Kinetics of Registry Selection of Chimeric Peptides Binding to MHC II†

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ABSTRACT: Major histocompatability complex type II proteins (MHC II) are $\alpha\beta$-heterodimeric glycoproteins that present peptides to the T cell receptor (TCR) of $CD4^+$ T-cells. This presentation may result in activation of these T-cells, depending on the nature of the peptide. Peptides interact specifically with MHC II with nine peptide amino acid positions, and the corresponding MHC II pocket positions are usually labeled P1−P9. However, the length of peptides binding to MHC II may be greater than nine amino acids, and therefore these peptides may potentially bind to the MHC II in more than one registry. To investigate the mechanism by which a long peptide binds to I-E^k, a murine MHC II, a chimeric peptide with two nonoverlapping registries, f-IAYLKQATKQLRMATPLLMR was designed. The IAYLKQATK peptide segment is based on moth cytochrome *c* 95−103 (MCC 95−103), and the QLRMATPLLMR segment is based on murine Ii CLIP 89−99 M90L (Ii CLIP 89−99 M90L). This chimeric peptide forms two isomeric complexes. The MCC and Ii CLIP registries dissociate from I-E^k with $t_{1/2}$ values of ≫800 and 4.94 h, respectively. The registry composition of this MHC II/chimeric peptide complex was found to change as a function of time in approaching thermodynamic equilibrium: the results are consistent with a kinetic model that involves no intramolecular isomer interconversion. The model depicts uncorrelated binding to the MHC II determined by relative association rates to the two registries. This is followed by dissociation and subsequent rebinding, leading ultimately to a preponderance of the most stable complex. Similar results were obtained at pH 5.3. The behavior of this chimeric peptide approximates the binding of a 1:1 solution mixture of two peptides to MHC II, where the more stable complex is selected over time. We have also found that a chimeric peptide and a human MHC II, HLA-DR4*0401, form isomers with relative association rates to DR*0401 at pH 5.3 of 15% for one isomer and 85% for the second isomer.

Major histocompatability class II molecules (MHC II)[1] are membrane-bound $\alpha\beta$-heterodimeric glycoproteins that present peptides to the $\alpha\beta$ T cell receptor of CD_4^+ helper T cells (*1*). The MHC II molecules are loaded in the endoplasmic reticulum with a membrane protein called the invariant chain. These complexes are transported to endosomes where sections of the CLIP protein are cleaved to release fragments of the invariant chain (Ii CLIP) 81−104 bound to the MHC II (*2*, *3*). After removal of the CLIP fragment catalyzed by HLA-DM, the MHC II are able to bind self- and antigenic peptides present in the endosome (*4*, *5*). According to crystallographic studies, these peptides bind to the MHC II in a polyproline type II conformation where nine peptide amino acids are located at MHC II pocket positions (*2*, *6*−*9*). However, peptides that bind to MHC II may be much longer than nine amino acids. Endogenous peptides ranging from 13 to 25 amino acids bound to MHC II have been found (*10*). In addition, the crystal structure of a complex of human invariant chain 81−104 peptide and HLA-DR3 has been determined. This shows that MHC II may bind a peptide much longer than nine amino acids (*2*). It is plausible that a peptide greater in length than nine amino acids may bind stably in more than one registry. There is an example of immunological importance in which a collagen II-derived peptide binds in a specific registry in one MHC II allele and in a different registry in another MHC II allele (*11*). Furthermore, it has been reported that a single peptide containing two different registries may bind to an MHC II in two different isomeric complexes (*12*). Another example of peptide/MHC II registry isomers is the ovalbumin 323−339/I-A^d complex where it was found using kinetic methods that the peptide may bind in two different registries to the MHC II, where one isomer stimulates a T-cell hybridoma (*13*). However, the mechanisms by which peptides are selected are not clear. Here we have investigated peptide registry selection for binding to MHC II, using in vitro kinetic methods.

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[1] Abbreviations: f, fluorescein; HCgp-39, human cartilage glycoprotein 39; HCgp-39 f327−337, HC-gp39 fluorescein-labeled wild-type peptide; f-peptide, fluorescein-labeled peptide; Ii CLIP, murine invariant chain; MHC, major histocompatability complex; HEPES, *N*-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HLA-DM, human leukocyte antigen DM; MCC, moth cytochrome *c*; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate buffered saline, 10 mM phosphate, 150 mM NaCl (pH 7.0); PBS/citrate pH 5.3 buffer, 9:1 PBS pH 7.0: 1 M NaCitrate, pH 4.8, by volume; TFA, trifluoroacetic acid; single letter abbreviations for amino acids used in this report are the following: A, *Ala*; C, *Cys*; D, *Asp*; E, *Glu*; F, *Phe*; G, *Gly*; H, *His*; I, *Ile*; K, *Lys*; M, *Met*; N, *Asn*; P, *Pro*; Q, *Gln*; R, *Arg*; S, *Ser*; T, *Thr*; V, *Val*; W, *Trp*; Y, *Tyr*.

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In addition to peptide/MHC II registry isomers, other kinetic isomers of peptide/MHC II complexes have been reported (*14*−*19*). In terms of MHC II/peptide isomer dynamics, Schmitt et al. have found that a particular isomer of a peptide/MHC II complex may interconvert to a second isomer without peptide dissociation (*20*, *21*). It is believed that these isomers have the same registry. There is additional evidence that a peptide may bind an MHC II in more than one conformation (*22*, *23*).

There is some evidence that peptide binds to MHC II through a random registry search (trial and error binding). It has been shown that the intrinsic rates of peptide association to the active form of sI-E^k (MHC II) of various peptides of equal length are similar (*24*). Increasing peptide length beyond 9−10 amino acids generally decreases the rate of peptide association. All these rates of peptide association are significantly slower than the diffusion limit. This was interpreted as a random and often unproductive search of all possible peptide registries that limits the association to the thermodynamically stable and final registry. However, the possibility that a peptide once bound to the MHC II groove may rearrange itself by "sliding" or "reptating" (without rebinding) along the groove has not been ruled out. Examples of a polymer or molecule sliding along the groove of a molecular structure are known. For example, repair enzymes slide along DNA to find damaged base pairs (*25*). RNA polymerase travels along DNA to make an RNA strand (*25*). Another example is the sliding of a ribosome along the messenger RNA to make polypeptides with the use of ATP (*25*). In polymers, it has been theorized and demonstrated that chains "reptate" through polymer media, resembling sliding motion through a tube (*26*, *27*).

