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Invariant chain peptides enhancing or inhibiting the presentation of antigenic peptides by major histocompatibility complex class II molecules

Two soluble invariant chain (Ii) peptides with overlapping sequences had contrasting effects on the presentation of antigenic peptides by murine A^d, A^k, E^d, and E^k major histocompatibility complex (MHC) class II molecules. Naturally produced class II-associated invariant chain peptides human (h)Ii81–104/murine (m)Ii80–103 inhibited antigen presentation on these MHC class II alleles in a manner consistent with competitive inhibition. The Ii-4 peptides hIi77–92/mIi76–91 enhanced presentation of antigenic peptides on I-E class II alleles by promoting the exchange of peptides at the cell surface. Treatment of antigen-presenting cells (APC) with Ii-4 before the addition of antigenic peptide greatly enhanced subsequent T cell responses, while treatment of APC with Ii-4 after antigenic peptide binding decreased subsequent T cell responses. The hIi81–104 and mIi80–103 peptides inhibited T cell responses in both types of assays. The binding of biotinylated antigenic peptide to MHC class II-transfected L cells, as measured by flow cytometry, was inhibited by mIi80–103 and enhanced by mIi-4. Segments of Ii fragments remaining associated with MHC class II, or released Ii peptides, appear to regulate the formation of stable antigenic peptide/MHC class II complexes either positively or negatively through interactions at or near the antigenic peptide binding site. These findings open a pathway for the design of novel therapeutics based on the structure and function of natural and rationally designed fragments of Ii.

1 Introduction

The invariant chain (Ii), or peptides derived from it, regulate the formation, trafficking, and antigen-presenting functions of MHC class II α,β complexes in several ways. Ii promotes efficient assembly of the MHC class II α,β heterodimers [1] and induces folding of MHC class II molecules into a conformation which subsequently leads to high-affinity antigenic peptide binding [2–4]. Ii also permits egress of MHC class II complexes from the endoplasmic reticulum (ER) [5, 6] and directs the intracellular transport of MHC class II molecules to a post-Golgi compartment [7–10], where both proteolysis of Ii and peptide charging occur [11, 12]. Prior to that point, Ii blocks the antigenic peptide-binding site on MHC class II molecules [13, 14]. Ii regulates presentation of endogenous antigens [15, 16] and its presence has a selective effect on the repertoire of antigenic determinants which are presented to T cells [17]. Antigenic peptide binding to purified MHC class II molecules is more efficient when the antigenic peptide is present during the cleavage and release of Ii from the MHC class II molecules by cathepsin B than when the antigenic peptide is added after the proteolytic release of Ii [18]. Analysis of human (h)Ii mutants, in which putative cathepsin B cleavage sites about hIi R^{78}, K^{80}, K^{82}, and K^{85} were altered, indicates that cleavages of Ii in that region are crucial for generating p6, which is the final [^{35}S] methionine-labeled Ii fragment coprecipitating with MHC class II α,β chains during cathepsin B cleavage of Ii [19, 20]. To better understand the role that Ii segments within the 76–103 region of murine (m)Ii play in the peptide-charging event, we have examined the biological activities of two overlapping Ii peptides, hIi81–104/mIi80–103 and hIi77–92/mIi76–91 (Ii-4), on the presentation of antigenic peptides by MHC class II molecules.

