Formation of Two Peptide/MHC II Isomers Is Catalyzed Differentially by HLA-DM†

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ABSTRACT: Major histocompatability class II proteins are transmembrane $\alpha\beta$-heterodimers that present peptides to T-cells. MHC II may bind exogenous peptides directly at the cell surface. Alternatively, peptides derived from processing of endosomal protein may bind to MHC II in endosomal compartments. There, HLA-DM catalyzes the formation of peptide/MHC complexes, which are then transported to the cell surface. Here we report evidence that the peptide Ii CLIP 81−104 binds to DR*0404 in two alternate registries, whose dissociation rates, while kinetically indistinguishable at pH 5.3 and 37 °C, are kinetically resolved in the presence of HLA-DM. In one registry isomer, CLIP Met 91 is placed in the N-terminal P1 pocket of DR*0404, and peptide dissociation is readily catalyzed by HLA-DM. In a second proposed registry, likely with CLIP Leu 97 in the P1 pocket, the complex is substantially less sensitive to HLA-DM catalysis. Without HLA-DM, or at pH 7, the fraction of each isomer formed in solution is relatively insensitive to the duration of incubation with peptide. However, with HLA-DM, the fraction of the DM-insensitive isomer is dramatically influenced by peptide incubation time. The mechanism of isomer formation appears to be determined by the HLA-DM-modified relative association to the two registries, followed by HLA-DM-catalyzed dissociation of each isomer and rebinding, leading to a final isomer composition determined by these kinetic constants. Intramolecular isomer interconversion does not appear to be involved. The behavior of these complexes may provide a model for peptide editing by DM in endosomes.

Major histocompatability class II proteins are transmembrane $\alpha\beta$-heterodimers that present self-peptides and exogenous peptides to the cellular immune system. Crystallographic studies of peptide/MHC II complexes have revealed the nature of the intermolecular interactions (*1*−*6*). The peptides bind to the MHC II protein through a conserved hydrogen bond network between the peptide backbone and certain amino acids in the MHC II molecule. Interactions between peptide side chains and MHC II occur at five approximately defined pockets (P1, P4, P6, P7, P9). In human MHC II alleles, pocket P1 is usually occupied by a hydrophobic peptide amino acid at the N-terminal region of the peptide.

In late endocytic compartments, antigenic and self-peptides are processed and loaded to an MHC II protein in the presence of a catalyst, HLA-DM. HLA-DM is a nonclassical MHC II heterodimer without apparent peptide binding capability (*7*). Peptide/MHC II complex formation is facilitated by HLA-DM, which accelerates the dissociation of the endogenous invariant chain fragment, CLIP[1] 81−104, from the MHC II, allowing efficient exchange with other peptides (*8*−*11*). The importance of HLA-DM in shaping the peptide repertoire of peptides bound to the MHC II has been demonstrated in various studies (*12*, *13*). HLA-DM appears to act as a peptide editor, biasing toward the formation of higher stability complexes through catalysis.

Evidence from in vitro kinetic studies and T-cell recognition assays suggests that some peptide/MHC II complexes may exist in more than one conformation (*14*−*18*). The nature of the molecular structure of these kinetic isomers has been investigated. In one example, a peptide binds to murine $I-E^k$ in a single registry, but in two distinct conformations as determined by kinetic studies and ^{19}F NMR spectroscopy (*14*, *15*, *19*). These isomers are believed to interconvert from one form to another without peptide release and rebinding (*15*). In a second study, the results suggested that an ovalbumin-derived peptide binds $I-A^k$ in two distinct registries (*20*). In a third case, myelin basic protein peptide (MBP) and $I-A^u$ form two isomeric complexes, where each isomer is recognized by a distinct T-cell clone (*21*).

On the basis of in vitro kinetic studies, we recently found that a complex between human invariant chain Ii CLIP peptide f81−104 and human DR4*0404 MHC II exists in two conformations, where one isomer is relatively insensitive to HLA-DM compared to the second isomer (*22*). The origin

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[1] Abbreviations: f, fluorescein; HCgp-39, human cartilage glycoprotein 39; f-peptide, fluorescein-labeled peptide; Ii, invariant chain; MHC(II), major histocompatability complex (class II); HLA, human leukocyte antigen; HPSEC, high-performance size exclusion chromatography; CLIP, class II-associated Ii peptide.

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of these isomers was not understood, since according to crystallographic studies of CLIP/DR3, one structure was observed where CLIP M91 is placed on the P1 pocket of the MHC (*5*). Here, we provide evidence that human Ii CLIP 81−104 may bind to DR4*0404 in two distinct registries. (Another possible interpretation of our results is discussed briefly.) We investigated the kinetic effects of HLA-DM on the process of isomer formation, and on the basis of these data, we propose a kinetic mechanism of catalyzed isomer formation.

MATERIALS AND METHODS

Expression and Purification of Recombinant Soluble HLA-DR4 Molecules. Soluble HLA-DR4*0404 molecules were isolated and purified as previously described (*23*, *24*). Briefly, Schneider-2 (S2) *Drosophila* melanogaster cells were cotransfected with pRmHA-3 containing a soluble DRA*0101 insert, soluble DRB1 insert (*0404) with epitope tag, and pUChs-Neo, using a calcium phosphate transfection kit (LTI). Transfected cells were cultured in Schneider's *Drosophila* medium containing 10% v/v FBS, 2 mM L-glutamine, and 50 μg/mL gentamycin and selected in 1.5 mg/mL active G418 (LTI). Selected cells were induced for 7 days with 1 mM $CuSO_4$, and DR expression was verified by Western blotting of tissue culture supernatants using an anti-DR antiserum (CHAMP; gift of L. Stern, MIT) and the epitope tag-specific monoclonal antibody (mAb), KT3. Cells expressing sDR*04 were cloned by limiting dilution (1 cell/well) in the presence of untransfected S2 feeder cells and 1.5 mg/mL G418. A clone with high expression was identified by Western blotting. For affinity purification, cultures were scaled up in 0.5 L spinner flasks.

The protocol for immunoaffinity purification of recombinant DR molecules was similar to that described by Gorga et al. (*25*). Briefly, the anti-DR mAb, L243, which recognizes assembled $\alpha\beta$-dimers, was coupled to CNBr-activated Sepharose 4B (Pharmacia), using the manufacturer's protocol. PMSF (1 mM final concentration) and, in some experiments, iodoacetamide (10 mM) were added to tissue culture supernatants after induction, and cell debris and insoluble material were removed by centrifugation, followed by filtration through a 0.20 μM CN membrane (Nalgene). Cleared supernatants were passed over the columns at least twice. After being washed in PBS, protein was eluted with 0.10 M Tris-HCl, pH 11.0, and neutralized immediately with 0.2 volume of 2 M Tris-HCl, pH 6.8. Protein-containing fractions, determined by absorbance at 280 nm, were pooled and concentrated by centrifugal ultrafiltration (Centricon-30, Amicon). Eluates were analyzed for purity by SDS−PAGE and Coomassie Blue staining; DR α- and β-chain bands comprised greater than or equal to 85% of total protein. Heterodimeric assembly was checked by native PAGE performed as described (*26*). Soluble DR protein (sDR) (*24*) was quantitated using the Bradford assay (Bio-Rad).

