Formation of a Highly Peptide-Receptive State of Class II MHC

Joshua D. Rabinowitz,* Marija Vrljic,* Peter M. Kasson,* Michael N. Liang,* Robert Busch,† J. Jay Boniface,‡ Mark M. Davis,§ and Harden M. McConnell* §

*Department of Chemistry
† Department of Pediatrics
‡ Howard Hughes Medical Institute
Stanford University
Stanford, California 94305

Summary

Peptide binding to class II MHC proteins occurs in acidic endosomal compartments following dissociation of class II-associated invariant chain peptide (CLIP). Based on peptide binding both to empty class II MHC and to molecules preloaded with peptides including CLIP, we find evidence for two isomeric forms of empty MHC. One (inactive) does not bind peptide. The other (active) binds peptide rapidly, with $k_{on}$ 1000-fold faster than previous estimates. The active isomer can be formed either by slow isomerization of the inactive molecule or by dissociation of a preformed peptide/MHC complex. In the absence of peptide, the active isomer is unstable, rapidly converting to the inactive isomer. These results demonstrate that fast peptide binding is an inherent property of one isomer of empty class II MHC. Dissociation of peptides such as CLIP yields this transient, peptide-receptive isomer.

Introduction

Class II MHC genes encode heterodimeric membrane glycoproteins that bind peptides and present them to CD4+ T cells. The predominant pathway that generates peptide/class II MHC complexes begins with the assembly of newly synthesized class II MHC with another membrane protein, invariant chain (ii), in the endoplasmic reticulum. Complexes of class II and ii assembled in the ER traverse the Golgi apparatus to endosomal compartments where ii is proteolyzed (reviewed in Pieters, 1997; Chapman, 1998). Following proteolysis of ii, a set of peptides derived from residues 81-104 of ii (class II-associated invariant chain peptides [CLIP]) remains bound to class II, filling the antigenic peptide binding cleft (Ghosh et al., 1995; Lee and McConnell, 1995; Sette et al., 1995). Dissociation of CLIP results in class II MHC heterodimers that bind antigenic peptides or other self-peptides present in the endocytic system. CLIP dissociation is accelerated by another membrane protein, HLA-DM (Denzin and Cresswell, 1995; Sherman et al., 1995; Sloan et al., 1995).

One approach to understanding peptide binding to class II MHC is to mix purified class II MHC with labeled peptides and measure peptide/MHC complex formation. Initial studies of this type were performed using detergent-solubilized class II molecules from B cell lines. These studies found that antigenic peptides bind to purified class II molecules to form stable complexes, but that this binding is very slow ($k_{on}$ $\approx$ 1-100 $M^{-1} s^{-1}$) (Buus et al., 1986; Roche and Cresswell, 1990; Roof et al., 1990). It was later found that these B cell-derived class II molecules contain a diverse array of endogenous peptides (Buus et al., 1988), and that the dissociation of these endogenous peptides limits the rate of labeled peptide association (Tampe and McConnell, 1991; Reay et al., 1992; Witt and McConnell, 1993). Expression of recombinant MHC lacking the transmembrane domain allowed purification of water-soluble class II molecules devoid of endogenous peptide (Wettstein et al., 1991; Stern and Wiley, 1992). Compared to detergent-solubilized class II-containing endogenous peptides, this water-soluble protein binds peptide slightly more rapidly ($k_{on}$ $\approx$ 10-100 $M^{-1} s^{-1}$). Nevertheless, this association rate is approximately 105-fold slower than that of an analogous peptide–protein reaction, the folding of the protein RNaseS (Goldberg and Baldwin, 1998), and is 104-fold slower than a recent measurement of peptide binding to class I MHC (Springer et al., 1998).

It has been suggested that the slow association rate of peptide to class II protein may be related to complexity in the peptide/class II reaction pathway (Sadegh-Nasseri and McConnell, 1989; Nasseri and McConnell, 1989). It was later found that these B cell-derived class II molecules contain a diverse array of endogenous peptides (Buus et al., 1988), and that the dissociation of these endogenous peptides limits the rate of labeled peptide association (Tampe and McConnell, 1991; Reay et al., 1992; Witt and McConnell, 1993). Expression of recombinant MHC lacking the transmembrane domain allowed purification of water-soluble class II molecules devoid of endogenous peptide (Wettstein et al., 1991; Stern and Wiley, 1992). Compared to detergent-solubilized class II-containing endogenous peptides, this water-soluble protein binds peptide slightly more rapidly ($k_{on}$ $\approx$ 10-100 $M^{-1} s^{-1}$). Nevertheless, this association rate is approximately 105-fold slower than that of an analogous peptide–protein reaction, the folding of the protein RNaseS (Goldberg and Baldwin, 1998), and is 104-fold slower than a recent measurement of peptide binding to class I MHC (Springer et al., 1998).

To understand better the reasons for this slow apparent rate, we have made a detailed kinetic study of peptide binding to water-soluble I-Ek derived from transfected Chinese hamster ovary cells (sEk) (Wettstein et al., 1991). Unlike several other empty class II molecules (Stern and Wiley, 1992; Germain and Rinker, 1993), empty sEk does not rapidly form aggregates. Furthermore, the circular dichroism spectrum of sEk closely resembles that of peptide-loaded I-Ek (Reich et al., 1997), suggesting the empty sEk is folded similarly to the peptide-loaded molecule (Fremont et al., 1996). Experiments measuring the binding of labeled peptide to empty versus peptide-loaded sEk provide evidence for two distinct isomers of empty sEk, a less stable active isomer and a more stable inactive isomer.
Immunity

700

Assuming this reaction scheme, dissociation of pre-bound peptide will likely, based on the principle of microscopic reversibility (Fersht, 1985), leave sEk in the active state. To investigate this possibility, we measured the binding of labeled MCC peptide to several preformed peptide/MHC complexes. In each case, the rate of labeled peptide binding is approximately equal to the rate of dissociation of the preformed complex (Figure 2), suggesting that dissociation of the preformed peptide/MHC complex is followed by rapid association of the second peptide (scheme 2).