The class II-associated invariant chain (CLIP) series of Ii peptides around h Ii80–106 [21–25] and m Ii80–101 [26, 27] have been acid-eluted from immunopurified MHC class II molecules. The nested series of peptides differ by the loss of N- and C-terminal amino acids and were derived from a region with a high degree of homology in murine and human Ii. The hIi81–104 peptide inhibits antigenic peptide binding to purified HLA-DR1 [21] and HLA-DR3 [24] MHC class II molecules. Surface expression of an N-terminally truncated form of Ii, with the CLIP region intact, is associated with inhibited peptide presentation by HLA-DR molecules [28], and CLIP region-derived peptides accumulate in cells exhibiting a deficiency in HLA-DM [29, 30]. In addition, the mIi85–101 peptide has high affinity for A^d but low affinity for E^d [26], demonstrating that this region of Ii has some apparent allelic selectivity in its binding to murine MHC class II molecules. Allelic selectivity in CLIP-region peptide binding to human MHC class II molecules has also been demonstrated. It has been shown that hCLIP-region peptides dissociate at different rates from HLA-DR3, HLA-DR4, and HLA-DR11 [31], and that HLA-DR1, HLA-DQ1, and HLA-DQ8 differ in CLIP-region peptide binding capacity [25].

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Abbreviations: **CLIP:** Class II-associated invariant chain peptide **h:** Human **HEL:** Hen egg lysozyme **Ii:** Invariant chain **m:** Murine **PGCC:** Pigeon cytochrome *c* **THMCC:** Tobacco hornworm moth cytochrome *c*

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The hIi-4 peptide Ii77–92, (mIi76–91), which has not been found naturally in association with MHC class II molecules, was synthesized to evaluate the possible roles of this Ii epitope in MHC class II antigen processing and presentation [32]. The hIi-4 sequence contains four spaced prolines, six positive and no negative charges, and is a palindrome allowing for chemical equivalence of side chains. By molecular modeling with Quanta, the Ii-4 peptide with the four prolines in *trans* configuration forms a polyprolyl type-II helix with axial alignment of the positive charges. The Ii-4 peptide induced a potent antiserum in each of two rabbits, reacting at 1:256 in ELISA to the immunizing peptide [33]. However, the antisera did not immunoprecipitate MHC class II α,β heterodimers, or free or complexed Ii chains. These findings led to the hypothesis that the Ii-4 peptide sequence was probably not positioned externally on MHC class II α,β-Ii trimers but instead turns inward to face the MHC class II α,β chains. Since the C-terminal sequence of Ii-4 overlaps the N terminus of hIi81–104 and mIi80–103 by 12 amino acids, the Ii-4 peptide was thought to be a useful tool, along with hIi81–104 and mIi80–103, for determining which amino acid sequences mediate binding of the mIi76–103 fragment to murine MHC class II molecules.

2 Materials and methods

2.1 Peptides

Peptides were synthesized using the solid-phase FMOC/o-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) method on a peptide synthesizer (430; Applied Biosystems, Foster City, CA) by Michael Berne at the Protein Chemistry Facility of Tufts Medical School, Boston, MA. For biotinylation, aliquots of resin-bound free N-terminal peptides were incubated overnight at room temperature with a 40-fold molar excess of biotin (Sigma, St. Louis, MO), which was activated with HBTU, in 50 % DMF/DMSO. Following coupling, the slurry was washed with DMF/DMSO, and the biotinylated peptides were cleaved and purified by reverse-phase high-pressure liquid chromatography to homogeneity. The following peptides were used in this study: HEL11–25, hen egg lysozyme 11–25, AMKRHGLDNYRGYSL; HEL 46–61, hen egg lysozyme 46–61, NTDGSTDYGILQINSR; HEL 106–116, hen egg lysozyme 106–116, NAWVAWRNRCK; PGCC 81–104, pigeon cytochrome *c* 81–104, IFAGIKKKAERADLIAYLKQATAK; THMCC 82–103, tobacco hornworm moth cytochrome *c* 82–103, FAGLKKANERADLIAYLKQATK; PH-1.0, prototypic helical peptide 1.0, LYQELQKLTQTLK; mIi 80–103, LPKSAKPVSQMRMATPLLMRPMSM; hIi81–104, LPKPPKPVSKMRMATPLLMQALPM; mIi-4, mIi76–91, YRMKLPKSAKPVSQMR; and hIi-4, hIi77–92, YRMKLPKPPKPVSKMR. The sequences of the synthetic mIi-4 and hIi-4 peptides have respective $L^{76}\rightarrow Y$ and $L^{77}\rightarrow Y$ substitutions. Each peptide was dissolved in PBS pH 7.2; the solutions were re-sterilized by filtration, and the peptide concentration and amino acid sequence integrity of each solution were determined by amino acid analysis (420A/130A derivatizer/HPLC after hydrolysis with 6 N HCl for 24 h *in vacuo*; Applied Biosystems at the Protein Chemistry Facility of Tufts Medical School, Boston, MA.