Expression and Purification of Recombinant Soluble HLA-DM Molecules. Unmodified sDM containing a FLAG epitope tag at the sDMa C terminus were purified from Schneider-2 supernatants by immunoaffinity chromatography on M2-agarose and FLAG peptide elution as described (*26*). Further purification was achieved by high-performance size exclusion chromatography (TSK G3000SWXL, TosoHaas) in 10 mM sodium phosphate, pH 7.0, 150 mM NaCl, and 0.02% NaN_3 with isolation of the sDM dimer peak.

Synthesis and Purification of Peptides. Peptides were synthesized with standard FMOC chemistry on an Applied Biosystems 431A peptide synthesizer (Applied Biosystems, Foster City, CA). When indicated, the peptide resins were labeled overnight at the free N-termini with a 5-fold molar excess of 5- (and 6-) carboxyfluorescein succinimidyl ester (Molecular Probes) in dimethyl sulfoxide and a catalytic amount of diisopropylethylamine. Acetylation of resins was done with a 10-fold molar excess of acetic anhydride and pyridine in dimethylformamide for 45 min. Resin cleaving was done for ~3 h at room temperature with trifluoroacetic acid in the presence of ~5% 1,2-ethanedithiol and 5% thioanisole as described elsewhere (*15*). The peptides were purified by HPLC (reversed-phase chromatography) and checked for identity and purity by mass spectrometry. The names and sequences of the peptides used in this study were the following: human fIi CLIP 81−104 (f-LPKPPKPVSKMRMATPLLMQALPM, f = fluorescein), human Ii CLIP 81−104 (LPKPPKPVSKMRMATPLLMQALPM), human fIi CLIP 81−104 L97A (f-LPKPPKPVSKMRMATPALMQALPM), human fIi CLIP 90−104 (f-KMRMATPLLMQALPM), human fIi CLIP 90−104 M93A (f-KMRAATPLLMQALPM), murine fIi CLIP 89−99 (f-QMRMATPLLMR), and murine Ii CLIP 85−99 V87A L96A M98F (Ac-KPASQMRMATPALFR, Ac = acetyl).

Preparation of Peptide/MHC II Complexes (See Table 1). HLA-DR4*0404 (1.7 μM) was incubated with excess peptide overnight (5 μM) in a 37 °C incubator at pH ~5.3. The buffer composition at pH 5.3 was 9 parts by volume of PBS, pH 7, buffer (PBS = 10 mM phosphate, 150 mM NaCl, 0.02% NaN_3) to 1 part of 1 M sodium citrate, pH 4.8, buffer (1 M sodium citrate, pH 4.8). The peptide/MHC II association solution was cooled to 4 °C. Then, the excess free peptide was separated from the complex with a small-scale Bio-Rad spin column at 4 °C. [Spin columns were packed with Sephadex G50 superfine (Pharmacia) and protein-blocked with 1% BSA/PBS solution to reduce MHC II binding to the column. Spin columns were then washed with excess PBS buffer, pH 7.0, before use.] The eluted solution containing the complex was brought to a final volume of ~800 μL with PBS/citrate for experiments at pH 5.3 unless indicated otherwise. Unlabeled human Ii CLIP 81−104 was also added (10 μM final concentration) to serve as a competitor, preventing fluorescent peptide rebinding during the dissociation kinetics. When indicated, a total concentration of 0.25 μM of a soluble form of HLA-DM was used to catalyze peptide dissociation.

Dissociation Kinetics Measurements. A kinetic experiment was started by injecting a 4 °C aliquot (45 μL) of the complex solution into a 5 μm particle size HPSEC (high-performance size exclusion chromatography) column (dimensions: 7.8 mm × 30 cm, G3000SWXL TSK-GEL; Tosohaas, Montgomeryville, PA) coupled to a fluorescence detector. The size exclusion column separates the f-peptide/MHC II complex from the f-peptide released by the MHC II. The complex elutes around 9 min, while the peptide elutes approximately 2 min later with a flow rate of 1.0 mL/min and a PBS, pH 7.0, mobile phase at 25 °C. The relative amount of labeled peptide/DR*04 complex was measured as a function of time by the height of the fluorescence signal

detected by a fluorometer with excitation wavelength set at 492 nm and emission detection set to 522 nm. Kinetic studies were done with a sensitive Shimadzu RF-10AXL fluorescence detector.

The half-life of the complex ($t_{1/2}$) was generally calculated from a single-exponential fit with the Kaleidagraph software (Synergy) of the normalized complex fluorescence versus time, according to the equation $F/F_0 = (e/2)\exp(-t/t_{1/2})$, where F is the complex fluorescence at time t, F_0 is the initial complex fluorescence, and $t_{1/2}$ is the half-life of the complex, which is related to the dissociation rate constant by $t_{1/2} = \ln 2/k$. A biphasic fit refers to need of the double-exponential function, according to the equation $F/F_0 = (e/2)A \exp(-t/t_{1/2}(\text{fast})) + (e/2)(1 - A)(\exp(-t/t_{1/2}(\text{slow}))$.

*Preparation of the Preloaded Complex AcMBP 84−99/DR*0404.* DR*0404 (1.7 μM) was incubated for 4 days in the presence of 200 μM unlabeled MBP peptide (AcMBP 84−99) at pH 5.3 and 37 °C in the presence of 0.25 μM DM and then stored at 4 °C until needed. Just before an experiment was started, the complex was separated from the excess free peptide with a small-scale spin column at 4 °C. AcMBP/0404 was used only in studies involving DM.

*Preparation of the "Preloaded" Murine CLIP 85−99 V87A L96A M98F/ DR*0404 Complex.* A murine CLIP 85−99 variant was designed to dissociate rapidly from DR*0404. The complex formed between the fluorescein-labeled peptide and DR*0404 dissociates with a half-life of 17 min. A small amount of a stable fluorescent complex (9% or less of the total complex, half-life $\gg$ 2 h) of unknown origin was detected. The overall low stability of the complex of DR*0404 and this peptide makes the peptide an ideal "preloader" for DR*0404. In this context, an MHC II is preloaded when it is bound by an unlabeled fast-dissociating peptide, allowing for a rapid exchange for other fluorescently labeled peptides present in excess in solution (*27*, *28*). Therefore, DR*0404 (1.7 μM) was incubated for 5−6 days in the presence of 200 μM unlabeled "fast"-dissociating peptide (murine CLIP Ac85−99 V87A L96A M98F) for 5 days at pH 5.3 and 37 °C and then stored at 4 °C until needed. The complex was separated from the excess free peptide with a spin column. The isolated complex is referred to as the "preloaded complex" as described. In practice, only 40−50% of DR*0404 gets loaded with this peptide, as described in the Results section. This complex was used in experiments with and without DM.

*Time-Dependent Association Reactions of Human Ii CLIP f81−104 with Preloaded DR*0404 in the Absence or Presence of Soluble Recombinant HLA-DM.* The isolated preloaded complex (murine AcCLIP/ 85−99 V87A L96A M98F/DR*0404, 0.50 μM) was incubated with 5.0 μM human Ii CLIP f81−104 for a specified amount of time (5 min, 10 min, 2 h, 4 h, ~20 h) in the presence or absence of 0.25 μM HLA-DM. The preloaded complex dissociates with a half-life of <10 min in the presence of 0.25 μM HLA-DM. The association reaction was stopped by cooling to 4 °C, and the complex was separated from the excess labeled peptide with spin columns. The dissociation kinetics of the recovered complex was measured at pH 5.3 (PBS/citrate) at an MHC II concentration of ~0.05 μM in the presence of 10 μM competitor peptide (unlabeled Ii CLIP 81−104) and 0.25 μM HLA-DM. The presence of DM allows the kinetic resolution of the two isomers, since one isomer is more DM-sensitive than the other. The normalized fluorescence dissociation kinetic data were fit to a double exponential. The preexponential weight of the fast phase corresponds to the relative amount of the DM-sensitive isomeric complex, and the preexponential weight of the slow phase corresponds to the relative amount of the HLA-DM "insensitive" isomer.