Figure 1. The Rate of Antigenic Peptide Binding to Empty sEk Is Not Accelerated by Increasing the Concentration of Added Peptide

The indicated concentration of fluorescently labeled MCC Ac95-103 was reacted with 100 nM sEki at pH 5.3, 37°C. After the indicated time, free peptide was separated from peptide/MHC complex by size exclusion chromatography, and labeled peptide/MHC complex formation was measured by a fluorescence detector.

does not bind peptide, while active binds peptide rapidly. The slow apparent rate of peptide binding to empty sEk is due to slow isomerization of inactive into active. This slow step is bypassed when CLIP-loaded sEk is used as the starting material, because CLIP dissociation releases the active isomer. The generality of this reaction scheme is supported by studies of labeled peptide binding to water-soluble DR1 (sDR1), and also of T cell stimulation by I-Ek on the surface of a fixed APC (see Appendix 2). Together these findings suggest that active and inactive isomers may be a general feature of class II MHC, and that a previously unrecognized function of CLIP is to promote endosomal peptide binding by release of the active isomer.

Results

Dissociation of a Prebound Peptide Generates Active sEk

We studied the reaction of the antigenic peptide moth cytochrome c Ac95-103 (MCC) with sEk that was purified from cells by affinity and size exclusion chromatography and stored at pH 7, 4°C until use. We use the symbol sEki to represent this initial ensemble of sEk molecules. Consistent with previous reports (Reay et al., 1992), we find that the binding of MCC to sEk is essentially irreversible (dissociation t1/2, at pH 5.3-7.0, 37°C > 200 hr), and that binding of MCC to sEki (sEki) slowly proceeds until most sEki is peptide bound. In addition, we find that the rate of peptide binding to sEki is nearly independent of the MCC peptide concentration for [MCC] > 100 nM at pH 5.3 (Figure 1) (Note: the reaction at pH 7.0 is much slower and requires micromolar peptide concentrations; see Appendix 1). One simple interpretation of this result is that peptide binding is preceded by a peptide-independent reaction of molecules in sEki to form active sEk (sEka) (scheme 1).

Assuming this reaction scheme, dissociation of pre-bound peptide will likely, based on the principle of microscopic reversibility (Fersht, 1985), leave sEk in the active state. To investigate this possibility, we measured the binding of labeled MCC peptide to several preformed peptide/MHC complexes. In each case, the rate of labeled peptide binding is approximately equal to the rate of dissociation of the preformed complex (Figure 2), suggesting that dissociation of the preformed peptide/MHC complex is followed by rapid association of the second peptide (scheme 2).

\[
\text{p} \quad \text{sEki} \quad \text{p/sEki} \quad \text{p} \quad \text{sEka} \quad \text{p} \\
\{\text{sEki}\} \overset{p}{\rightarrow} \text{sEka} \quad \overset{p}{\rightarrow} \text{p/sEki}
\]
Isomers of Empty Class II MHC

sEka are isomers with identical covalent structure. A potential complication to this reaction scheme is that the slow peptide binding to both sEki and $\tilde{s}$Eki is better described by a sum of two exponential binding curves than a single exponential curve, indicating there may be more than one isomer of inactive sEki (data not shown).

Association Rate of Different Peptides to Active sEki

The ability to generate a sample containing a significant fraction of active, empty sEki (~25%) by dissociation of the 8-mer/sEki complex for 5 min in the absence of added peptide allowed direct measurement of the rate of labeled peptide binding to active sEki (Figure 4). The rate of peptide binding was determined in two ways, either by measuring the peptide concentration dependence of binding over a fixed time interval (3 min, Figure 4), or by measuring the time dependence of binding of a fixed concentration of peptide (data not shown). In both cases, the results are consistent with reaction scheme (3), with a rate of peptide binding of $\sim 5 \times 10^2 \ M^{-1} s^{-1}$ found for the MCC 95-103 peptide, CLIP, and a CLIP mutant in which the P1 and P9 methionine anchor residues have been replaced by leucine. This association rate is $\sim 4 \times 10^3$-fold faster than previous estimates of the peptide-sEki association rate. In addition, it is interesting to note that the different peptides studied all bind with very similar association rate constants despite varying in their dissociation rates by $\sim 10^3$-fold (dissociation $t_{1/2}$ 200 hr for MCC versus 3 min for CLIP; Figure 2 and Reay et al., 1992; Liang et al., 1995). To confirm that these brief reactions of MCC with active sEki do indeed produce stable complexes, we measured the dissociation rate of complexes formed in this way. We find that most such complexes are stable, with <20% dissociating in 24 hr at pH 5.3, 37°C (data not shown).