MATERIALS AND METHODS

Synthesis and Purification of Peptides. All peptides described were synthesized with standard FMOC chemistry on an Applied Biosystems 431A peptide synthesizer (Applied Biosystems, Foster City, CA). The peptides f-IAYLKQATKQMRMATPLLMR (f-MCC 95−103 Ii CLIP 89−99, f = carboxyfluorescein), f-IAYLKQATKQLRMATPLLMR (f-MCC 95−103 IiCLIP 89−99 M90L), f-SAYLKQATKQLRMATPLLMR (f-MCC 95−103 I95S Ii CLIP 89−99 M90L), f-IAYLKQATK−NH_2 (f-MCC 95−103), f-QLRMATPLLMR (f-Ii CLIP 89−99 M90L), and f-GYDNQESVKSKGYDDQESVKSK (HCgp-39 327−337 D330N 327−337, HCgp-39 = human cartilage glycoprotein) were all labeled with the same technique. Briefly, the peptide resin was labeled overnight (room temperature) at the free N-termini with 5-fold excess 5- (and 6-) carboxyfluorescein succinimidyl ester (Molecular Probes) in dimethyl sulfoxide and a catalytic amount of diisopropylethylamine. The cleaving of the resin 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 (*20*). After the resin was cleaved, the labeled peptide was purified by HPLC (reversed-phase chromatography) and checked for identity and purity by mass spectrometry. The purity of all chimeric peptides in this study was greater than or equal to 95%. The competitor peptide AcMCC 95−103 (AcIAYLKQATK) was acetylated at the N-terminus with 10-fold molar excess acetic anhydride in dimethylformamide and a catalytic amount of pyridine for 45 min at room temperature.

Soluble MHC II Protein. The particular MHC II used mostly in this study has been the water-soluble murine sI-E^k prepared as described in previous work (*28*). sI-E^k was a gift from Dr. Mark M. Davis. sI-E^k has been studied and characterized previously in terms of kinetics (*5*, *20*). HLA-DR4*0401 MHC II and soluble HLA-DM (sDM) were a gift from Dr. Elizabeth D. Mellins (*29*, *30*).

Preloading of sI-E^k. Soluble I-E^k was incubated with an excess of unlabeled murine Ii CLIP Ac85−99 at pH 5.3 (37 °C) for a period of 30−50 h, similar to procedures found in the literature (*5*). The buffer system employed was nine parts PBS pH 7 (PBS = 10 mM phosphate, 150 mM NaCl, 0.02% NaN_3) to one part 1 M sodium citrate buffer (pH 4.8) resulting in a final pH of 5.3. The final concentration of sI-E^k was ∼0.80 mg/mL (or ∼16 μM), and the concentration of the unlabeled peptide was ∼190 μM. After the incubation period, the solution was stored at 4 °C and used when needed.

Isolation of Preloaded sI-Ek Complex. A solution of 10 μL of preloaded sI-Ek/Ac Ii CLIP 85−99 (Ac = acetyl at N-terminus of peptide) was combined with 40 μL of PBS, pH 7, and delivered to a small scale Bio-Rad spin column at 4 °C for separation of the excess free peptide from the preloaded complex. (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 pH 7.0 buffer before use.) Then, 300 μL of PBS pH 7 was added to wash down the spin column. After draining the sample, an additional 230 μL of PBS pH 7 was added to the spin column, and the eluate was collected at 4 °C. The purpose of this procedure is to separate the free unlabeled peptide from the complex. The dissociation half-life of the preloader Ac Ii CLIP 85−99 from sI-E^k is approximately 9 min at pH 7 and 37 °C based on dissociation kinetic measurements of the fluorescently labeled peptide fIi CLIP 85−99 from sI-E^k.

Incubation of Preloaded sI-E^k with 3.0 μM Peptide Followed by Dissociation Kinetics. A volume of 220 μL of the eluate (with complex separated from free peptide as previously described) containing preloaded I-E^k was combined with 27 μL of PBS pH 7 and 17 μL of a 46.6 μM aqueous stock solution of the carboxyfluorescein labeled chimeric peptide at 4 °C. The solution was then incubated at 37 °C for a specified amount of time at pH 7 (i.e., 1 h, 13 h, 24 h). The concentration of the MHC and the labeled peptide during the incubation were ∼300 nM and ∼3.0 μM, respectively. Then the reaction was cooled in a 4 °C water bath for ∼1 min. Then 60 μL of reaction aliquots were placed into each of two pH 7 PBS spin columns at 4 °C. Then, 300 μL of PBS pH 7 was added to each of the spin columns. After elution, an additional 230 μL of PBS pH 7 was added to each of the spin columns, and the eluate was collected. This procedure was done to remove the excess of free labeled peptide from solution. Then a total of 440 μL of the eluate was combined with 220 μL of PBS pH 7, 15 μL of 500 μM Ac-MCC 95−103 competitor, and 46.4 μL of water. The purpose of adding the water was to maintain an exact solvent composition as the incubated mixture since the peptide stock solution was made in water. Then the dissociation kinetics of the labeled peptide/sI-E^k complex (total initial volume of

720 μL, pH 7.0) at 37 °C was followed by measuring the relative change in fluorescence of the complex as a function of time. Briefly, a 45 μL aliquot of the labeled peptide/MHC II solution was injected into a 5 μ particle size HPSEC (high performance size exclusion) column (dimensions: 7.8 mm × 30 cm, G3000SWXL TSK-GEL, Tosohaas, Montgomery, PA) which separates the complex from the free labeled peptide, and this column is coupled to a fluorescence detector (SHIMADZU RF-10AXL). The resolved complex is excited at 492 nm, and fluorescence is detected at 522 nm. The complex elutes at around 8.5 min, while the peptide elutes ~2.5 min later with a flow rate of 1.0 mL/min and a PBS pH 7.0 mobile phase at 25 °C. The half-life or half-lives of the complex were generally calculated from a single or double exponential fit of the normalized complex fluorescence decay versus time with the Microsoft Kaleidagraf software or the Mathematica v. 3.0 (WOLFRAM RESEARCH) software package when indicated. Kinetic simulations that are discussed in Results were also done with the Mathematica v. 3.0 software.

Determination of the Association Constant of the Chimeric Peptide to I−E^k. A volume of 20 μL of "preloaded" 11.5 μM sI-E^k was combined with 30 μL of PBS pH 7 and passed through a sephadex G-50 (superfine) spin column (same as kinetic procedure) to separate unlabeled excess "CLIP" peptide from the MHC II. The final 230 μL of spin column eluate containing preloaded sI-E^k was combined with an additional volume of PBS pH 7 buffer to give a final concentration of ~0.25 μM sI-E^k. Then a small volume of the preloaded complex solution (75 μL) was incubated at 37 °C for 5 min, followed by addition of 25 μL of a stock solution of the labeled chimeric peptide (f-IAYLKQATKQLRMATPLLMR) to obtain a desired final peptide concentration (3.33, 1.67, 0.833, 0.417, and 0.208 μM) and ~0.20 μM MHC II. The labeled peptide and I-E^k solution was then incubated at the specific peptide concentration for 3 min, and the relative fluorescence of the peptide/MHC II complex was determined with the same approach as the kinetics procedure (size exclusion column). The relative concentration of peptide/MHC complex formed was based on the fluorescence of the complex. The association constant (k_{on}) of the peptide to I−E^k was calculated by fitting the data to the expression

$$MP^* = MP^*_{max}(1 - \exp(-k_{on}t_{inc}[P]))$$

where MP* is the amount of complex, MP^*_{max} is the amount of complex formed at the peptide saturation limit of I-E^k, k_{on} is the association rate constant of peptide "P", t_{inc} is the association incubation time (180 s), and [P] is the concentration of the peptide.