Similar procedures were used for the kinetic studies of human Ii CLIP f90−104 with preloaded DR*0404 and for time-dependent association reactions of human Ii CLIP f81−104 with preloaded AcMBP 84−99/DR*0404 in the presence of 0.25 μM soluble recombinant DM.

Kinetic Simulations. The kinetic simulations were done with the Mathematica Software, version 3.0 (Wolfram Research).

Determination of Best Fit Half-Lives of Isomers (for Studies Summarized in Figures 4 and 5). For studies based on the DR*0404 preloaded complexes + DM (corresponding to Figures 4 and 5), the best fit dissociation half-lives of the fast- and slow-dissociating isomers of CLIP f81−104/DR*0404 (and CLIP f90−104/DR*0404) were determined from a "concensus fit" of the dissociation curves in each individual study, some of which are shown in Figure 4. In this procedure, the dissociation curves were simultaneously fit, with concensus values of $t_{1/2}$(fast) and $t_{1/2}$(slow), while the preexponential coefficients were allowed to vary independently (*29*).

*Calculation of Relative Association and Dissociation Rate Enhancements. (A) f81−104/DR*0404.* The uncatalyzed dissociation half-life estimated from a monophasic fit is ~11 h. The dissociation half-lives in the presence of DM are ~0.3 and ~4.3 h for the fast- and slow-phase isomers, respectively (two isomers, based on studies corresponding to Figure 4). Therefore, the dissociation enhancements are 37- and 2.6-fold, respectively, and the relative enhancement of dissociation is 37/2.6 = 14 (DM-sensitive vs DM-insensitive isomers).

In this work, the relative on-rate constants in the absence of DM are $0.74k_{on}$ and $0.26k_{on}$ for the DM-sensitive and DM-insensitive isomers, respectively. This is based on short incubation time studies shown in Figure 5A (squares). See Results section for discussion of how the isomers were resolved. In the presence of 0.25 μM DM, the relative association rates are $0.917k_{on}'$ and $0.083k_{on}'$ for the DM-sensitive and DM-insensitive isomers, respectively. Therefore, the relative enhancement of association is $(0.917k_{on}'/0.74k_{on})/(0.083k_{on}'/0.26k_{on}) = 3.9$ (DM-sensitive vs DM-insensitive isomers).

*(B) f90−104/DR*0404.* The uncatalyzed dissociation rate estimated from a single-exponential fit is ~40 h. The dissociation half-lives in the presence of DM are ~0.3 and ~7.1 h (two isomers, based on studies corresponding to Figure 5B). Therefore, the dissociation enhancements are 133- and 5.6-fold, respectively, and the relative enhancement of dissociation is 133/5.6 = 24 (DM-sensitive vs DM-insensitive isomers).

The relative on-rate constants in the absence of DM are $\sim 0.5k_{on}'$ and $0.5k_{on}$ for the DM-sensitive and DM-insensitive isomers, respectively. This is based on short incubation time studies shown in Figure 5B (squares). Refer to the Results section for discussion of how the isomers were resolved. In the presence of 0.25 μM DM, the relative association rates are $\sim 0.8k_{on}'$ and $0.2k_{on}'$ for the DM-sensitive and DM-

Table 1: Dissociation Kinetic Half-Lives of the Fluorescently Labeled CLIP Peptide Variant/DR*0404 Complexes in the Presence and Absence of 0.25 μM HLA-DM[a]

peptide/MHC II complex	peptide sequence[b]	$t_{1/2}$, h (−HLA-DM)	$t_{1/2}$(fast), h (+HLA-DM)	$t_{1/2}$(slow), h (+HLA-DM)	% slow phase (+HLA-DM)
Ii CLIP f81−104/DR*0404	f-LPKPPKPVSK**M**RMATPLLMQALPM	11.2	0.25	3.5	34
Ii CLIP f90−104/DR*0404	f-K**M**RMATPLLMQALPM	39.6	0.32	8.0	57
Ii CLIP f90−104 M93A/DR*404	f-K**M**RAATPLLMQALPM	73[c]	0.50	10.1	60
Ii CLIP f81−104 L97A/DR*0404	f-LPKPPKPVSK**M**RMATPALMQALPM	6.0	0.27[d]		
murine Ii CLIP f89−99/DR*0404	f-Q**M**RMATPLLMR	17.5	0.20[d]		

[a] The half-lives of the complexes were calculated from a best fit to a single-exponential decay function of the normalized fluorescence decay of the complex versus time, unless indicated otherwise. Each complex was prepared and isolated as described in Materials and Methods. Dissociation kinetics, with half-lives indicated in the third column, were done with PBS/citrate, pH 5.3, buffer. Columns 4−6 correspond to best fit kinetic parameters of the complexes dissociating at pH 5.3 with 0.25 μM HLA-DM. Some of these kinetics were best described by a double-exponential fit, where the half-lives of the fast- and slow-dissociating components are given in columns 4 and 5, respectively. The sixth column is the percentage of the slowly dissociating component. All dissociation kinetics were measured in the presence of an unlabeled peptide competitor to prevent labeled peptide rebinding to DR*0404. [b] The putative peptide P1 anchor is shown in bold. Mutations relative to wild-type human Ii CLIP 81−104 peptide are underlined. [c] A slightly better fit may be obtained by a biphasic decay function. [d] Kinetics adequately described with a single-exponential function.

insensitive isomers, respectively. Therefore, the relative enhancement of association is $(0.8k_{on}'/0.5k_{on})/(0.2k_{on}'/0.50k_{on}) = 4$ (DM-sensitive vs DM-insensitive isomers).

RESULTS

*Alternative Registries for CLIP Peptides Bound to DR*0404.* In this study, complexes are formed between the soluble form of DR4*0404 MHC II and fluorescently labeled peptides. The soluble form of HLA-DM is used to enhance the dissociation of these complexes. The dissociation kinetics of the fluorescently labeled CLIP peptide 81−104 ("long CLIP") or CLIP variants from DR4*0404 in the presence or absence of 0.25 μM HLA-DM were fit to either a single-exponential or a double-exponential form, as appropriate (see Materials and Methods). The term *monophasic* is used for dissociation kinetics of the peptide/MHC II complex adequately described by a single-exponential function, while the term *biphasic* is used for kinetics described by a double-exponential function. We refer to a complex as "HLA-DM sensitive" if $t_{1/2}$(in)/$t_{1/2}$(obs) is large (relative to values from a second complex), where $t_{1/2}$(in) is the intrinsic dissociation half-life of the complex and $t_{1/2}$(obs) is the half-life of the complex in the presence of 0.25 μM HLA-DM. The term "slow phase" refers to the peptide/MHC II isomer whose dissociation is relatively HLA-DM insensitive; the term "fast phase" refers to the peptide/MHC II isomer whose dissociation is relatively HLA-DM sensitive.