Dissociation of Prebound Peptide Generates Active sDR1

To investigate whether reaction scheme (3) might also apply to other class II MHC molecules, we studied the rate of peptide binding to empty sDR1 purified from transfected insect cells (Stern and Wiley, 1992; Sloan et al., 1995), frozen, and thawed immediately before use (sDR1). Unlike sEki, empty sDR1 forms oligomers (Stern and Wiley, 1992); therefore, reaction scheme (3) is an incomplete description of its reactions. However, similar to sEki, sDR1 preloaded with a rapidly dissociating peptide (8-mer) binds peptide more rapidly than sDR1. Moreover, lower concentrations of antigenic peptide (hemagglutinin 306-318, HA) are required to achieve maximal binding to 8-mer/sDR1 complex than slightly faster than peptide dissociation. This is likely because the unlabeled 8-mer peptide dissociates slightly faster than the labeled 8-mer peptide, presumably due to a hydrogen bond between the label and the MHC protein predicted based on the I-Ek crystal structure (Fremont et al., 1996). Also in (C), the association axis extends only to 0.6 units, because only half of the sEki produced by 8-mer dissociation is highly peptide receptive. (D) is a negative control which shows that a stable preformed peptide/MHC complex will not bind the added labeled peptide.
Figure 4. Rapid Binding of Several Different Peptides to Active sEk

A solution containing approximately 25% active sEk (total sEk concentration 8 nM) was formed by allowing 8-mer/sEk complex to dissociate in the absence of added peptide for 5 min at pH 5.3, 37°C. At this point, the labeled peptide indicated in the figure legend was added and allowed to bind for 3 min. Lines indicate a single exponential fit to the data with $k_{on} \approx 5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ (range 4-6 $\times 10^5 \text{M}^{-1} \text{s}^{-1}$) for each peptide (see Experimental Procedures).

Figure 3. Reversible Inactivation of Active sEk

(A) Formation of active sEk by peptide/MHC complex dissociation is followed by rapid sEk inactivation. 8-mer/sEk complex was incubated at pH 5.3 and 37°C for the indicated "delay time" in the absence of added peptide. The amount of active sEk present after the delay was measured by the binding of 5 nM labeled MCC for 5 min. The solid line indicates the analytical solution to the system of differential equations describing reaction scheme (3) (see Experimental Procedures), with the half-life of active sEk of 13 min. Errors bars indicate the standard error of triplicate measurements after delays of 10 min and 40 min. (B) Inactive sEk slowly reverts to active sEk. Shaded bars depict the binding of labeled MCC to inactive sEk formed by incubating CLIP/sEk complex in the absence of peptide for 2 hr at pH 5.3, 37°C prior to the MCC addition. Solid bars are the positive control depicting the binding of labeled MCC to CLIP/sEk complex. CLIP/sEk is at 50 nM; labeled MCC at 100 nM; similar data have been obtained for MCC concentrations up to 50 nM. Error bars indicate the standard error of duplicate measurements.

Discussion

In this report we deduce the existence of two isomers of empty class II MHC that have very different peptide binding capacities. One isomer (active) binds peptide rapidly at endosomal pH ($k_{on} = 10^4 \text{M}^{-1} \text{s}^{-1}$). The other isomer (inactive) does not itself bind peptide but can slowly ($t_{1/2} = 3 \text{ hr}$) isomerize into the active molecule. In the absence of peptide, the active isomer is unstable, inactivating with a half-time of a few minutes. These isomers appear to be a general feature of I-Ek, as they are formed both by a water-soluble version of the I-Ek protein (sEk) and by I-Ek on the surface of a fixed B lymphoma cell (see Appendix 2). In addition, the peptide binding characteristics of water-soluble DR1, although complicated by oligomerization, are consistent with an active DR1 isomer.

The isomerization reactions of empty class II MHC are a notable example of how the activity of a protein can be controlled by its folding kinetics. In many cases,
proteins fold rapidly to a single state that performs the function of the protein. This state may represent the thermodynamic global minimum for a particular protein covalent structure. In other cases, intermediates in the protein folding pathway may persist for a long enough time to be biologically relevant (Baker and Agard, 1994). One important instance of such folding is the isomerization of normal proteins into prions (reviewed in Harrison et al., 1997). Another well-studied example is the folding of the serpin family of protease inhibitors. Some serpins, such as plasminogen activator inhibitor 1, fold first to a state that is an active protease inhibitor (Wang et al., 1996). This active isomer then converts to a more stable, but inactive, isomer over a period of several hours. Reversion of the stable, inactive isomer to the less stable, active isomer has not been observed. Analogous to the serpins, empty class II MHC folds into two isomers, with the less stable isomer performing the peptide binding function of the protein. In contrast to the serpins, however, the isomers of empty class II reversibly interconvert. Based on the rates of interconversion, the free energy difference between the isomers is approximately 2 kcal/mol. Experiments measuring the temperature dependence of the rate of sE1 isomerization have been performed to investigate the enthalpy and entropy of the transition state in this reaction (J. R., Gordana Konjevic, and H. M. M., unpublished results). The finding that this rate decreases greatly with decreasing temperature suggests that the transition state is high in both entropy and enthalpy.

An intriguing question is whether protein isomerization is biologically useful. In the case of the prions, isomerization of the native state into the prion form appears to be a disadvantage. In the case of the serpins, however, it is unclear whether the irreversible inactivation reaction is merely a result of intrinsic protein instability or whether it is of value as a regulatory mechanism. For class II molecules, arguments can be made for either viewpoint. On the one hand, removal of the peptide from the crystal structure of peptide/MHC complexes would result in a potentially unstable structure, with a cleft between the helices and exposed hydrophobic pockets (Stern et al., 1994; Ghosh et al., 1995; Fremont et al., 1996). This raises the possibility that the inactivation of the peptide-receptive state of empty class II MHC may be an inevitable consequence of the general fold of the protein. On the other hand, it is possible that the general fold of MHC has been evolutionarily selected in part because the instability of empty, active MHC is advantageous. One effect of reversible MHC inactivation is to maintain a steady but low number of active cell surface binding sites (see Appendix 2). This may promote immune surveillance while preventing the binding of large numbers of self-peptides against which tolerance is weak or not established.