2.2 Cell lines

The TPc9.1 hybridoma specific for pigeon cytochrome *c* 81–104 on E^k was a gift from Dr. Susan Pierce (Northwestern University, Evanston, IL). The TPc9.1 hybridoma responds heteroclitically to tobacco hornworm moth cytochrome *c* 82–103 on E^k. The 3A9 T hybridoma specific for hen egg lysozyme 46–61 on A^k was a gift from Dr. Paul Allen (Washington University School of Medicine, St. Louis, MO). The 9.30.B2 and G28.C9 T hybridomas were gifts from Dr. Eli Sercarz (University of California, Los Angeles, CA). The 9.30.B2 hybridoma is specific for hen egg lysozyme 11–25 on A^d, and the G28.C9 hybridoma is specific for hen egg lysozyme 106–116 on E^d. The A20 and CH27 B cell lymphoma lines (H-2^d and H-2^k, respectively) were from the ATCC. The following L cell lines, each stably transfected with a single murine MHC class II allele [34–36], were gifts from Dr. Ronald Germain (NIH): RT2.3.3H-D6 (A^d), RT7.3H3 B4.5 (A^k), RT10.3B-C1 (E^d), and DCEK Hi7 (E^k).

2.3 Measurement of antigenic peptide-specific T cell activation

Fixed CH27 (A^kE^k) or A20 (A^dE^d) APC were generated by treating 1×10^6 cells/ml for 5 min with 0.5 % paraformaldehyde (Sigma) in PBS pH 7.2, followed by two washes with four volumes of DMEM-10 % FCS/10 mM Hepes. Unfixed (or live) mitomycin C-treated CH27 or A20 APC were generated by incubating 5×10^6 cells/ml for 20 min at 37 °C with 0.025 mg/ml of mitomycin C (Sigma) in DMEM/10 mM Hepes, followed by two washes with four volumes of DMEM-5 % FCS/10 mM Hepes. T cell hybridomas were irradiated (2200 rad) before each assay.

For simultaneous competition assays, 5×10^4 fixed or live APC, 5×10^4 T hybridoma cells and a submaximal concentration of antigenic peptide were cultured with and without serial fourfold dilutions of each effector (Ii) peptide from 64 μM to 1 μM at pH 7.2–7.4, in complete DMEM-5 % FCS, 10 mM Hepes, 1 × nonessential amino acids (Sigma), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 5×10^{-5} M 2-ME. Wells containing only T+APC were included to monitor background T cell activation, and wells containing T+APC+Ii peptide were included to monitor nonspecific T cell activation by each Ii peptide. Co-culture supernatants (75 μl) were removed after 24 h and were assayed for their ability to maintain the growth of 1×10^4 IL-2/IL-4-dependent HT-2 cells (added in 75 μl complete DMEM-5 % FCS), as measured by incorporation of [^{3}H] thymidine, added at 1 μCi/well during the last 5 h of a 24-h HT-2 assay.

Simultaneous competition assays designed to test for competitive *vs.* noncompetitive inhibition were carried out under the same conditions as described above with the following modifications. T cells and fixed APC were co-cultured with eight serial twofold dilutions of HEL11–25 (5 μM – 0.039 μM) for A^d; and seven serial twofold dilutions of PGCC81–104 (24 μM – 0.375 μM) for E^k; without and with 1, 4, 16 and 64 μM of mIi80 – 103 for A^d, and 16 and 64 μM PH-1.0 for E^k.