In recent experiments, the dissociation kinetics of the human Ii CLIP f81−104/DR*0404 complex were measured (*22*). The dissociation kinetics of the complex are accurately described with a single-exponential function with a half-life of ∼11 h. In contrast, the dissociation kinetics of this complex in the presence of HLA-DM are biphasic. The biphasic components of the HLA-DM-enhanced kinetics include ∼70% of a short-lived species ($t_{1/2}$ ∼15 min) and ∼30% of a longer lived species ($t_{1/2}$ ∼4 h). This corresponds to a 40-fold dissociation enhancement of one isomer by HLA-DM and a 3-fold enhancement of dissociation for a second isomer. The kinetic behavior of this complex is unusual, since monophasic dissociation kinetics of peptide/MHC II complexes is usually found along with monophasic kinetic behavior in the presence of HLA-DM (*30*).

To identify the CLIP sequences required for this kinetic behavior, a series of human Ii CLIP peptide variants were synthesized, and the dissociation kinetics of the corresponding complexes in the presence and absence of HLA-DM were measured. The variants include truncations of the "parent" peptide Ii CLIP f81−104 as summarized in Table 1. The dissociation kinetics of the complex Ii CLIP f90−104/DR4*0404 (f90−104 = "short CLIP") is monophasic at pH 5.3 to within experimental error and biphasic in the presence of HLA-DM (Table 1). The interpretation of this result is that the N-terminal peptide residues 81−89 are not essential for the isomer formation. In a similar experiment, the dissociation kinetics of the complex Ii CLIP f90−104 M93A/DR*0404 were slightly biphasic at pH 5.3 and biphasic in the presence of HLA-DM as well, arguing that neither residues 81−89 or Met 93 are essential for the isomer formation. The dissociation kinetics of the complex Ii CLIP 81−104 L97A/DR4*0404 are monophasic to within experimental error at pH 5.3 as well as monophasic in the presence of HLA-DM. The dissociation of the complex is also relatively HLA-DM sensitive (22-fold dissociation enhancement). These results imply that the Leu 97 peptide side chain is essential for the formation of the second isomer. One interpretation of these data is that human Ii CLIP f81−104 is able to bind DR*0404 in two registries. In peptide/DR*04 complexes, a peptide hydrophobic residue at the P1 pocket position is essential for their stability (*31*, *32*). Further, in the Ii CLIP 81−104 peptide/DR3 structure residue Met 91 occupies the P1 pocket (*5*). Thus it is likely that, in one isomer, peptide Met 91 occupies the P1 pocket; however, we propose that in a second isomer, peptide Leu 97 occupies the P1 pocket (Figure 1).

Additional evidence supports this conclusion. The dissociation kinetics of murine Ii CLIP 89−99/DR*0404 are monophasic in the presence and absence of HLA-DM. The dissociation of the complex is also relatively sensitive to HLA-DM catalysis (86-fold dissociation enhancement). The murine peptide (QMRMATPLLMR) essentially contains the "core" binding registry of human CLIP for binding to DR*0404 (MRMATPLLM). An interpretation of this result is that peptide C-terminal residues 101−104 in human CLIP are essential for the formation of the second isomer, consistent with the assignment of L97 as the P1 residue of the second isomer.

On the basis of these results, CLIP 81−104 binds to DR*0404 in the MRMATPLLM registry, as well as in the

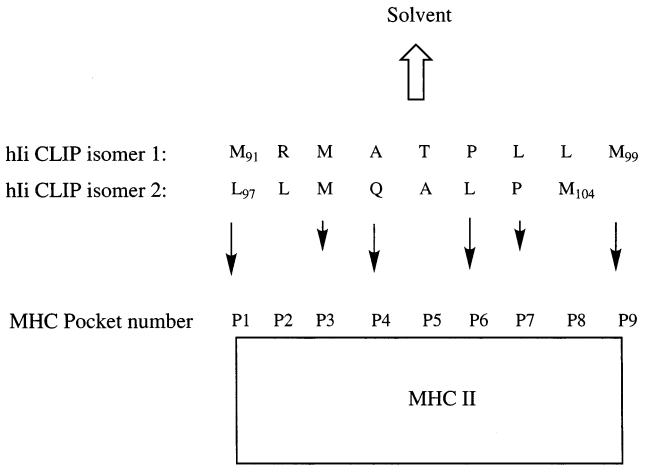

FIGURE 1: Schematic representation of the predicted structures of the two isomeric complexes of human Ii CLIP f81−104/DR*0404. Isomer 1 binds DR*0404 in a registry where peptide Met 91 is placed in the P1 pocket of DR*0404. Isomer 2 is predicted to bind DR*0404 in a registry where Leu 97 is placed in the P1 pocket of the MHC II. Only eight MHC II pockets are occupied by the peptide in this isomer. However, the possibility that the DM-insensitive isomer binds in a registry other than the one shown cannot be ruled out. The size of arrows indicates the qualitative importance of peptide anchor residues in the peptide/MHC II binding interactions.

LLMQALPM registry. The dissociation of the CLIP/DR*0404 isomer corresponding to the MRMATPLLM registry is HLA-DM sensitive relative to the isomer corresponding to the LLMQALPM registry. The LLMQALPM registry is somewhat unusual in that the peptide interacts with the MHC II at pockets P1−P8 as opposed to the common P1−P9 binding interaction. However, peptide/MHC II complexes have been previously studied where the peptide interacts mainly with the P1−P8 pockets of MHC II (*27*, *33*). A second possibility that we cannot rule out is that the two isomers have identical registries but have different molecular conformations. If this is the case, formation of these isomers is sensitive to the C-terminal peptide sequence and length.

*Influence of HLA-DM on the Formation of Ii CLIP f81−104/DR*0404 Isomers.* The influence of DM on the formation of peptide/MHC II isomers has not been previously studied to our knowledge. This influence may have important immunological consequences in the editing of peptide/MHC II conformations which are ultimately presented to helper T-cells. Therefore, we investigated the DM effect on the formation of Ii CLIP f81−104/DR*0404 isomers. The general strategy in this work is to first react DR*0404 with excess unlabeled preloader for a relatively long period of time (≥1 day). The excess unlabeled peptide is then removed by gel filtration. Then labeled CLIP f81−104 (or CLIP f90−104) peptide is reacted with the peptide-receptive state released by the complex upon preloader dissociation in the presence of DM. At any given point in time the reaction may be stopped, the excess labeled peptide is removed, and the relative fractions of each isomer may be kinetically resolved by dissociation at pH 5.3 in the presence of DM.

Here we present a series of studies corresponding to the reaction of f-IiCLIP 81−104 and f90−104 with preloaded DR*0404. The studies based on the reaction of fIi CLIP 81−104 and the DR*0404/AcMBP 84−99 complex in the presence of HLA-DM are presented first (preloader = AcMBP 84−99). This is followed by studies of the reaction of fIi CLIP 81−104 and f90−104 with a relatively fast dissociating complex DR*0404/(murine AcCLIP 85−99 V87A L96A M98F). In these studies, one of the purposes of using a preloaded complex is that it allows for the rapid formation of a measurable amount of complex upon reaction with a labeled peptide (*27*, *28*).