*Experimental Procedures for Studies with HLA-DR4*0401 pH Jump Experiments.* Preloaded soluble DR*0401 was prepared by incubating 1.67 μM DR*0401 with 200 μM AcKPASQMRMAAPLLFR peptide (murine Ii CLIP Ac85−99 V87A T94A M98F, Ac = acetyl) at pH 5.3, 37 °C for ~5 days. The half-life of dissociation of AcKPASQMRMAAPLLFR from DR*0401 is ~10 min. The reaction was stopped by cooling the solution to 4 °C, and the complex was separated from the free unlabeled peptide with a 4 °C PBS pH 7.0 Sephadex G-50 spin column. Then, the chimeric peptide f-GYDNQESVKSKGYDDQESVKSK (HCgp-39 327−337 D330N 327−337, HCgp-39 = human cartilage glycoprotein) was allowed to react at pH 5.3 with the preloaded form of soluble HLA-DR4*0401 for 1 h at 37 °C. The labeled peptide and preloaded DR*0401 concentrations were 10 and 0.20 μM, respectively. The reaction was stopped by cooling to 4 °C and the complex was separated from the labeled free peptide by with a Sephadex G-50 spin column preequilibrated at pH 8.0 with a HEPES/MES buffer. Dissociation kinetics were done with procedures very similar to the I-E^k study, except, this time, HA 306−318 (HA = influenza haemagglutinin) was used as a competitor, since the HA peptide binds very well to DR*0401.

Kinetic Resolution of Isomers with HLA-DM at pH 5.3. Since both MHC/peptide isomers of the chimeric peptide and DR*0401 dissociate slowly at pH 5.3 (results not shown), an attempt was made to kinetically resolve them with HLA-DM. First, DR*0401 was preloaded, and then the isolated (preloaded) complex was reacted at pH 5.3 with the labeled chimeric peptide for 1 h as described above. Then the reaction was stopped by cooling to 4 °C, and the labeled peptide/MHC II complex was isolated with a Sephadex G-50 spin column preequilibrated with PBS pH 7.0. The solution was acidified to pH 5.3 with sodium citrate (as described previously), and dissociation kinetics were measured at pH 5.3 and 37 °C in the presence of soluble HLA-DM (0.375 and 0.50 μM).

RESULTS

Chimeric Peptide Forms Kinetic Isomers with Distinct Registries. A trial chimeric (or hybrid) peptide with a fast dissociating registry and a slow dissociating registry was designed based on the moth cytochrome *c* registry, IAYLKQATK (MCC 95−103) and the murine invariant chain CLIP registry MRMATPLLM (murine Invariant chain Ii CLIP). f-MCC 95−103 has a dissociation half-life from sI-E^k ($t_{1/2}$) of much greater than 500 h (~2000 h) at pH 7, while f-Ii CLIP 85−99 has a $t_{1/2}$ of approximately 9 min (CLIP registry 90−98). The chimeric peptide synthesized was based on the junction of the sequences of MCC 95−103 and murine Ii CLIP 89−99 (f-IAYLKQATKQMRMATPLLMR). During this and other experiments, the peptide is allowed to bind to the *active state* of sI-E^k by preloading sI-E^k with a fast dissociating unlabeled peptide such as murine CLIP and incubating the preloaded complex with labeled peptide for a specific amount of time in a procedure similar to that used by Rabinowitz et al (*5*). The less stable isomer (presumably Ii CLIP registry) in the chimeric peptide fMCC 95−103 Ii CLIP 89−99 dissociates rapidly (~36% component) from sI-E^k as shown in Figure 1, after prior incubation with the MHC II for 30 min at 37 °C. The half-life of the unstable isomer is approximately 10 min. This presents problems in terms of difficulty of obtaining accurate values for the $t_{1/2}$ of the fast component, as well as minimizing the time for potential intramolecular "interconversion" or sliding. To reduce the dissociation rate of the Ii CLIP registry in the chimeric peptide, the P1 anchor was mutated from *Met* to *Leu*. The peptide f-QLRMATPLLMR has a dissociation half-life on the order of ~25 h. This peptide mutation at the P1 position has also been found to increase the affinity of a peptide/MHC II complex (*31*). Thus, the chosen chimeric peptide having a more stable isomer is f-IAYLKQATKQ*L*-

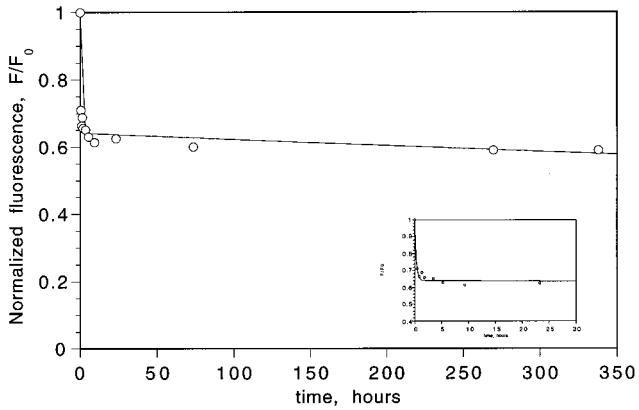

FIGURE 1: Dissociation kinetics of chimeric f-MCC 95−103 Ii CLIP 89−99 from I-E^k measured in PBS pH 7.0, 37 °C (PBS = phosphate buffered saline) in the presence of 10 μM unlabeled MCC competitor. The solid line is a double exponential fit to the data (circles). The fast phase isomer has a best fit $t_{1/2}$ of ∼10 min and represents 36% of the total complex. The complex was prepared by incubation of 3 μM labeled peptide with preloaded I-E^k (∼0.3 μM) for 30 min at pH 7.0 followed by separation of the preloaded complex from excess peptide prior to the kinetic measurement. [Preloaded I-E^k was prepared as described in Materials and Methods.] Inset: early time dissociation behavior of the peptide from I−E^k (0−30 h).

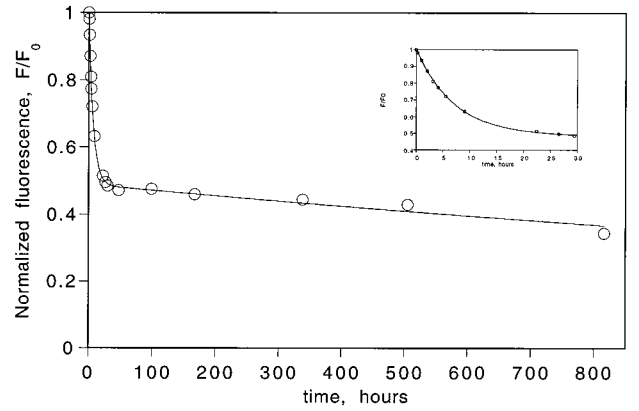

FIGURE 2: Dissociation kinetics of chimeric f-MCC 95−103 Ii CLIP 89−99 M90L from I-E^k measured in PBS pH 7.0, 37 °C (PBS = phosphate buffered saline) in the presence of 10 μM unlabeled MCC competitor. The solid line is a double exponential fit to the data (circles). The fast phase isomer has a $t_{1/2}$ of ∼5 h and represents ∼51% of the total complex. The complex was prepared by incubation of 3 μM labeled peptide with preloaded I-E^k (∼0.3 μM) for 1.08 h at pH 7.0 followed by separation of the preloaded complex from excess peptide prior to the kinetic measurement. [Preloaded I-E^k was prepared as described in Materials and Methods.] Inset: early time dissociation behavior of the peptide from I-E^k (0−30 h).