Antigenic peptide prepulse assays were carried out under essentially the same conditions as described for simultaneous competition with the following modifications. Fixed APC were first incubated for 6 h at 1×10^6 cells/ml in complete DMEM-5 % FCS in 24-well plates (1 ml/well) with antigenic peptide, followed by four washes with ten volumes of DMEM-5 % FCS. A single dose of 64 μM of each Ii peptide was used. The baseline T cell response was measured by culturing T hybridoma cells with the antigenic peptide-prepulsed APC in the absence of Ii peptides.

Effector (Ii) peptide prepulse assays were carried out in the A^d and E^d systems as described for simultaneous competition assays with the following modifications. Fixed APC were first incubated for 6 h at 0.5×10^6 cells/well in 0.5 ml complete DMEM-5 % FCS with 64 μM of each Ii peptide or with PBS. The APC were then washed four times with ten volumes of DMEM-5 % FCS, and were cocultured with T hybridoma cells and same submaximal doses of antigenic peptides used in simultaneous competition assays. The baseline T cell response was measured by culturing T hybridoma cells with antigenic peptide and PBS-pretreated APC.

2.4 Biotinylated peptide binding to MHC class II-positive L cells

The relative affinity of mIi80–103 and mIi-4 for A^d, A^k, E^d, and E^k was determined using biotinylated forms of each peptide and MHC class II-positive L cell lines using basic techniques described elsewhere [37], with the following modifications. Unfixed L cells (in DMEM-5 % FCS) were incubated for 16 h at 37 °C with PBS or with 1, 4, 16 and 64 μM of biotinylated mIi80–103 or mIi-4 (prepared in PBS). Following incubation and washing, the L cells were fixed with 0.5 % paraformaldehyde-PBS for 5 min, followed by washing. Surface-bound biotinylated peptides were detected by incubating the L cells with 1 μg per 1×10^6 cells of streptavidin-R-phycoerythrin (Life Technologies, Gaithersburg, MD) in 200 μl of PBS/2 % FCS/0.2 % sodium azide (FACS buffer) for 1.5 h on ice, followed by washing. The L cells were then fixed with 1 % paraformaldehyde-PBS for 1 h, followed by washing. The L cells were subjected to standard cytofluorometry using a FACS scanner (Becton Dickinson, Raritan, NJ). MHC class II-negative cells were used to monitor for nonspecific biotinylated peptide binding. The data presented are the mean fluorescence channel shift (log scale) of 5000 events from the biotinylated peptide-treated cells minus the mean fluorescence channel shift (log scale) of 5000 events from the PBS sham-treated control. Fluorescence was measured on a 0–1000 scale.

Comparison of biotinylated peptide binding capacity of the four murine MHC class II alleles was done following normalization of MHC class II expression levels among the four different L cell lines at the time of biotinylated peptide binding. For class II staining, L cells were incubated for 20 min on ice with TIB-120 for A^d, E^d, and Ek (rat IgG_{2b}, ATCC); and TIB-92 for A^k (mouse IgG_{2a}, ATCC), followed by washing and incubation for 20 min on ice with biotinylated rabbit anti-rat IgG (Pierce, Rockford, IL) and biotinylated rat anti-mouse IgG (Pierce), respectively, followed by PE-avidin staining.

mIi80–103 and mIi-4 effects on biotinylated antigenic peptide binding to the A^d- and the E^d-positive L cell lines were determined as described above, with the following modifications. mIi80–103 inhibition was determined by incubating A^d-positive L cells with 100, 40, and 10 μM of biotinylated HEL11–25 antigenic peptide without and with serial twofold dilutions of mIi80–103 (20 to 0.625 μM). mIi-4 enhancement was determined by incubating E^d-positive L cells with 10 μM and 20 μM of biotinylated HEL106–116 antigenic peptide without and with the mIi-4 peptide at 20, 40, and 80 μM. Sham-treated cells were incubated with PBS only. Surface-bound biotinylated peptide was detected by PE-avidin staining.