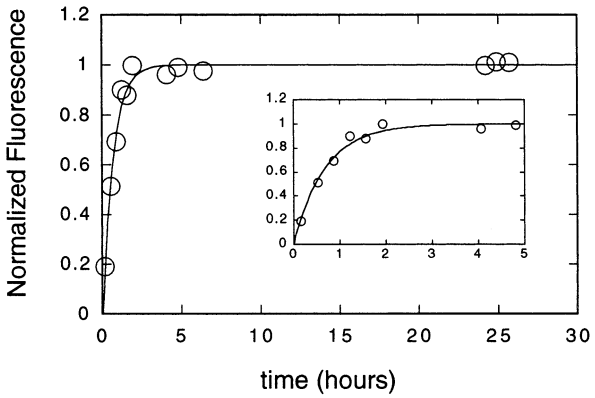

FIGURE 2: Kinetics of association of a fluorescently labeled CLIP peptide (f-Ii CLIP 81−104, f = fluorescein) to the active state of DR*0404 generated from dissociating the preformed AcMBP 84−99/DR*0404 complex (open circles) in the presence of 0.25 μM DM. Inset: early time association kinetics (0−5 h). The AcMBP 84−99/0404 complex was isolated with a Sephadex G-50 size exclusion column and was subsequently reacted with 5 μM f-CLIP peptide for the indicated amount of time (*x*-axis) at pH 5.3, 37 °C, and 0.25 μM DM. The amount of the CLIP f-Ii 81−104/DR*0404 complex formed at a given time in this reaction was determined by injecting an aliquot into an HPSEC column to resolve the complex from the excess peptide, and the fluorescence of the complex was then measured by excitation at 492 nm and detection at 522 nm. The open circles are the normalized fluorescence of the complex (normalized by asymptotic fluorescence value), and the solid line is a single-exponential fit of the data with a half-life of ∼0.46 h.

*Reactions of Ii CLIP f81−104 with the AcMBP 84−99/DR*0404 Complex in the Presence of HLA-DM.* The preloaded complex DR*0404/AcMBP 84−99 releases the peptide-receptive state of DR*0404 very slowly with a half-life of ∼30 h (results not shown). However, with DM-catalyzed preloader dissociation, the peptide-receptive state of DR*0404 is released significantly faster. Specifically, in the presence of 0.25 μM DM, the MBP/0404 complex dissociates with an apparent half-life of 0.46 h, allowing fIiCLIP 81−104 to bind to 0404 as shown in Figure 2. The f-peptide binding to 0404 is described by a single-exponential function to within experimental error. In the absence of preloader, the time scale of formation of the active state of DR*0404 in the presence of DM is significantly slower, with a $t_{1/2}$ of ∼2 h (unpublished observation). As will be seen, the time scale of interconversion of fCLIP/0404 isomers is coincidentally on the order of 2 h as well. Therefore, it is advantageous to have a preloaded complex that releases the active state with a half-life of significantly less than 2 h in the presence of DM.

The kinetics of formation of human Ii CLIP 81−104/DR*0404 isomers were studied by incubating Ii CLIP f81−104 with preloaded DR*0404 in the presence of DM. The reaction was then stopped at a specified time of incubation, and the complex was isolated. The complex was then dissociated at pH 5.3 in the presence of 0.25 μM HLA-DM,

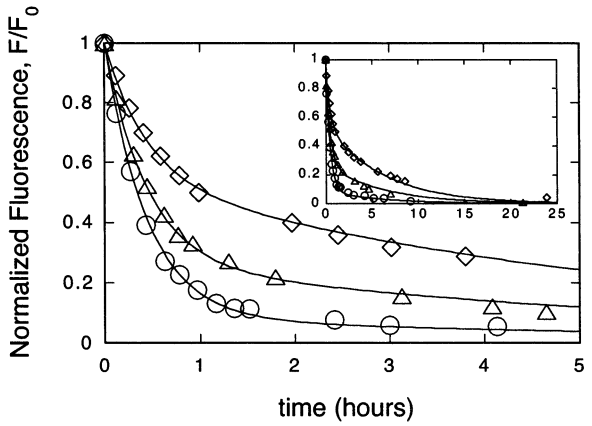

FIGURE 3: Dissociation kinetics of fluorescently labeled CLIP f81−104/DR*0404 in the presence of 0.25 μM HLA-DM are sensitive to preparation history. Dissociations of fluorescently labeled 81−104 human invariant chain peptide (f81−104 Ii CLIP) from HLA-DR4*0404 MHC II were measured at 37 °C, pH 5.3, in the presence of 0.25 μM HLA-DM. The complexes were prepared by incubation of Ii CLIP f81−104 with preloaded AcMBP 84−99/DR*0404 (see Materials and Methods) at pH 5.3, 37 °C, in the presence of 0.25 μM HLA-DM for 20 min (circles), 2.0 h (triangles), and 21.3 h (diamonds). The fluorescence decay of the complex is normalized by the initial fluorescence value ($t = 0$). Each solid line is a fit of the data to a double-exponential decay function. An excess of an unlabeled peptide competitor was used to prevent labeled peptide from rebinding to the MHC II. Inset: Long time dissociation behavior of the f-CLIP/DR*0404 complex.

and the relative amount of each isomer was resolved from a double-exponential fit of the normalized fluorescence versus time:

$$\frac{F}{F_0} = C\left[\exp\left(\frac{-t \ln 2}{t_{1/2}(\text{fast})}\right)\right] + (1 - C)\left[\exp\left(\frac{-t \ln 2}{t_{1/2}(\text{slow})}\right)\right] \quad (1)$$

In this equation, F/F_0 is the normalized fluorescence, C is the fraction of the fast-dissociating isomer with half-life $t_{1/2}$(fast), and $t_{1/2}$(slow) is the half-life of the slow-dissociating isomer. Representative dissociating kinetic curves of the complex Ii CLIP f81−104/DR*0404 in the presence of HLA-DM (0.25 μM) are shown in Figure 3. The fraction of the slow-phase CLIP/DR*0404 isomer formed after a given incubation time of fIi CLIP 81−104 with MBP/DR*0404 in the presence of DM is shown in Figure 4 (circles). The best fit half-life for the slow- and fast-phase isomers are 4.3 and 0.30 h, respectively (see Materials and Methods).

Scheme 1

$$\text{M/Prel} \underset{k_{\text{on-prel}}}{\overset{k_{\text{off-prel}}}{\rightleftharpoons}} \text{M}_\text{a}$$

$$(\text{MP})_1 \underset{k_{\text{on1}}}{\overset{k_\text{f}}{\rightleftharpoons}} \text{M}_\text{a} + \text{P} \underset{k_\text{s}}{\overset{k_{\text{on2}}}{\rightleftharpoons}} (\text{MP})_2$$

The kinetic behavior of these isomers in the presence of DM was simulated in terms of the kinetic mechanism shown in Scheme 1. In this scheme, M is the MHC II that converts to the active state (M_a) upon dissociation of the preloader peptide (prel) with dissociation and association rate constants of $k_{\text{off-prel}}$ and $k_{\text{on-prel}}$, respectively. The CLIP peptide (P) is able to form two isomeric complexes (MP_1 and MP_2). The dissociation and association rate constants corresponding to complex $(MP)_1$ are k_f and k_{on1}, respectively, where f signifies