*RMATPLLM*R (fMCC 95−103 Ii CLIP 89−99 M90L). The incubation of preloaded sI-E^k with 3.0 μM of this chimeric peptide for 1 h leads to biphasic dissociation kinetics shown in Figure 2. This may be fit to a double exponential

$$F/F_0 = A\exp(-k_{fast}t) + (1\text{-}A)\exp(-k_{slow}t) \qquad (1)$$

where F/F_0 is the normalized fluorescence of the complex, A and (1-A) are the relative amounts of fast and slow phase components respectively, and k_{fast} and k_{slow} are the fast and slow dissociation rate constants, respectively. The fast phase isomer has a half-life of ∼5 h and forms about 50% of the

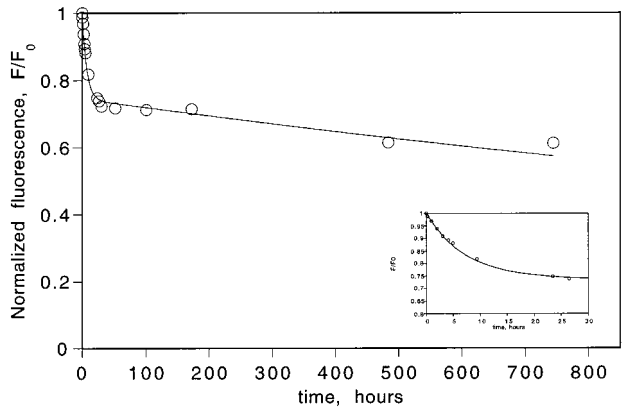

FIGURE 3: Dissociation kinetics of chimeric f-MCC 95−103 Ii CLIP 89−99 M90L from I-E^k MHC II measured in PBS pH 7.0, 37 °C (PBS = phosphate buffered saline) in the presence of 10 μM unlabeled MCC competitor. The solid line is a double exponential fit to the data (circles). The fast phase isomer has a $t_{1/2}$ of ∼5 h and represents ∼25% of the total complex. The complex was prepared by incubation of 3 μM labeled peptide with preloaded I-E^k (∼0.3 μM) for 13.3 h at pH 7.0 followed by separation of the preloaded complex from excess peptide prior to the kinetic measurement. [Preloaded I-E^k was prepared as described in Materials and Methods.] Inset: early time dissociation behavior of the peptide from I-E^k (0−30 h).

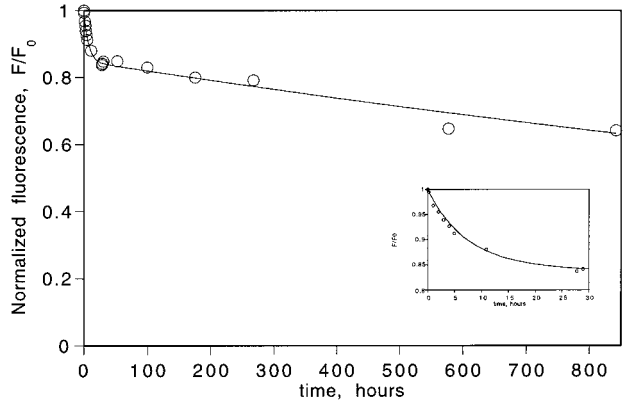

FIGURE 4: Dissociation kinetics of chimeric f-MCC 95−103 Ii CLIP 89−99 M90L from I-E^k MHC II measured in PBS pH 7.0, 37 °C (PBS = phosphate buffered saline) in the presence of 10 μM unlabeled MCC competitor. The solid line is a double exponential fit to the data (circles). The fast phase isomer has a $t_{1/2}$ of ∼5 h and represents ∼25% of the total complex. The complex was prepared by incubation of 3 μM labeled peptide with preloaded I-E^k (∼0.3 μM) for 24.2 h at pH 7.0 followed by separation of the preloaded complex from excess peptide prior to the kinetic measurement. [Preloaded I-E^k was prepared as described in Materials and Methods.] Inset: early time dissociation behavior of the peptide from I-E^k (0−30 h).

total complex after 1 h incubation. This is an example of a peptide/MHC II kinetic isomer. Duplicate experiments were done for ∼13 and 24 h incubation. Representative curves are shown in Figures 2, 3, and 4. An observed trend is that the percent of the *fast* phase species decreases with incubation time resulting in an increase in the fraction of the most thermodynamically stable slow phase. Thus, an incubation time of 1 h results in less than 50% slow phase. In contrast, a 1-day incubation results in approximately 85% slow phase. To obtain a best fit estimate of the lifetime of the fast and slow phase isomer, a fit of the data shown in Figures 2−4 and another set of duplicate experiments (not shown) was done, with the constraint of a concensus value of k_f and k_s

Scheme 1

$$(MP)_1 \underset{k_{on1}}{\overset{k_f}{\rightleftharpoons}} M_a + P \underset{k_s}{\overset{k_{on2}}{\rightleftharpoons}} (MP)_2$$

Scheme 2

$$(MP)_1 \rightleftharpoons (MP)_2$$

for all the data using the *FindMinimum* option in Mathematica v. 3.0. The *FindMinimum* option finds the local minimum value for a function (*32*). Therefore, the minimum least-squares fit set of parameters was found, assuming that k_f and k_s are independent of incubation time. Various reasonable starting guesses for the fitting parameters were used, and the final optimized parameters were quite similar, implying that a global minimum fit was found. On the basis of the fit, a consensus $t_{1/2}$ of 4.94 h for the fast phase ($t_{1/2f}$) and a $t_{1/2}$ of ~1990 h for the slow phase ($t_{1/2s}$) were found. Statistically speaking, the confidence intervals for these two values were 4.67−5.21 h for $t_{1/2f}$ and 1850−2120 h for $t_{1/2s}$ (NonlinearRegress option, Mathematica v. 3.0).

To interpret these results, we analyze the data in terms of the kinetic scheme 1, where P is a chimeric peptide able to bind the MHC in registry 1 or 2 to form complexes $(MP)_1$ or $(MP)_2$, respectively, k_{on1} and k_{on2} are the association rate constants of registries 1 and 2 to the MHC, respectively, and k_f and k_s are the dissociation rate constants of registries 1 and 2 from the MHC, respectively (subscripts f = fast dissociating, s = slow dissociating), and M_a is the active state of MHC II able to bind peptides.