PH-1.0 peptide and THMCC82–103 antigenic peptide effects on biotinylated mIi80–103 and mIi-4 binding to E^k-positive L cells were determined as described above, with the following modifications. The L cells were incubated with 16 μM of the biotinylated mIi80–103 and mIi-4 peptides without and with 4, 16, and 64 μM of PH-1.0 and THMCC82–103. Sham-treated cells were incubated with PBS only. Surface-bound biotinylated peptide was detected by PE-avidin staining.

The relative dissociation rates of biotinylated mIi80–103, mIi-4, and HEL11–25 from A^d-positive L cells and of biotinylated mIi80–103, mIi-4, and HEL106–116 from E^d-positive L cells were determined as described above, with the following modifications. L cells were incubated with 40 μM of each biotinylated peptide, in duplicate, or with PBS for the sham control. The L cells were then washed, resuspended in DMEM-5 % FCS, and were re-incubated at 37 °C for the following time points: 0, 1, 2, 6, 12 and 24 h after peptide washout. Surface-bound peptides were detected by PE-avidin staining. The data presented represent the average mean fluorescence channel shift (log scale) of the duplicate samples minus the mean fluorescence channel shift (log scale) of the sham control.

3 Results

3.1 Competitive inhibition of antigenic peptide presentation to T cells by the hIi81–104 and mIi80–103 peptides

The effects of the hIi81–104 and mIi80–103 peptides were determined on the presentation of antigenic peptides by murine MHC class II alleles A^d, A^k, E^d, and E^k to respective, restricted T cell hybridomas. The hIi81–104 or mIi80–103 peptides inhibited antigenic peptide presentation in the A^d and A^k systems with paraformaldehyde-fixed APC, and inhibited in all four allelic systems with mitomycin C-treated APC (Table 1). The PH-1.0 peptide also inhibited the T cell response to antigenic peptides presented by E^k MHC class II molecules. When serial dilutions of the mIi80–103 peptide were tested against serial dilutions of the HEL11–25 antigenic peptide in the A^d allelic system (Fig. 1A), the dose-response curves at individual concentrations of mIi80–103 were parallel, had similar slopes, and joined at a common value at high concentrations of HEL11–25. Such a pattern is consistent with that produced by a competitive inhibitor, for which increasing concentrations of ligand (antigenic peptide) overcome inhibition (by the mIi80–103 peptide) [38, 39]. A similar pattern consist-

Table 1. Potencies of inhibitor peptides

Peptide	Allele	IC_{50} a)	Peptide Ratio b)	Antigen System c)
Fixed APC Assays				
PH-1.0	Ek	18 μM	6	PGCC81-104, 3 μM
	Ek	7 μM	47	THMCC 82-103, 0.15 μM
mIi80-103	Ad	0.3 μM	1	HEL 11-25, 0.3 μM
	Ak	9 μM	180	HEL 46-61, 0.05 μM
	Ek	>64 μM	(>20)	PGCC 81-104
hIi81-104	Ad	0.3 μM	1	HEL 11-25
	Ak	10 μM	200	HEL 46-61
Live APC Assays				
PH-1.0	Ek	6 μM	15	PGCC 81-104, 0.4 μM
	Ek	9 μM	120	THMCC 82-103, 0.075 μM
mIi80-103	Ad	2 μM	40	HEL 11-25, 0.05 μM
	Ak	30 μM	600	HEL 46-61, 0.05 μM
	Ed	48 μM	120	HEL 106-116, 0.4 μM
	Ek	19 μM	48	PGCC 81-104
	Ek	15 μM	200	THMCC 82-103
hIi81-104	Ad	2 μM	40	HEL 11-25
	Ak	32 μM	640	HEL 46-61
	Ed	>64 μM	(>160)	HEL 106-116
	Ek	8 μM	20	PGCC 81-104
	Ek	10 μM	133	THMCC 82-103

a) The inhibitor peptide concentration (μM) required for 50 % inhibition of the T cell response to antigenic peptide (a 50 % response).
b) The ratio of [inhibitor peptide (μM)/ antigenic peptide (μM)] at the point of IC50.
c) The antigenic peptide concentration used in the T cell functional assays.