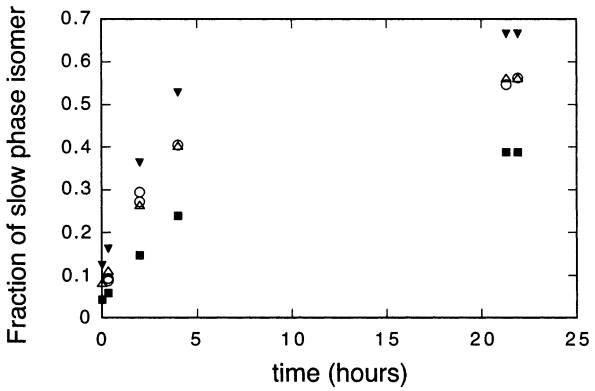

FIGURE 4: Effect of HLA-DM on the formation kinetics of two isomers of the human Ii CLIP f81−104/DR*0404 complex. Human fIi CLIP f81−104 (5 μM) was incubated with preloaded DR*0404 (AcMBP 84−99/0404; see Materials and Methods) for a specified amount of time (x-axis) in the presence of 0.25 μM HLA-DM. The newly formed complex was separated from the excess labeled peptide, and then the complex was dissociated in the presence of 0.25 μM HLA-DM at pH 5.3 and 37 °C. The relative fractions of the slow and fast phase isomers were determined from the preexponential weights of the double-exponential fit of the dissociation kinetics (circles). These data (circles) were fit to a kinetic model based on Scheme 1 (triangles) using the representative half-lives of the slow- and fast-phase isomer and the binding behavior of this peptide to 0404 (see Figure 2). In this simulation, the only adjustable parameter is the relative association rate of the two isomers in the presence of HLA-DM, which is set to $0.083k_{\text{on}}$ and $0.917k_{\text{on}}$ for the relative on-rate of the slow and fast phase, respectively (see Materials and Methods and Results). To get a qualitative measure of the sensitivity of the simulation to the adjustable parameter, simulations were done with relative on-rates (slow phase/fast phase isomer) of $0.043k_{\text{on}}$:$0.957k_{\text{on}}$ (filled squares) and $0.123k_{\text{on}}$:$0.877k_{\text{on}}$ (inverted filled triangles).

that the complex dissociates relatively fast in the presence of HLA-DM. Similarly, the dissociation and association rate constants corresponding to complex $(MP)_2$ are k_s and k_{on2}, respectively, where s signifies that the complex is slow dissociating in the presence of HLA-DM. Two important assumptions are made in this simulation:

(a) The first assumption is that $(MP)_1$ does not isomerize in the presence of HLA-DM to form $(MP)_2$ according to Scheme 2.

Scheme 2

$$(\text{MP})_1 \rightleftharpoons (\text{MP})_2$$

(b) A second assumption is that the inactivation of the peptide-receptive form of the MHC II (M_a) may be neglected. This implies that once DR*0404 has exchanged the preloader peptide for the CLIP 81−104 peptide, the total complex concentration (CLIP/DR*0404) is constant. This is true according to Figure 2, where the unlabeled MBP preloader dissociates with an apparent half-life of ~0.46 h, allowing the CLIP f81−104 peptide (5 μM concentration) to bind to the active state of the MHC II. At incubation times ≫0.5 h, the total complex concentration is approximately constant. In this simulation, the overall association rate of CLIP to DR*0404 is approximated as 1×10^4 μM^{-1} s^{-1} based on peptide association measurements to the active state of sI−E^k (*34*).

Finally, the magnitude of the overall CLIP f81−104 association rate constant is taken as 1×10^4 M^{-1} s^{-1}, and

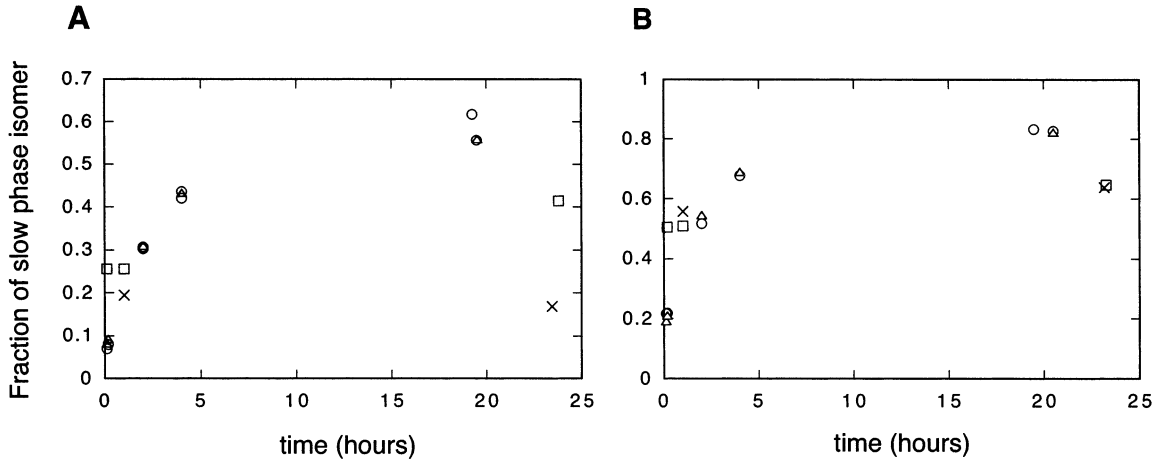

FIGURE 5: Effect of HLA-DM on the formation kinetics of two isomers of the human Ii CLIP f81−104/DR*0404 complex (A) and human Ii CLIP f90−104/DR*0404 complex (B). Human fIi CLIP f81−104 (5 μM) (or f90−0404) was incubated with DR*0404 preloaded with murine CLIP Ac85−99 V87A L96A M98F/0404 (see Materials and Methods) for a specified amount of time (*x*-axis) in the presence or absence of 0.25 μM HLA-DM. The newly formed complex was separated from the excess labeled peptide, and then the complex was dissociated in the presence of 0.25 μM HLA-DM at pH 5.3 and 37 °C. The relative fractions of the slow- and fast-phase isomers were determined from the preexponential weights of the double-exponential fit of the dissociation kinetics (open circles). The data obtained in the presence of DM (open circles) were fit to a kinetic model based on Scheme 1 (triangles) using the average half-lives of the slow- and fast-phase isomer and the binding behavior of these peptides to 0404 (see Figure 6). In this simulation, the only adjustable parameter is the relative association rate of the two isomers in the presence of HLA-DM, which is set to $0.075k_{on}$ and $0.925k_{on}$ for the relative on-rate of the slow and fast phase, respectively (f81−104/DR*0404, Figure 5A; see Materials and Methods and Results). For studies corresponding to the f90−104/DR*0404 complex (panel B), the relative association rate in the presence of HLA-DM is set to $0.18k_{on}$ and $0.82k_{on}$ for the slow- and fast-phase isomers, respectively. The relative fractions of each isomer formed by preparing the complex in the absence of HLA-DM at pH 5.3 and 7.0 were also determined (squares and ×, respectively).

under these conditions the results of the simulation are insensitive to the values of this rate constant. The relative association rate to form $(MP)_1$ and $(MP)_2$ is the only adjustable parameter used to obtain a fit between the simulated and observed fraction of slow phase as a function of incubation time in the presence of DM. On the basis of these constraints, the model prediction of the isomer composition as a function of incubation time agrees well with the experiments as shown in Figure 4 (triangles). The best fit relative association rates of the DM-sensitive and DM-insensitive isomers to DR*0404 are $0.083k_{on}$ and $0.917k_{on}$, respectively, where k_{on} is a rate constant not determined in this experiment. These results are therefore consistent with a mechanism where DM promotes isomer interconversion without enabling intramolecular isomerization in the MHC II groove (reptation, Scheme 2). In earlier experiments, this mechanism of uncorrelated isomer binding and dissociation also described well the process of isomer formation of a chimeric peptide binding to $sI-E^k$ in the absence of DM (*29*).