Regulation by accessory molecules may enhance the
advantageous aspects of protein isomerization reactions while minimizing their negative consequences. At least two accessory molecules, invariant chain (Ii) and HLA-DM, are required for the normal function of class II MHC (Bikoff et al., 1993; Viville et al., 1993; Karlsson et al., 1994; Morris et al., 1994). Ii binds to newly synthesized class II MHC in the endoplasmic reticulum and remains bound until the Ii/class II MHC complex reaches the endosome. There, Ii is proteolyzed to CLIP, and CLIP release is catalyzed by HLA-DM, which remains bound to empty class II MHC preventing its inactivation and aggregation (Denzin et al., 1996; Kropshofer et al., 1997).

We propose that the precise effect of HLA-DM is to prevent the isomerization of active class II MHC to the inactive state. This proposal is consistent with Kropshofer et al.'s data showing that in the absence of HLA-DM the loss of class II peptide binding capacity occurs before the accumulation of aggregated MHC (compare Figures 1A and 3C of Kropshofer et al., 1997) and that this loss of peptide binding capacity is prevented by HLA-DM.

In addition to peptide binding to newly synthesized class II MHC as described above, peptide can also bind to recycled MHC via an alternative pathway independent of Ii and DM (Pinet et al., 1995; Zhong et al., 1997). Peptide epitopes processed in late endosomes are presented via the Ii- and DM-dependent pathway; epitopes processed in early endosomes are presented via the recycling pathway. A common feature of both pathways is that binding of antigenic peptide follows dissociation of a prebound peptide. Therefore, our finding that dissociation of a prebound peptide releases an active state of class II MHC provides a common mechanism for the effective loading of class II in both cases. The active state of class II MHC binds a diverse array of peptides with a similar, rapid association rate (Figure 4). This property should facilitate the generation of a diverse repertoire of peptide/MHC complexes that is restricted solely by the set of peptides present when the active class II isomer is formed and by the stability of the resulting complexes.

Appendix 1: Reactions of Soluble Class II MHC at pH 7.0

Consistent with previous work (Wettstein et al., 1991; Reay et al., 1992; Sette et al., 1992; Boniface et al., 1993), we do not observe any major pH effect on the reactions of sDR1, but we do find that peptide binding to sEk is much more rapid at acidic than neutral pH (compare Figure 2 and Figure 6A). One common feature of peptide binding to empty sEk at pH 7.0 and pH 5.3 is that dissociation of a preformed peptide/sEk complex results in active sEk (Figures 2 and 6A). The slower rate of dissociation of an active sEk at pH 5.3 (approx. 3 hr at pH 5.3 versus 26 hr at pH 7.0, Figure 1 and data not shown) are slower at pH 7.0 than at pH 5.3.

Appendix 2: Reactions of Cell Surface I-Ek

As Measured by T Cell Stimulation

Exposure of a mixture of T cells and APCs to antigenic peptide results in rapid increases in T cell acid release that are class II restricted and peptide specific (Rabinowitz et al., 1996). The kinetics of the T cell response reflect the kinetics of the reactions of peptide with cell surface class II (McConnell et al., 1995; Beeson et al., 1996; Rabinowitz et al., 1997). Thus, APCs must have a pool of active, empty class II molecules on their surface that can be rapidly loaded with peptide to stimulate T cells. We have undertaken experiments aimed at understanding the source of this pool of empty, cell surface class II. In addition, these experiments were designed to investigate whether our reaction scheme for sEk could be generalized to cell surface I-Ek.

Our experimental approach was to manipulate cell surface I-Ek by preloading it with nonantigenic peptides that bind either transiently or stably, and thus might either enhance or inhibit subsequent binding of antigenic peptide. The binding of the antigenic peptide MCC was measured by the acid release of MCC-specific T cells. Preliminary experiments using live CH27 B lymphoma cells as APCs did not reveal any substantial effect of preloading the APCs with different peptides. This likely reflects both the rapid turnover of the I-Ek on the live CH27 cell surface and the difficulty of effectively

Figure 6. Reactions of sEk at pH 7.0

(A) Dissociation of a preformed peptide/sEk complex generates active sEk. Analogous to Figure 2B, starting material is 50 nM of the complex between sEk and CLIP M90L M98L. The filled circles indicate complex dissociation; the open circles, the association of 5 μM labeled MCC Ac95-103. Solid lines are a single-exponential fit to the plotted data. For comparison, 5 μM MCC Ac95-103 binding to empty sEk is shown as the open squares and dashed line.