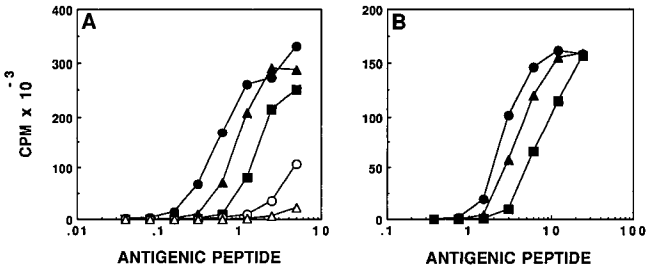

Figure 1. Simultaneous competition assays using serial dilutions of the antigenic peptide (μM) and different concentrations of Ii effector peptide revealed competitive inhibition kinetics. (A) mIi80–103 in the HEL11–25/A^d system: 0 μM mIi80–103 (solid circle), 1 μM mIi80–103 (solid triangle), 4 μM mIi80–103 (solid square), 16 μM mIi80–103 (open circle), 64 μM mIi80–103 (open triangle). (B) PH-1.0 in the PGCC81–104/E^k system: 0 μM PH 1.0 (solid circle), 16 μM PH-1.0 (solid triangle), 64 μM PH-1.0 (solid square). The data shown are each representative of two independent experiments which yielded similar results. Each data point is the mean of triplicate wells with a mean SEM of less than ± 10 %.

ent with competitive inhibition was obtained with PH-1.0 tested against serial dilutions of the PGCC81–104 antigenic peptide (Fig. 1B).

3.2 Enhancement of antigenic peptide presentation by the hIi-4 and mIi-4 peptides

The murine and human Ii-4 peptides enhanced T cell responses in the E^d and E^k systems as much as 12 times when the effector peptide was added simultaneously with limiting concentrations of the antigenic peptide (Table 2). The PH-1.0 peptide also enhanced T cell responses in the A^d and E^d systems. The Ii-4 peptides did not enhance T cell responses in the A^d/HEL11–25 and the A^k/HEL46–61 systems. None of the peptides altered T cell responses to the anti-CD3 mAb 2C11 in the presence of APC and the enhancing peptides did not stimulate the T cell hybridomas in the absence of antigenic peptide (data not shown).

Table 2. Potencies of enhancer peptides

Peptide	Allele	EC200 a)	Peptide Ratio (200) b)	Antigen System c)
Fixed APC Assays				
mIi-4	Ed	<1 μM	(<0.7)	HEL 106-116, 1.5 μM
	Ek	19 μM	6	PGCC 81-104, 3 μM
	Ek	16 μM	107	THMCC 82-103, 0.15 μM
hIi-4	Ed	2 μM	1	HEL 106-116
	Ek	>64 μM	(>20)	PGCC 81-104
	Ek	19 μM	127	THMCC 82-103
PH-1.0	Ad	36 μM	120	HEL 11-25, 0.3 μM
	Ed	48 μM	32	HEL 106-116
Live APC Assays				
mIi-4	Ed	2 μM	5	HEL 106-116, 0.4 μM
	Ek	60 μM	150	PGCC 81-104, 0.4 μM
	Ek	33 μM	440	THMCC 82-103, 0.075 μM
hIi-4	Ed	62 μM	155	HEL 106-116
	Ek	>64 μM	(>160)	PGCC 81-104
	Ek	>64 μM	(>853)	THMCC 82-103
PH-1.0	Ad	>64 μM	(>1280)	HEL 11-25, 0.05 μM
	Ed	>64 μM	(>160)	HEL 106-116

a) The enhancer peptide concentration (μM) required to double the baseline T cell response to antigenic peptide (a 200 % response).
b) The ratio of [enhancer peptide (μM)/antigenic peptide (μM)] at the point of EC200.
c) The antigenic peptide concentration used in the T cell functional assays.