In a second series of similar experiments, DR*0404 was preloaded with the peptide Ac-KPASQMRMATPALFR whose complex with 0404 dissociates with a half-life of ∼20−30 min in the absence of DM (fast CLIP) and <10 min in the presence of 0.25 μM DM. The isomer composition of fIi CLIP 81−104/DR*0404 and f90−104/DR*0404 complexes as a function of time of reaction of the peptides with fast CLIP/DR*0404 and DM was determined as shown in panels A and B of Figure 5, respectively (open circles). The relative amount of complex formed versus incubation time of the fIi CLIP 81−104 and fIi CLIP 90−104 peptides with preloaded DR*0404 in the presence of DM is shown in panels A and B of Figure 6, respectively. Clearly, there is an initial binding "burst" of ∼30−50% of the complex, followed by a slower binding reaction. The kinetic analysis of these reactions is complicated by the biphasic binding profile of the peptide to DR*0404 in the presence of DM. We propose that this biphasic behavior is due to incomplete binding of the fast CLIP preloader to 0404. In this scenario, the initial binding burst is due to rapid DM-catalyzed displacement of the bound preloader followed by f-CLIP binding, and the slow- phase binding represents DM-catalyzed binding of f-CLIP to empty DR*0404 (otherwise, mostly inactive). We have also observed this behavior when preloading 0404 with other fast-dissociating peptides (results not shown). The kinetic behavior of formation of the short CLIP/0404 and long CLIP/0404 isomeric complexes with DM is still consistent with the model [compare triangles (simulated) versus open circles (experiment) in panels A and B of Figure 5]. In these simulations of Scheme 1 for fIi CLIP 81−104/DR*0404 reactions in the presence of DM, the experimentally determined input parameters are $t_{1/2}$(slow) = 4.1 and $t_{1/2}$(fast) = 0.25 h (see Materials and Methods). The experimentally observed time dependence of the relative amount of complex is also taken into account in the simulation (solid line, Figure 6A). In this simulation, the MHC II is initially bound by two hypothetical preloaders which, upon dissociation with two dissimilar time constants, release the active state of the MHC, leading to the double-exponential binding profile of f-CLIP in Figure 6A (solid line). The adjustable parameter is the relative association rate of the two isomers to 0404, which was set to $0.075k_{on}$ and $0.925k_{on}$ for the slow- and fast-phase isomers, respectively. Similarly, the experimentally determined input parameters for the simulation of fIi CLIP 90−104/DR*0404 reactions in the presence of DM are $t_{1/2}$(slow) = 7.1 h and $t_{1/2}$(fast) = 0.30 h (see Materials and Methods). The experimentally observed time dependence of the relative amount of complex

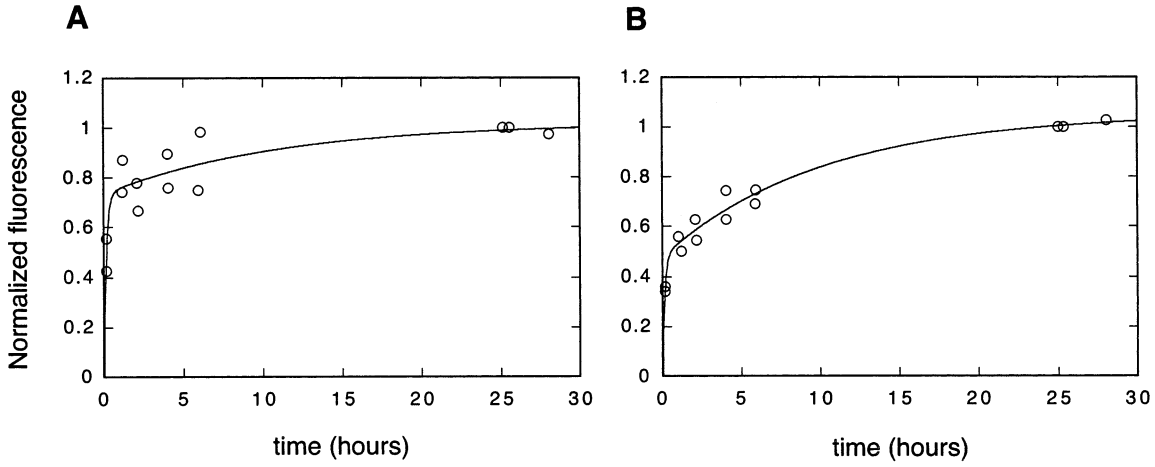

FIGURE 6: Relative amount of the human Ii CLIP f81−104/DR*0404 complex (A) and Ii CLIP f90−104/DR*0404 complex (B) formed versus incubation time of the preloaded DR*0404 complex (murine CLIP Ac85−99 V87A L96A M98F/0404; see Materials and Methods) with labeled Ii CLIP (5 μM) at pH 5.3 and 37 °C in the presence of 0.25 μM HLA-DM. Solid lines are fits to a double-exponential function whose best fit parameters are used in the respective kinetic simulations. After reaction, the human Ii CLIP f81−104/DR*0404 complex was separated from the excess free peptide with a size exclusion spin column, and an aliquot of the isolated complex was then injected into an HPSEC column, and the relative amount of the complex was measured by a fluorescence detector (excitation 492 nm, emission 522 nm).

is also taken into account in the simulation (solid line, Figure 6B). The adjustable parameter is the relative association rates of the two isomers which were set to $0.18k_{on}$ and $0.82k_{on}$ for the slow- and fast-phase isomers, respectively. From these results we may conclude that the mechanism of isomer formation is not sensitive to the sequence of the preloader peptide.

Similar studies in the absence of DM reveal that the isomer composition is relatively insensitive to reaction time of these CLIP f-peptides with preloaded 0404 at pH 5.3 and especially at pH 7.0 [see squares (pH 5.3) and × (pH 7.0) for Ii CLIP f81−104 (Figure 5A) and Ii CLIP f90−104 (Figure 5B)].

DISCUSSION

We have investigated the unusual behavior of the human Ii CLIP f81−104/DR*0404 complex, where, in the absence of HLA-DM, the dissociation profile of the complex is monoexponential but turns biphasic in the presence of HLA-DM. The experimental evidence summarized in Table 1 is consistent with a molecular picture where human Ii CLIP f81−104 binds to DR*0404 in two distinct registries with apparently similar dissociation rates in the absence of HLA-DM. One registry places Met 91 in the P1 pocket of DR*0404, and the dissociation of this isomer is relatively sensitive to HLA-DM. In contrast, the dissociation of the second isomer is relatively insensitive to HLA-DM. This registry likely places Leu 97 in the P1 pocket of DR*0404, where only pockets P1−P8 of DR*0404 are occupied. Although not common, peptide binding to MHC II has been observed for peptides occupying less than nine pockets of the MHC II (*27*, *35*, *36*).