(B) Peptide concentration dependence of sEk binding at pH 7.0. The indicated concentration of fluorescently labeled MCC Ac95-103 was reacted with 100 nM CLIP M90L/sEk complex at 37°C, and the binding after 20 min was compared to the binding of saturating concentrations of labeled MCC (100-300 μM).
loading I-Ek at neutral pH. To avoid these problems, we simplified the system by fixing the CH27 cells. Fixing the APCs resulted in an approximately 2-fold increase in the concentration of MCC peptide required to produce a given T cell response (data not shown). Such shifts in the T cell dose response likely reflect reciprocal changes in the amount of active I-Ek on the APC surface, based on the equation

\[ \frac{[p/Ek]}{[Eka]} = k_{on} \cdot t \cdot [p] \cdot [Eka] \]  

where \([p/Ek]\) is the number of peptide/I-Ek complex on the APC surface, \([p]\) the concentration of peptide in solution, and \([Eka]\) the number of active I-Ek molecules on the APC surface. This equation is valid when the product \(k_{on} \cdot t \cdot [p] \ll 1\) (i.e., when peptide binding does not begin to saturate Eka). In our T cell stimulation experiments, \(>3 \mu M\) peptide is added for 3 min at pH 7.0, satisfying these criteria (Figure 6B). Before preloading the fixed CH27 cells with peptide, we wanted to test whether leaving the APCs at pH 5.3, 37°C in the absence of peptide would affect their ability to present peptide antigen. In these experiments, we found that the T cell dose response to peptide presented by freshly fixed APCs was identical to that of the APCs left for 1-2 days in the absence of peptide (data not shown). This is consistent with the previous finding that a nearly stable level of class II peptide binding capacity is maintained when either purified MHC or fixed APCs are incubated in the absence of peptide (Jensen, 1990, 1991).

The first peptide preloading experiment with fixed APCs tested whether dissociation of peptide from the cell surface I-Ek results in active I-Ek. The design of the experiment was analogous to the experiment with purified sEk in Figures 2B and 6A, where the effect of preloading sEk with the mutated CLIP peptide M90L M98L was measured. The mutated CLIP peptide was used instead of wild-type CLIP, because the APCs are left in the absence of peptide at pH 7.0 during the setup of the experiment (approximately 40 min), and wild-type CLIP would have dissociated greatly during that time. As shown in Figure 7A, preloading of the fixed CH27 cells with mutated CLIP peptide results in an enhancement of the acid release response to MCC peptide. The data in Figure 7A are summarized in Figure 7B, which shows the acid release response versus the log of the MCC peptide concentration added. The plot shows that 10-fold less MCC is needed when presented by APCs pretreated with the mutated CLIP peptide versus those pretreated in the absence of peptide. Based on equation I, this implies that the mutated CLIP treated APCs have 10-fold more active I-Ek on their surface than the no peptide treated APCs (see Experimental Procedures). Therefore, dissociation of preformed peptide/MHC complex results in active, cell surface I-Ek. This may explain why some peptides that bind weakly to class II MHC augment T cell responses to other, unrelated peptides (Feng and Lai, 1994).

The stability of the active, cell surface I-Ek was investigated by allowing fixed CH27 cells preloaded with the mutated CLIP peptide to incubate in the absence of peptide at pH 5.3, 37°C for 1 hr. This treatment results in a decrease in the amount of active I-Ek on the cell surface as measured by the T cell activation dose response (Figure 7C and data not shown). In four independent experiments this decrease was always \(>3\)-fold, indicating that the half-life of the active, cell surface I-Ek must be \(<40\) min.

The experiments described above demonstrate that active I-Ek on the cell surface is unstable, but that incubating freshly fixed APCs in the absence of peptide does not markedly reduce the amount of active I-Ek on their surface. To investigate this possibility is reactivation of cell surface I-Ek does not occur, and that the steady level of active I-Ek on the APC surface is due solely to slow release of endogenous peptide. The final set of APC preloading experiments was designed to test these possibilities. The approach in these experiments was to reduce the amount of empty, inactive I-Ek on the cell surface and see whether this would affect the amount of active I-Ek present. If a substantial fraction of active I-Ek is formed by reactivation of inactive, empty molecules, then a decrease in active I-Ek, as measured by the T cell dose response, would be observed. In contrast, if all active I-Ek on the cell surface is formed by dissociation of endogenous peptides and inactive I-Ek does not reanimate, no effect of reducing the inactive pool would be observed.

The amount of inactive I-Ek on the cell surface was reduced by loading all empty I-Ek formed by dissociation of endogenous peptides with a peptide that binds stably to I-Ek, preventing it from forming the inactive, empty molecule. The rationally designed peptide used in these experiments, which we term binds Ek peptide (BEK), has a dissociation half-time \(>200\) hr from I-Ek (data not shown) but has no similarity to MCC at its T cell receptor contacts (see Experimental Procedures). It therefore should only affect MCC-specific T cell stimulation by MHC blockade. Pretreatment of fixed CH27 cells with the BEK peptide results in an approximately 3-fold reduction in the amount of active I-Ek on the cell surface as measured by the T cell dose response (data not shown). This reduction is maintained even if BEK is washed away 100 min prior to use of the APCs (Figure 7D). This delay is sufficient for most of the active I-Ek on the cell surface to inactivate, as the half-life of the active I-Ek molecule is \(<40\) min. Therefore, the BEK treatment cannot directly reduce the amount of active I-Ek by binding to it. Instead, it must reduce the amount of active I-Ek by decreasing the pool of inactive molecules that can reanimate. Thus, the steady level of active I-Ek on the surface of a fixed APC is maintained by a slow reactivation of inactive, empty I-Ek, as well as by dissociation of endogenous peptide/I-Ek complexes.