This simple analytical model entails two important assumptions:

(i) The first assumption is that once the chimeric peptide binds the MHC II in one registry, it does not rearrange itself to bind in the second registry. This means that there is no reaction (Scheme 2):

(ii) A second assumption is that the inactivation of the active state form of the protein may be neglected. That is, during the incubation of the chimeric peptide f-**I**AYLKQAT-KQ**L**RMATPLLMR with sI-E^k, there is a steady state of peptide/MHC II complex such that whenever a peptide falls off sI-E^k, another peptide replaces it. Therefore, there is a constant amount of complex ($[(MP)_1] + [(MP)_2]$) at all times. In the case where sI-E^k is incubated with 3.0 μM peptide concentration, the total amount of complex is approximately constant after and including 1 h of incubation at pH 7.0 as shown in Figure 5. This implies a steady-state amount of complex after 1 h of incubation with a 3.0 μM peptide concentration during the incubation and may be interpreted as a steady state of the active and empty form of I-E^k:

$$d[E]/dt = -k_{on}[E][P] + k_f[(MP)_1] + k_s[(MP)_2] \sim -k_{on}[E][P] + k_f[(MP)_1] = 0 \quad (2)$$

where [P] is the peptide concentration, [E] is the concentration of empty and active sI-E^k, k_{on} is the association rate constant of the peptide to sI-E^k, k_f and $[(MP)_1]$ are the dissociation rate constant and concentration of the fast phase species, respectively, and k_s and $[(MP)_2]$ are the dissociation rate constant and concentration of slow phase species, respectively. In this model, an approximation is made where the slowly dissociating registry $(MP)_2$ is assumed not to dissociate within the time scale of the experiment (i.e., $k_s \sim 0$). This approximation is valid since the $t_{1/2}$ of the slow registry is ≫800 h. The rate constant of association of the chimeric peptide to sI-E^k was calculated as described in Materials and Methods. Briefly, a given amount of the active form of sI-E^k was associated with varying concentrations of peptide as shown in Figure 6. The calculated k_{on} at pH 7 is 0.9×10^4 M^{-1} s^{-1}. As mentioned earlier, at a peptide concentration of 3.0 μM, the nearly constant amount of complex shown in Figure 5 is interpreted as a very fast rebinding of f-peptide in solution to the newly formed active sI-E^k created by the dissociation of f-peptide from MHC II. In other words, the rate of f-peptide association to newly formed active I-E^k is greater than the rate of inactivation of sI-E^k.

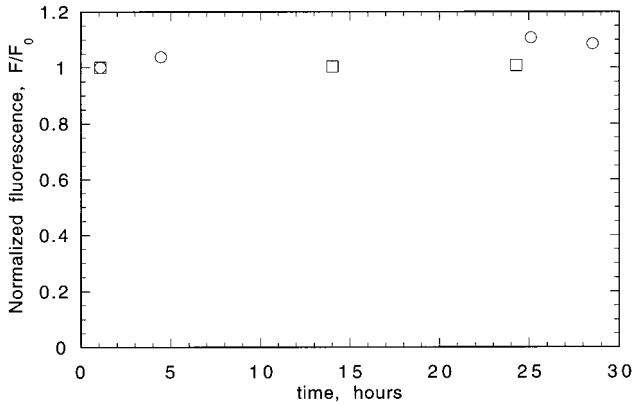

FIGURE 5: Binding saturation of 3 μM f-MCC 95−103 Ii CLIP 89−99 M90L peptide to preloaded I-E^k (~0.3 μM) at pH 7.0 and 37 °C. Preloaded I-E^k was prepared as described in Materials and Methods. The preloaded complex was then incubated with the fluorescently labeled peptide (3.0 μM, 37 °C, pH 7.0) for a given amount of time (*x*-axis). The fluoresecently labeled peptide/I-E^k complex was then separated from the excess free peptide by direct injection of an aliquot into an HPLC column, and the relative amount of complex (*y*-axis) was detected by a fluorescence detector with excitation and emission wavelengths set at 492 and 522 nm, respectively. Squares and circles represent data for two separate experiments.

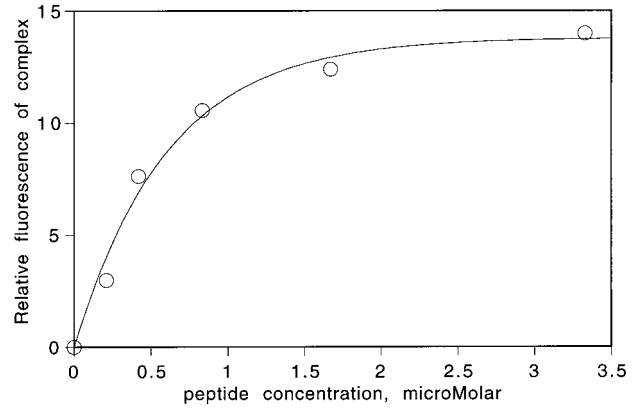

FIGURE 6: Determination of the association rate constant (k_{on}) of chimeric f-MCC 95−103 Ii CLIP 89−99 M90L to the active state of I−E^k (preloaded) at pH 7.0, 37 °C as described in Materials and Methods. The *y*-axis is the fluorescence of the f-peptide/MHC II complex formed from a 3-min incubation of the preloaded complex with a specified f-peptide concentration (*x*-axis). Data were fit to an exponential form given in Materials and Methods. The best fit association rate constant is 0.9×10^4 M^{-1} s^{-1}.

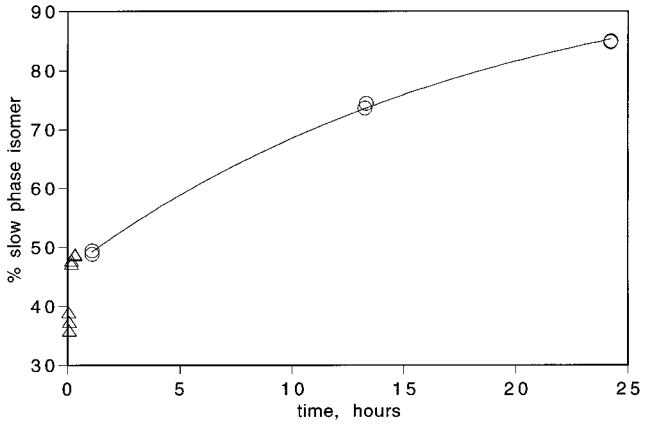

FIGURE 7: Exponential fit (eq 5) of the percentage of slow dissociating f-MCC 95−103 Ii CLIP 89−99 M90L/I-E^k isomer as a function of incubation time (greater than or equal to 1 h, circles) at pH 7.0 and 37 °C. The best fit half-life for this process is 12.9 h, and the intercept is 46% slow phase ($1 - F = 0.46$) which is interpreted as the initial ($t = 0$) fraction of slow phase. The time-zero percent slow phase was also calculated using eq 6, yielding $S = 0.38$ (a corrected value of $S = 0.41$ may be calculated as discussed in Results). For relatively short incubation times (especially smaller than 10 min), the presence of a third very short-lived kinetic intermediate complicated the kinetic analysis without affecting the overall interpretation of the results, and therefore these data (shown in triangles) were not included in the fit.