It is not obvious why the dissociation of one isomer is more sensitive to HLA-DM than the second isomer. Clearly, in this example, differences in intrinsic stability are not required for differences in DM susceptibility, in agreement with previous studies (*30* and references cited therein, *37*, *38*). Recently, we have shown that the dissociation enhancement of peptide/MHC II complexes by HLA-DM is dependent on the sequence of the peptide (*30*) and that the peptide C-terminal residues may be important factors. The CLIP/DR*0404 isomers appear to involve different peptide sequences. A speculation is that the lack of a P9 pocket occupancy of DR*0404 by the CLIP peptide in one of the isomers may contribute to the unusually low HLA-DM dissociation enhancement relative to the other isomer.

The kinetic mechanism of CLIP f81−104/DR*0404 isomer formation (and CLIP f90−104/DR*0404) was investigated in the absence and presence of HLA-DM. In the absence of HLA-DM, the isomer composition of the complex is relatively insensitive to the incubation time with CLIP f81−104 peptide at pH 5.3 and especially at pH 7.0 (Figure 5A, squares and ×, respectively). In contrast, when DM is present, the formation of the isomer sensitive to DM-enhanced dissociation is favored at early incubation times (circles, Figures 4 and 5A) relative to binding in the absence of DM. At longer incubation times (~20 h), a slight majority of the isomer insensitive to DM-enhanced dissociation is formed. The interpretation of this result is that DM affects differentially the association as well as the dissociation of these isomers.

To our knowledge, this is the first report of an effect of DM on the relative association rates of intramolecular peptide epitopes to MHC II. The DM effect on the relative formation rates of the isomers implies that DM stabilizes the peptide-receptive state of MHC II or the transition state for the bimolecular peptide association step, or both.

Consistent with a catalytic mechanism, the DM dissociation enhancement of one isomer relative to the second isomer qualitatively mirrors the relative DM association enhancement. According to the principle of microscopic reversibility, however, the dissociation enhancement by a catalyst should be equivalent to the association enhancement if the association and dissociation steps are single steps. Experimentally, we find that the relative enhancement of association of the isomers resembles the trend of dissociation enhancement but is not in agreement to within a factor of approximately 4−6 (f81−104/DR*0404, relative association rate enhancements by DM ~3.9:1; relative off-rate enhancements ~14:1; f90−104/DR*0404, relative association rate enhancements by DM ~4:1, relative off-rate enhancements by DM 24:1; see

Materials and Methods). There are several complications that could explain this apparent result. One likely factor is that, in the absence of DM, the off-rates of the two isomers may differ within a factor of 2 or 3 but may still be described reasonably well by a single exponential, introducing an error in the estimation of the dissociation rate enhancement. Another possibility is that the mechanism by which DM catalyzes complex formation is a multistep process in which the macroscopic on- and off-rate constants do not necessarily reflect the individual microscopic rate constants.

On the basis of the apparent "relative" association rates and the dissociation rates of these two isomers in the presence of HLA-DM, a kinetic simulation was used to predict the isomer composition versus incubation time of the two CLIP peptides to preloaded DR*0404. One of the key assumptions in this simulation is that the two registries do not isomerize in the MHC II groove. The simulation is in good agreement with the experimental results (Figures 4 and 5**,** circles vs triangles). Thus, our data are consistent with a mechanism where once the isomers are "locked" into place, further rearrangement in the MHC groove does not take place. We cannot rule out the possibility that, in the initial step of peptide binding to the MHC II groove, sliding may affect the observed relative association rates of both isomers. It is also conceivable that, in the presence of DM, peptide sliding in the MHC II groove is the rate-limiting step for release of the DM-resistant isomer or that it occurs on a time scale significantly slower than the off-rates of either isomer. However, we can eliminate the possibility that peptide sliding in the MHC II groove occurs much faster than the dissociation of the DM-resistant isomer. DM-mediated changes in abundance of alternate peptide registers in longer peptides/polypeptides have not been widely studied. Other peptide/MHC II complexes with multiple binding registers will need to be studied to evaluate the generality of the dissociation/rebinding mechanism described here.

There is ample evidence that HLA-DM edits the repertoire of peptides other than CLIP in vivo (*39*). Comparisons of the class II-associated peptide repertoire in DM^+ and DM^- APC, using mass spectrometry or alloreactive T-cells, indicate gain and loss changes in the peptide repertoire imposed by HLA-DM (*12*, *13*, *40*). Both DM-susceptible and DM-enhanced peptides are present on normal APC from mouse spleen (*12*). It may seem difficult to reconcile these observations with the notion that DM merely removes unstable and/or DM-susceptible peptides by accelerating dissociation. Our data illustrate how, in a model system comprising one peptide binding in alternative registries, the duration of exposure to DM can dictate the relative abundance of the two alternative isomers in a manner consistent with the gain and loss changes seen in vivo. The only requirement seems to be that DM differentially catalyzes association and dissociation of the two isomers; short DM exposures will then favor DM-susceptible isomers, whereas long DM exposures will favor DM-resistant ones, even in the absence of differences in intrinsic stability. Similar mechanisms may also account for intermolecular peptide editing and editing of peptides differing in intrinsic stability (*30*).

How likely is intramolecular DM editing to occur in vivo? Potential motifs for binding to the same MHC II allele have been found to interdigitate or be adjacent in the primary sequence of antigenic proteins (*20*, *41*). In addition, MHC class II molecules have been shown to bind longer polypeptide precursors in several settings. In the absence of Ii, MHC class II molecules bind polypeptides in the ER, with or without subsequent endosomal trimming or exchange, although this pathway is largely suppressed by Ii in normal APC (*42*, *43*). Pathways also exist for polypeptide binding to MHC class II molecules at the plasma membrane and/or in the endocytic pathway (*44*−*46*). However, whether such complexes are edited by DM, and whether the same editing mechanism prevails in these settings, may be difficult to assess in the face of competing proteolysis.

DM affects the relative amounts of the two peptide/MHC II isomers in a highly time-dependent manner. The less stable isomer is more prevalent at short incubation times. In contrast, the absence of DM may lead to a relatively static isomer composition far from equilibrium. Thus, the immunogenicity of an isomer will be sensitive to the processing pathway and the duration of each processing step. The site of peptide encounter with MHC, at the cell surface, generally without DM, or in endosomes with DM, will greatly influence isomer presentation. Recent work has described a phenomenon in which a peptide/MHC complex generated after whole antigen immunization was distinguished (by T-cells) from a complex of the same peptide and MHC generated by immunization with peptide (*47*−*49*). It is attractive to consider the possibility that this differential recognition may reflect DM-dependent differential presentation of registry isomers, similar to those described here.

This study examines the behavior of a naturally occurring long peptide, generated during proteolysis of the dedicated class II chaperone, invariant chain. However, it should be noted that the CLIP peptide initially interacts with MHC class II molecules in the context of full-length invariant chain. Thus, in antigen-presenting cells, naturally processed CLIP peptide is likely bound initially in the canonical register, which would be imposed by its position within whole Ii. Whether the existence of a DM-resistant isomer allows accumulation of CLIP in an alternative register for DR*0404 molecules is an open question. However, in regard to this study, CLIP serves as an example of any peptide that may bind MHC II molecules in alternate registers in endosomes.

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