**Experimental Procedures**

**MHC Proteins and Peptides**

Soluble I-Ek was expressed as glycosylphosphatidylinositol-anchored molecules in Chinese hamster ovary cells, affinity purified as described (Wettstein et al., 1991; Reay et al., 1992), further purified by high performance size exclusion chromatography (HPSEC), and stored at 4°C prior to use. The duration of storage at 4°C (up to 1 year) did not affect the peptide binding kinetics (data not shown). Soluble DR1, expressed by S2 cell transformants, affinity purified
were washed and mixed with 5 C.C.T cells. The T cells response the presence or absence of a peptide that binds stably to I-Ek, BEK Peptides described above. Incubation of the APCs in the absence of peptide active I-Ek on the cell surface (summary of data presented in [A]). independent experiments. (B) Dissociation of a preformed peptide/MHC complex generates sured as described above. Similar results were obtained in three conditions, in the absence of BEK, and T cell stimulation was mea-

103 peptide was then measured based on increases in the rate of then washed and incubated for additional 100 min, under the same 

m absence of 200 $^{\text{C}}$ for 20 hr in either presence or (D) Inactive I-Ek on the cell surface slowly reverts to active I-Ek. Fixed CH27 cells were incubated at pH 5.3, 37 $^{\text{C}}$ for 30 hr in the presence of 200 $^{\text{M}}$ CLIP M90L M98L. The APCs were then washed and incubated for additional 60 min, under the same conditions, in the absence of BEK, and T cell stimulation was mea-

95-103 (AcIAYLKQATK); MCC 95-103 (IAYLKQATK); MCC Ac96-103 (AcAYLKQATK); iI85-99 “CLIP” (KPVSQMNSTMP LLRR); ii 85-99 M90L M98L “CLIP M90L M98L” (KPVSQMNRATPLLL R); HA 306-318 (PKYVKQNTKLAT); BEK (ANERADLISYTTQADK). Ac represents an acetyl group; the other letters are standard one letter abbreviations for the amino acids. The 8-mer peptide binds to I-Ek in the same register as full-length MCC, as it can stimulate cytokine production by MCC-specific T cells (J. R., unpublished results; Schwartz et al., 1985). The 8-mer peptide also likely binds in the groove of sDR1, as its binding to sDR1 is blocked by addition of unlaube HA (data not shown). The BEK peptide sequence was designed based on the I-Ek binding motif described by Reay et al. (1994). It is identical to MCC 88-103 except for mutations at the p2, p5, and p8 T cell receptor contacts (A96S, K99T, and T102D) and the p4 residue (L98T).

Peptide labeling was performed as follows: all peptides except MCC Ac95-103 and MCC Ac96-103 were labeled while on the resin by reaction of their free N termini with 5-(and 6-)carboxyfluorescein succinimidyl ester in DMSO with a catalytic amount of disopropyl-ethylamine. MCC Ac95-103 and MCC Ac96-103 were labeled at the C terminus as follows: in the standard FMCOC synthesis, an additional C-terminal amino acid was added, N- -Fmoc-N-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl-L-lysine (Novabiochem). This resulted in a resin-bound peptide with MCC lysine 103 fixed at the C terminus with an additional lysine protected with the -Dde functionality. With the peptide still on resin and the N terminus Fmoc protected, the -Dde group was selectively cleaved with 2% hydrazine in DMF, and carboxyfluorescein was coupled to the ω-amino group of the additional C-terminal lysine as described above.

Peptide-MHC Reaction Kinetics Labeled peptide and class II MHC were incubated together in PBS (150 mM sodium chloride, 10 mM sodium phosphate, and 0.02% sodium azide) at pH 7.0, or PBS containing 100 mM sodium citrate at pH 5.3. Complex association was measured by taking an aliquot from the peptide-MHC mixture and separating free peptide from peptide-MHC complex by passage down a spin column (small scale Sephadex G50-SF size exclusion column pretreated with 10 mg/mL BSA solution for >10 hr to minimize nonspecific binding to the column; similar results were also obtained with spin columns blocked with reconstituted nonfat dried milk) at 4 $^{\text{C}}$, pH 7.0. The eluted peptide/MHC complex was then further purified by HPSEC.

Data points represent the percent increase in acid release rate 12 min after addition of MCC peptide. The line indicates a log-linear fit to the data. The label “12.6-fold” indicates that a 12.6-fold greater concentration of peptide was required to produce a given level of acid release using the no peptide treated APCs as compared to the mutated CLIP treated APCs. Similar results were obtained in three independent experiments.

(C) Formation of active I-Ek by peptide/MHC complex dissociation is followed by rapid I-Ek inactivation. Fixed CH27 cells were incubated at pH 5.3, 37 $^{\text{C}}$ for 20 hr in the presence of 200 $^{\text{M}}$ CLIP M90L M98L. The APCs were then washed and incubated for additional 60 min, under the same conditions, in either the presence or absence of 200 $^{\text{M}}$ of this peptide, and T cell stimulation was measured as described above. Incubation of the APCs in the absence of peptide for 60 min resulted in a 3-fold shift in the T cell dose response in four independent experiments.

(D) Inactive I-Ek on the cell surface slowly reverts to active I-Ek. Fixed CH27 cells were incubated at pH 5.3, 37 $^{\text{C}}$ for 30 hr in either the presence or absence of a peptide that binds stably to I-Ek, BEK (for details on BEK, see Experimental Procedures). The APCs were then washed and incubated for additional 100 min, under the same conditions, in the absence of BEK, and T cell stimulation was measured as described above. Similar results were obtained in three independent experiments.

