determined by experiments performed in the absence of DM or by extrapolation of DM-dependent rate profiles.

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crystals (WT: 0.1 × 0.05 × 0.05, F54C: 0.6 × 0.07 × 0.07 mm) using an ADSC Q315 detector were processed with HKL2000. Initial phasing was obtained by molecular replacement using Phaser (38) with coordinates from another HLA-DR1 structure (PDB code: 1PWW) as a search model. Multiple rounds of refinement and building were carried out using Phenix (39) and COOT (40). PDB ID codes are 3QDX (F54C-CLIP) and 3QXA (WT-CLIP).

H/D Exchange. WT and ΔF54C loaded with HA peptide were transferred to PBS pH 7.2 and concentrated to 55 μL. To initiate H/D exchange, MHC II-peptide complexes or HA peptide alone were diluted 1:10 into PBS in D₂O, followed by incubation at 25 °C. At each time interval we quenched the reaction by diluting the exchange mixture 1:10 into 1 M glycine pH 2.7 at 4 °C and flash-freezing at −40 °C. All components of the LCMS instrument (Thermo Scientific) in contact with the sample were chilled to 4 °C before data collection. Samples were diluted twofold in ice cold buffer A (0.1% TFA in 2% ACN) immediately before injection (50 μL/min) onto 1 × 15-mm C4 PepMap300 and C18 PepMap100 columns in the presence of HLADRM. With transfer to 25% ACN the MHC II is retained and the peptide is released for infusion into the mass spectrometer, using capillary temperature 275 °C and source voltage 4.5 kV. Spectra were acquired from m/z 450–660 with a time of −1 s. Average m/z values were calculated by summing each peak in the isotope distribution weighted by its relative abundance, and converted to fractional H/D exchange by reference to the equation, which assumes equal probability of exchange at each position:

\[ P(k) = \sqrt{k/(n-k)!} \left(1-p\right)^{(n-k)} \]

where \( n \) is the number of hydrogens exchanged.

Electron Transfer-Induced Dissociation MS. Peptides eluted as described above were introduced into an amaZon-ETD quadrupole ion-trap instrument (Bruker Daltonics) operating in positive ion mode and subjected to ETD fragmentation. ETD spectra were processed and assigned to c- and z-series ions using Bruker Daltonics Data Analysis and BioTools software. Average peptide fragment masses observed at various times in D₂O were compared with H₂O values to determine HΔMW. Sequential c-series or z-series ions were subtracted to give ΔHΔMW values, which represent the amount of deuterium incorporation per amide (or pairs of amides) at each time point.

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10. Chou CL, Sadeqzadeh S (2000) HLA-DR molecules recognize the major histocompatibility complex class II antigen presentation can be altered by manipulation of MHC-peptide kinetic stability and building were carried out using Phenix (39) and COOT (40).