Without any further simplifications, the differential equation relating the slow phase species concentration $[(MP)_2]$ with time is

$$\frac{\partial[(MP)_2]}{\partial t} = \frac{k_f k^s_{on}}{k^f_{on} + k^s_{on}} \left\{ c_0 - \left(1 + \frac{K_f}{K_s}\right)[(MP)_2] \right\} \quad (3)$$

where c_0 is the sum of the concentrations of the slow $(MP)_2$ and fast $(MP)_1$ phase species, respectively, and K_f and K_s are the stability constants for the fast phase and slow phase, respectively. Since $K_f \ll K_s$, a solution to eq 3 in terms of the time dependence of the fraction of slow phase species ($[(MP)_2]/c_0$) is

$$\frac{[(MP)_2]}{c_0} = 1 - \frac{k^f_{on}}{k^f_{on} + k^s_{on}} \exp\left(\frac{-k_f k^s_{on}}{k^f_{on} + k^s_{on}} t\right) \quad (4)$$

In fitting the experimental data in Figure 7, eq 4 is written in terms of two parameters, F and t_s. F was determined by

$$\frac{[(MP)_2]}{[c_0]} = 1 - 2^{-t/ts} F \quad (5)$$

the proportion of slow and fast phase found at "zero time". The best fit value for F was 54% (intercept). The quantity t_s was determined from the build-up of slow phase for incubation times longer than 1 h (see below). The best fit t_s is 12.9 h (confidence interval: 12.27 − 13.54 h). From eq 6, we then determine $S = 0.38$ using $k_f = 0.140$ h^{-1} ($t_{1/2f} = 4.94$ h) for the fast phase dissociation constant. It will be

$$t_s = \frac{\ln 2}{k_f S} \quad (6)$$

seen that $S + F = 1$ according to eqs 4 and 5, while the experimental results yield $F = 0.54$ and $S = 0.38$. However,

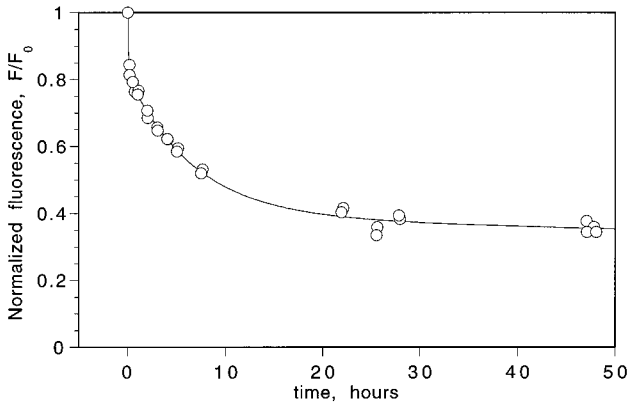

FIGURE 8: Dissociation kinetics of chimeric f-MCC 95−103 Ii CLIP 89−99 M90L from I-E^k measured in PBS pH 7.0, 37 °C (PBS = phosphate buffered saline) in the presence of 10 μM unlabeled MCC competitor. The solid line is a triple exponential fit to the data (circles). The isomer composition is ~19% of a very short-lived complex ($t_{1/2} \sim 4$ min), ~42% of an intermediate short-lived isomer, and ~39% of a long-lived isomer. The complex was prepared by incubation of 3 μM labeled peptide with preloaded I-E^k (~0.3 μM) for 5 min at pH 7.0 followed by separation of the complex from excess peptide prior to the kinetic measurement. [Preloaded I-E^k was prepared as described in Materials and Methods.]

there is a small but measurable push-off effect that leads to a slightly larger value of S, $S = 0.41$, as discussed below.

The competitor peptide AcMCC 95−103 has a measurable effect on the dissociation kinetics. The experiments used to determine the equilibration time t_s in eq 5 are necessarily carried out in the absence of competitor peptide, whereas most of our kinetic dissociation experiments are routinely carried out in the presence of the competitor peptide AcMCC 95−103. This peptide has a measurable "push-off" effect on the dissociation. In the absence of competitor peptide, the half-time observed is 5.2 h ($k_f = 0.132$ h^{-1}). With this value of k_f, one obtains $S = 0.41$ from eq 6. (As expected, the enhancement of the off-rate is proportional to the competitor peptide concentration, the half-time for dissociation in the presence of 30 μM competitor being 3.3 h.)

Note that all the parameters in eq 5 and eq 6 are determined from independent experimental observations. That is, k_f is a measured off-rate, t_s is a measured time constant, and S then follows directly from eq 6, and F is measured from the fit to eq 5 at time zero. That is, the parameter set is overdetermined. Even so, the parameter set fits the data nicely to within experimental error.

Using kinetic simulations, we have investigated the question of whether intramolecular rearrangement could possibly contribute to the equilibration of the two isomers and still be consistent with the experimental data. Using a larger parameter set so as to include Scheme 2 kinetics, we nonetheless find that only slow (and negligible) intramolecular equilibration yields kinetics consistent with the observed results. Thus, intramolecular rearrangement does not contribute significantly to the equilibration between the fast and slow dissociating isomers to within the experimental errors.

At short incubation times such as 5 min, ~19% of a very short-lived (transient) species is detected with a half-life of approximately 4 min as shown in Figure 8. Such very short-lived complexes have been observed previously with cyto-

chrome *c* peptide/I-E^k complexes (*20*, *21*, *14*). Data obtained at incubation times shorter than 1 h were not included in the analysis of the present results. The very short-lived species do not persist after 10 to 20 min incubation with I-E^k as shown in the dissociation kinetics in Figures 2−4. (For early incubation times (<10 min), the percent slow phase is less than 40% and increases to slightly below 50% after 10 min of incubation (Figure 7).) This very short-lived isomer clearly does not play an limiting role in the equilibration of the two chimeric isomers, based on the fit of the data to our model. This is not surprising in view of the short lifetime of this isomer.

To compare the binding behavior of two separate peptides to sI-E^k in contrast to the chimeric peptide, a 50% f-MCC 95−103, 50% f-Ii CLIP 89−99 M90L solution was incubated with sI-E^k for ~1 and ~63 h at a total peptide concentration of 3.0 μM, followed by dissociation kinetic measurements. The dissociation data were fit to a biphasic curve using the accurate single peptide dissociation half-lives as fixed parameters and treating the fraction of *fast phase* peptide as an adjustable parameter. Thus, the half-life of the murine fIi CLIP 89−99 M90L peptide was fixed at 27 h and the half-life of f-MCC 95−103-NH$_2$ was fixed at 2000 hours. The best fit fast phase percentages were 76.8 and 56.8% after 1 and 63 h incubation, respectively. Using the time-zero percentage of fast phase as 76.8%, a value of 52.5% fast phase is calculated for 63 h of incubation based on eq 4. This is in reasonable agreement with the observed experimental value of 56.8%. Therefore, the 1:1 peptide binary mixture binding to I-E^k behaves similarly to the chimeric peptide. However, there is a significant difference between the initial fractions of MCC and CLIP M90L bound to sI-E^k in the free peptide form relative to the chimeric form.