Figure 7. T Cell Stimulation by Fixed APCs Preloaded with Various Peptides

(A) Enhanced T cell acid release by APCs preloaded with a peptide that rapidly dissociates from I-Ek, CLIP M90L M98L. Fixed CH27 cells were incubated at pH 5.3, 37 $^{\text{C}}$ for 20 hr in either presence or absence of 200 $^{\text{M}}$ of this CLIP mutant. After this time, the APCs were incubated at pH 5.3, 37 $^{\text{C}}$ for 30 hr in either presence or (B) Formation of active I-Ek by peptide/MHC complex dissociation is followed by rapid I-Ek inactivation. Fixed CH27 cells were incubated at pH 5.3, 37 $^{\text{C}}$ for 20 hr in the presence of 200 $^{\text{M}}$ CLIP M90L M98L. The APCs were then washed and incubated for additional 60 min, under the same conditions, in either the presence or absence of 200 $^{\text{M}}$ of this peptide, and T cell stimulation was measured as described above. Incubation of the APCs in the absence of peptide for 60 min resulted in a 3-fold shift in the T cell dose response in four independent experiments. (D) Inactive I-Ek on the cell surface slowly reverts to active I-Ek. Fixed CH27 cells were incubated at pH 5.3, 37 $^{\text{C}}$ for 30 hr in either the presence or absence of a peptide that binds stably to I-Ek, BEK (for details on BEK, see Experimental Procedures). The APCs were then washed and incubated for additional 100 min, under the same conditions, in the absence of BEK, and T cell stimulation was measured as described above. Similar results were obtained in three independent experiments.

Immunity

706
using a 7.5 mm x 60 cm TSK G3000SW column (Toso Haas, Montgomeryville, PA) with a running buffer of pH 7.0 PBS at 25°C. The flow rate was 1 ml/min. Peptide/MHC complex eluted at 16 min for sEk complexes and 18 min for sDR1 complexes. The HPSEC column was connected to a fluorescence detector and a UV detector in series. Concentrations of fluorescent peptide/MHC complex were determined by comparison of the peak height of the complex to the peak height of known concentrations of carboxyfluorescein. For Figures 3B and 4, the aliquot of labeled peptide and MHC was injected directly onto the HPSEC without initial purification on the spin column. This was possible because of the low labeled peptide concentrations used in these experiments.

In many experiments, empty sDR1 or sEk was preloaded with an unlabeled peptide prior to adding the labeled peptide. Preloading was for 36 hr at pH 5.3, 37°C with 1.2 μM MHC and 200 μM peptide (sEk) or 1 nM peptide (sDR1). Following this loading reaction, the reaction mixture was stored at pH 5.3, 4°C until immediately prior to use, when free peptide was separated from peptide/MHC complex by passage down a spin column at 4°C, pH 7.0 to isolate the MHC for the subsequent association reaction with labeled peptide.

For measurements of peptide/MHC complex dissociation, labeled complex was isolated from a mixture of free peptide and MHC using a spin column as described above. After complex isolation (final complex concentration = 100 nM MHC), unlabeled peptide (MCC 88-103 or MCC Ac95-103 for sEk, HA 306-318 for sDR1) was added to 10 μM final concentration to prevent rebinding of labeled peptide, citrate was added for dissociation reactions at pH 5.3, and the complex was placed at 37°C. After the indicated time, complex was injected on the HPSEC apparatus, and the amount of labeled peptide/MHC complex remaining was measured based on the height of the fluorescence signal.

Inactivation Rate Constants
The inactivation rate of active sEk and sDR1 was computed using the data shown in Figures 3A and 5C. For these computations, we assume the validity of reaction scheme (4) shown below.

\[
p/M \xrightarrow{k_{off}} M_{a} \xrightarrow{k_{in}} M_{i} \quad \text{(4)}
\]

\(p/M\) is a preformed peptide/MHC complex with dissociation rate \(k_{off}\); \(M_{a}\) and \(M_{i}\) are active and inactive class II (either sDR1 or sEk); \(k_{in}\) is the rate of inactivation of \(M_{a}\); \(k_{on}\) is the rate of formation of \(M_{a}\) from \(M_{i}\). This scheme is identical to scheme (3) except that we assume no free peptide is present. The differential equations corresponding to scheme (4) are the following:

\[
\frac{dp[M]}{dt} = -k_{off}[p[M]] \quad \text{(Ila)}
\]

\[
\frac{d[m][M]}{dt} = k_{off}[p[M]] - k_{on}[M_{a}] + k_{in}[M_{i}] \quad \text{(Iib)}
\]

\[
\frac{d[M][i]}{dt} = -k_{on}[M_{a}] - k_{in}[M_{i}] \quad \text{(Iic)}
\]

This system of equations can be simplified if the formation of \(M_{a}\) from \(M_{i}\) is assumed to be either (i) low relative to the formation of \(M_{a}\) from \(p/M\) (which is true for sEk; Figure 3A and additional data not shown) or (ii) approximately constant with respect to time (which is true for sDR1, Figure 5 and additional data not shown). Based on these assumptions, equations Ila and Iib can be solved analytically for \([M_{a}]\) as a function of time with \(k_{on} = 0\) to give the equation

\[
[M_{a}(t)] = \frac{k_{on}[M_{i}][k_{on} - k_{off}] \exp(-k_{off}t) + C}{k_{off} - k_{on}} \quad \text{(III)}
\]

where \(C\) is a constant added to represent the plateau binding level after long delay times (C = 0.2 for 8-mer/Ek, Figure 3A; C = 0.4 for 8-mer/DR1, Figure 5C). \(k_{on}\) can be directly measured based on the data shown in 2C and 5B (\(k_{off} = 0.14\) min⁻¹ for 8-mer/Ek; \(k_{in} = 0.033\) min⁻¹ for 8-mer/DR1). \(k_{on}\) was calculated by obtaining the best fit to equation (III). For the fit, the first term in equation (III) was multiplied by an arbitrary constant to account for the binding units, and \(t\) was taken as the delay time plus the 5 min binding time used to measure \([M_{a}]\), as dissociation of \(M/p\) continues during the binding reaction.