Identity Verification of the "Slow" and "Fast" Phase Isomer. To verify that the slowly dissociating isomer is the MCC registry in the chimeric peptide, a mutant peptide was made, where the P1 *Ile* anchor of the MCC registry was mutated to serine, a bad P1 anchor (f-**S**AYLKQATKQ**L**RMATPLLMR). The dissociation kinetics of this peptide (squares) is compared to the *reference* CLIP M90L registry mutant used earlier (f-**I**AYLKQATKQ**L**RMATPLLMR, diamonds) as shown in Figure 9. The kinetics of f-**S**AYLKQATKQLMATPLLMR are monophasic within experimental error with a $t_{1/2}$ of 6.80 h after 1 h 5′ incubation. This time is comparable to the 4.94 h half-life of the fast phase of the *reference* peptide, consistent with an assignment of the stable isomer to be **I**AYLKQATK/I-E^k. Similarly, the *wild type* chimeric peptide (f-**I**AYLKQATKQ***M***RMATPLLMR) weakens the CLIP registry relative to the *reference* peptide (diamonds) as shown in Figure 9 (circles) as expected since *Met* has been shown to be a poor P1 anchor for sI-E^k relative to *Leu* (*31*). Consistent with these arguments, after 1 h incubation, the wild type chimeric peptide has only 13.7% fast phase with a $t_{1/2}$ of ~13 min under the same conditions. The interpretation of this result is that the fast phase species of the *reference* peptide is the **L***RMATPLLM* /I-E^k isomer.

It should be pointed out that there is a difference in the dissociation half-life of registries in the chimeric form relative to the "free" peptide form. Specifically, there is an intramolecular destabilizing effect of the N-terminal f-MCC sequence on the Ii CLIP M90L registry of the chimeric peptide which results in the decrease of the dissociation half-life of the CLIP registry from ~26 h in the free CLIP M90L peptide/sI-E^k complex (fIi CLIP 89−99 M90L/sI-E^k) to ~5 h in the chimeric peptide f-IAYLKQATKQLRMATPLLMR/sI-E^k complex. This effect is similar to the observations by Strominger et al. (*12*) who found that an N-terminal overhang in a peptide destabilizes the C-terminal peptide isomer bound to MHC II.

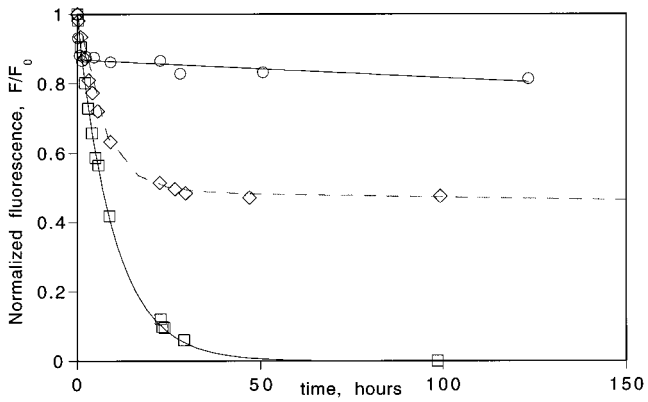

FIGURE 9: Comparative dissociation kinetics of chimeric f-MCC 95−103 Ii CLIP 89−99 (circles), f-MCC 95−103 Ii CLIP 89−99 M90L (diamonds), and f-MCC 95−103 I95S Ii CLIP 89−99 M90L (squares) from I-E^k at pH 7.0 and 37 °C. Kinetic f-peptide/I-E^k complexes were prepared by incubating 3.0 μM f-peptide with ~0.30 μM preloaded I-E^k (see Materials and Methods) at pH 7.0 and 37 °C for 1 h, followed by excess peptide separation using Sephadex G-50 spin columns. The dissociation kinetics are consistent with the assignment of the MCC registry as the slow dissociating isomer and the CLIP registry as the fast dissociating isomer. Specifically, mutation of the putative P1 anchor of the MCC registry from Ile (diamonds) to Ser (squares) virtually eliminates this isomer as expected. Similarly, mutation of the putative P1 anchor of the CLIP registry from Met (circles) to Leu (diamonds) increases the stability of the fast dissociating isomer as expected.

pH 5.3 Behavior of the Chimeric Peptide. In addition to cell surface MHC II binding, peptides also bind to MHC II in acidic endosomal compartments. To address this physiologically relevant condition, the chimeric peptide f-MCC 95−103 Ii CLIP 89−99 M90L was incubated with sI-E^k for 10 min and 3.5 h at pH 5.3. The best fit half-lives for the fast and slow dissociating isomers were 1.58 and 145 h, respectively (results not shown). The relative amounts of the slow dissociating isomer after 10 min and 3.5 h incubation were 35.8 and 61.2%, respectively. These results are consistent with eq 5 and Scheme 1 where it is calculated that there should be 62.9% slow phase after 3.5 h of incubation. Therefore, the kinetic behavior at pH 7.0 and pH 5.3 for the peptide/MHC II complex is consistent with the model described by eq 5. Short incubation times (10 min) at pH 5.3 clearly favor the formation of the fast dissociating isomer over the slow dissociating species (64.2% fast phase). For comparison, at pH 7.0, formation of the fast dissociating species is only slightly favored after 10 min of incubation time (52−53%).

Evidence for a Biased Binding Mode of Registry Isomers in an MHC II. On the basis of the results presented thus far, it is apparent that the relative association rates of the two registries in the chimeric peptide f-MCC 95−103 Ii CLIP 89−99 M90L to I-E^k are not identical, especially at pH 5.3. A striking example of a difference of this kind was found for complexes formed between the chimeric peptide f-

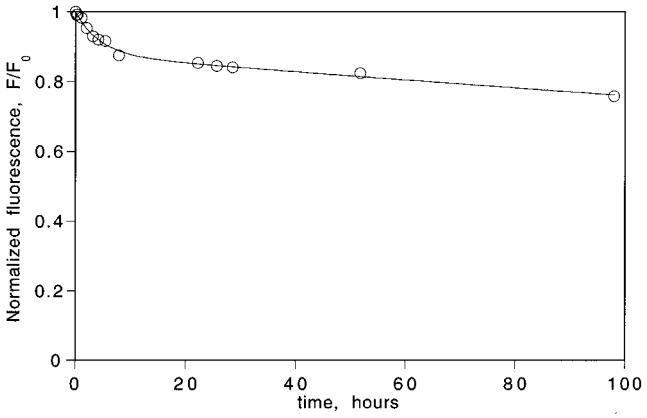

FIGURE 10: Representative dissociation kinetics of chimeric HCgp-39 327−337 D330N 327−337/DR*0401 at pH 8.0 (HEPES/MES buffer) in the presence of excess competitor peptide (10 μM). The solid line is a double exponential fit to the data (circles). The fast phase isomer has a best fit half-life of 3 h and represents 12% of the total complex. The half-life of the slow phase is greater than 200 h. The complex was prepared by incubating the chimeric peptide (10 μM) with preloaded DR*0401 (0.20 μM) for 1 h at pH 5.3 (PBS/citrate) and 37 °C. Both isomeric complexes are very stable at pH 5.3 (half-life ≫2 days), but the dissociation of one registry isomer is especially enhanced at pH 8 while the other registry isomer is relatively stable at both pH 5.3 and 8.0 (see Results). An interpretation of this experiment is that the relative association rates of each registry to preloaded DR*0401 is 7:1 at pH 5.3, in favor of the isomer whose dissociation is relatively insensitive to pH.