Association Rate Constants
The association rate of peptide to active sEk was calculated by fitting the data in Figure 4 to the equation

\[
[p/M](t) = \frac{[p/M]_{max} (1 - \exp(-k_{on}[p][t])}{t} \quad \text{(V)}
\]

t = 3 min and the peptide concentration variable. Analogous experiments performed with [p] = 50 nM and data points taken at 1, 2, and 3 min yielded identical association rates.

The association rate of peptide to active sDR1 was approximated based on the minimal concentration of HA required to fill most sDR1a formed by 8-mer dissociation (100 nM HA, Figure 4B). As shown in scheme (5) below, sDR1a formed by 8-mer/DR1 complex (8/DR1) dissociation can react to form sDR1i with the rate constant \(k_{on}\).

\[
8/DR1 \xrightarrow{k_{on}} DR1a \xrightarrow{8} HA/DR1 \quad \text{(5)}
\]

Alternatively, sDR1a can react with HA to form HA/sDR1 with the rate constant \(k_{on}\). As shown in Figure 5B, 100 nM HA is sufficient for slightly more than half of the sDR1a to bind HA rather than inactivate. Thus \(k_{on} = 0.14\) min⁻¹ known from Figure 5C.

\[
8/DR1 \xrightarrow{k_{on}} DR1a \xrightarrow{HA} HA/DR1 \quad \text{(5)}
\]

Amount of Active I-Ek on the Surface of an APC
Estimation of the amount of active I-Ek on the surface of an APC is based on T cell acid release measurements with 5C.C7 T cells and CH27 APCs performed as previously described (Rabinowitz et al., 1996). When indicated in the text, CH27 cells were fixed with 0.1% glutaraldehyde in PBS (pH 7.4), 37°C for 2 min, followed by immediate addition of a 2-fold volume excess of DMEM 0.2 M L-lysine. The fixed CH27 cells were then washed once in RPMI medium 1640 with 10% fetal calf serum prior to use.

Similar to previous work (Beeson et al., 1996), we observe that the acid release response to peptide over a broad range of peptide concentrations can be related to the log of the peptide concentration added (Figure 7). This suggests that the number of peptide/I-Ek complexes per APC (Ep/Ek) and the T cell acid release response (A) can be related by the following equation:

\[
A[Ep/Ek] = C_{1} + C_{2} \log [Ep/Ek] \quad \text{(IV)}
\]

where the constants \(C_{1}\) and \(C_{2}\) depend on the peptide, the T cells, and the APCs, but not on the APC preloading.

As described in the Appendix, we also find that for 100 nM ≤ [MCC] ≤ 3 μM,

\[
[p/Ek] = k_{on} \cdot t \cdot [p] \cdot [Ek] \quad \text{(I)}
\]

Combining the above, we get

\[
A[Ep/Ek] = A[k_{on} \cdot t \cdot [p] \cdot [Ek]]
\]

\[
= C_{1} + C_{2} \log [k_{on} \cdot t \cdot [p] \cdot [Ek]]
\]

\[
= C_{1} + C_{2} \log [k_{on} \cdot t \cdot [p] + C_{1} \log [Ek] + C_{2} \log [p/p_{p}] + C_{1} \log [p/p_{p}] + C_{2} \log [p/p_{p}]]
\]

where \(p_{p}\) is an arbitrary, fixed peptide concentration (1 μM) needed for each of the terms under the logarithm function to be dimensionless. For a fixed APC pretreatment (i.e., fixed [Ek]), this implies that the T cell acid release response (A) is related to the concentration of peptide added by the equation:

\[
A[p] = C_{1} + C_{1} + C_{2} \log [p/p_{p}] + C_{1} \log [p/p_{p}] + C_{1} \log [Ek] + C_{2} \log [p/p_{p}] + C_{1} \log [p/p_{p}] + C_{2} \log [p/p_{p}]
\]

The lines in Figures 7B, 7C, and 7D indicate the best fit to the experimental data using equation (V). Note that the X intercept (i.e.,
\[ \frac{[p]}{[\ell]} = \exp \left( \frac{C_i - C_{ii}}{C_j} \right) = \frac{[Eka]}{[Eka]} \]  

(VII)

Thus, the ratio of the X intercepts of the dose-response plots shown in Figures 7B, 7C, and 7D is inversely proportional to the ratio of the amount of Eka on the surface of the APCs. These comparisons are valid only for experiments done simultaneously (i.e., within a single panel of Figure 7) because of small differences in the condition of the T cells that affect their dose response (i.e., values of \( C_i \) and \( C_j \)) from day to day.

Acknowledgments

We thank Betsy Mellins and Denis Zaller for providing the recombinant DR1, and Alex Cao and Patricia Jones for help growing the SC.C7 T cells. We also thank Tom Andersen, Lutz Schmitt, and Betsy Mellins for many helpful discussions. Grant support was provided by the National Institutes of Health (H. M. M.) and the Howard Hughes Medical Institute (M. M. D.). J. R. was supported by the Medical Scientist Training Program.

Received August 27, 1998; revised October 7, 1998.

References


Isomers of Empty Class II MHC