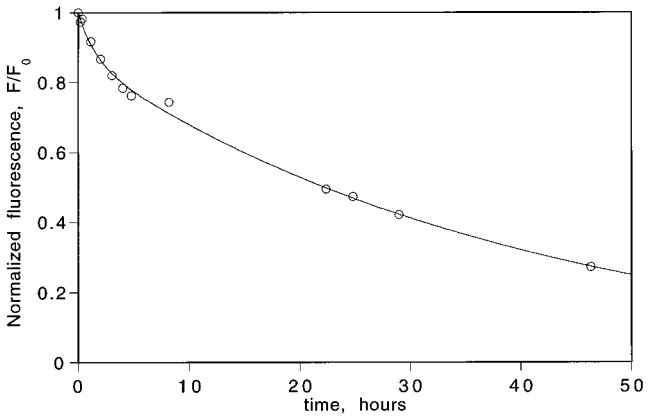

FIGURE 11: Representative dissociation kinetics of chimeric HCgp-39 327−337 D330N 327−337/DR*0401 at pH 5.3 (PBS/citrate buffer) in the presence of 0.38 μM HLA-DM and excess competitor peptide (10 μM). The solid line is a double exponential fit to the data (circles). The fast phase isomer has a best fit half-life of 1 h and represents 13% of the total complex. The half-life of the slow phase is 28 h. The complex was prepared by incubating the chimeric peptide (10 μM) with preloaded DR*0401 (0.20 μM) for 1 h at pH 5.3 (PBS/citrate) and 37 °C. Both isomeric complexes are very stable at pH 5.3 (half-life ≫ 2 days in the absence of HLA-DM) but are kinetically resolved at pH 5.3 in the presence of HLA-DM. An interpretation of this experiment is that the relative association rates of each registry to preloaded DR*0401 is 7:1 at pH 5.3, consistent with the pH jump experiment summarized in Figure 10 (see Results.)

G**Y**DNQESVKSKG**Y**DDQESVKSK (HCgp-39 327−337 D330N 327−337) and HLA-DR4*0401 (human MHC II). This chimeric peptide was designed such that it may bind to human DR*0401 MHC II in two different registries. The two putative P1 anchors are indicated in bold, and this peptide may potentially bind to DR*0401 with the **Y**DNQESVKS registry in one isomer form and with the **Y**DDQESVKS registry in a second isomer form. The dissociation kinetics of the C-terminal registry (HCgp-39 327−337) are expected to be sensitive to pH while the N-terminal registry is not according to previous studies of the free peptides (*30*). Specifically, the GYDDQESVKSK/DR*0401 complex has a much shorter half-life at pH 7 than at pH 5.3 (factor of 30), while the GYDNQESVKSK/DR*0401 complex has comparable half-lives at pH 7.0 and pH 5.3. The half-lives of both complexes are on the order of weeks at pH 5.3. As expected, the HCgp-39 327−337 D330N 327−337/DR*0401 complex(es) is very long-lived at pH 5.3 and 37 °C ($t_{1/2}$ ≫ 2 days, results not shown). In contrast, association at pH 5.3 of the chimeric peptide to preloaded DR*0401 for 1 h (preloader: AcKPASQMRMAAPLLFR, Ac = acetyl) followed by dissociation at pH 8 leads to approximately 12−15% of a relatively unstable species ($t_{1/2}$ ∼ 3.4 h) based on the weights in a double exponential fit as shown in Figure 10. The half-life of the slow phase is greater than 200 h. An incubation time of 1 h of the peptide with the MHC II at pH 5.3 is much shorter than the time scale of dissociation of either isomeric complex at pH 5.3 (≫2 days), and the relative amount of each isomer formed under this conditions is a measure of the relative association rates of each registry to DR*0401. The interpretation of these experiments is that the short-lived species at pH 8 is most likely the YDDQESVKS/DR*0401 registry isomer. The relative association rates of each registry to preloaded DR*0401 is 7:1 at pH 5.3, in favor of the YDNQESVKS/DR*0401 registry isomer.

To attempt to kinetically resolve these isomers at pH 5.3, the complex was incubated with preloaded DR*0401 for 1 h at pH 5.3, followed by dissociation of the complex at pH 5.3 in the presence of varying concentrations of HLA-DM. At concentrations of HLA-DM of 0.375 and 0.50 μM, the two isomers are kinetically resolved and the results are consistent with the pH switching experiments. Specifically, at a HLA-DM concentration of 0.375 μM, ∼13% fast phase is detected with fast and slow phase half-lives of 1.0 and 28 h, respectively, as shown in Figure 11. Similarly, at a HLA-DM concentration of 0.50 μM, ∼13% of fast phase is detected with fast and slow phase half-lives of 0.89 and 23 h, respectively (results not shown).

In this experiment as well as the other experiments with the chimeric peptides, we have not ruled out the possibility that the quantum fluorescence yield may be different for each isomer, but we believe that in both registry isomers, the fluorescent dye is sufficiently solvent exposed to prevent any significant differences in local environment.

DISCUSSION

We have shown that registry isomers of a chimeric peptide bound to sI-E^k only interconvert by dissociation and rebinding. At early times, the registry with the highest association rate to the MHC II dominates. In this study, the CLIP M90L registry in the chimeric peptide appears to have a slightly greater association rate to the active state of sI-E^k than the MCC registry at pH 7.0. In other words, at short incubation times, a slightly greater proportion of the CLIP M90L registry occupies the sI-E^k binding groove (>50%) despite the fact that MCC registry is more stable overall. However, after long incubation times, the thermodynamically favored

isomer (MCC) is formed in increasing amounts. As a control, it is found that a 1:1 mixture of peptides incubated with sI-E^k also equilibrates according to this mechanism. The CLIP M90L peptide has a significantly greater association rate than the MCC peptide to the active state of I-E^k based on the these experiments. The kinetic simulations show that at pH 7.0 a given registry does not isomerize to form the other registry. The kinetic experiments at pH 5.3 for the chimeric peptide/I-E^k complex are also consistent with this behavior. The difference in the relative association rates of each registry in the chimeric peptide to I-E^k is more pronounced at pH 5.3 than at pH 7.0. Kinetic experiments with a chimeric peptide and DR*0401 are also consistent with a significant difference in the relative association rates of both registry isomers (~12−15% for one isomer). This shows that the initial binding of the peptide to the MHC II is registry selective.

This study describes the equilibration kinetics of two peptide epitopes binding to an MHC II protein. The associations and dissociations of registry isomers may be important in determining which registry isomer is presented to T-cells at early times after exposure to the peptide antigen. In previous work, MHC II/peptide isomers have been identified that may not involve different registries (*20*, *21*, *33*). In these and other studies of MHC II/peptide isomers, the method used in this work might be used to test for equilibration by Scheme 1 or Scheme 2.

Finally, the role of catalysts such as HLA-DM in isomer selection of multiple registry peptides needs investigation. One would expect HLA-DM to accelerate the dissociation of both registry isomers from the MHC II, decreasing the time of formation of the most thermodynamically stable isomer(s). This could lead to differences in the time of formation of the most stable registry isomer between peptides loaded in endosomal compartments and peptides loaded at the cell surface. We cannot of course rule out the possibility that HLA-DM may catalyze intramolecular isomerization of a peptide with two isomeric registries.

Our study indicates that if there is registry selection in endosomal compartments of a cell, then that selection persists at the cell surface. This is because registry interconversion requires peptide dissociation from the MHC II for which there is a low probability of rebinding in the absence of high extracellular peptide concentrations.

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