3.3 hIi-4/mIi-4 and hIi81–103/mIi80–103 inhibition of the presentation of preformed antigenic peptide/MHC class II complexes

APC were incubated with different sub-saturating concentrations of antigenic peptide to form peptide/MHC class II molecule complexes. Unbound antigenic peptide was removed by washing and the APC were cultured with T hybridoma cells and 64 μM of each Ii peptide (Table 3). Both hIi-4 and mIi-4 peptide then inhibited T cell responses to E^d/HEL106–116 and E^k/PGCC81–104 complexes. The T cell response to APC prepulsed with 0.75 μM of HEL 106–116 was reduced from 100 % to 37 % by 64 μM of mIi-4. Similarly, the T cell response to APC prepulsed with 10 μM PGCC81–104 was reduced from 100 % to 17 % by 64 μM of hIi-4. In the E^k antigen systems, inhibition by Ii-4 depended on the concentration of the antigenic peptide during prepulse (17 % response with 10 μM PGCC81–104 *vs.* 39 % response with 20 μM PGCC81–104) and on the affinity of the antigenic peptide for E^k. That is, inhibition was seen with the lower-affinity PGCC81–104 peptide, but not seen with the higher-affinity THMCC82–103 peptide [40], even though the THMCC82–103 peptide concentration was 50 times less.

Similarly, hIi81–104 and mIi80–103 inhibition of T cell responses to preformed complexes of antigenic peptide was seen in the A^d, A^k, E^d, and E^k systems. For example, in the A^k/HEL46–61 system, 64 μM hIi81–104 reduced the T cell responses to antigenic peptide-prepulsed APC from 100 % to 24 % and 37 %; and in the Ek/PGCC81–104 system, 64 μM of hIi81–104 reduced the T cell responses to 55 % and 42 %.

3.4 hIi81–104/mIi80–103 pretreatment decreases, and hIi-4/mIi-4 pretreatment enhances presentation of antigenic peptides

APC of the A^d and E^d systems were incubated with 64 μM of each Ii peptide or with PBS prior to washing and a second incubation with antigenic peptide and specific T hybridoma cells. APC prepulsed with 64 μM of either mIi-4 or hIi-4 had enhanced presentation of subsequently added HEL106–116, as indicated by respective responses of 590 % and 927 % (Table 4). No significant degree of enhancement was seen with the other peptides tested. In contrast, pre-incubation of the APC with either mIi80–103 or hIi81–104 peptide reduced presentation of HEL11–25. Only a 36 % response was obtained with APC prepulsed with 64 μM mIi80–103, and a 38 % response was seen with APC prepulsed with 64 μM of hIi81–104. In addition, the PH-1.0 peptide, which enhanced A^d- and E^d-restricted antigenic peptide-specific responses in assays with simulta-

Table 3. Antigenic peptide prepulse assay

Allele/Antigenic Peptide	No Peptide a)	mIi-4 b)	hIi-4	mIi 80-103	hIi 81-104	PH-1.0	PBS
Ad							
0.3 μM HEL 11-25 c)	100	82±12 d)	88±16	51±9	41±4	89±2	97±9
0.15 μM	100	114±9	124±19	32±13	41±6	127±1	122±25
Ak							
0.4 μM HEL 46-61	100	98±2	76±8	54±7	24±17	88±7	75±13
0.2 μM	100	76±7	80±6	56±10	37±1	84±4	94±2
Ed							
1.50 μM HEL 106-116	100	63±8	65±2	110±15	121±3	122±2	96±1
0.75 μM	100	37±12	64±7	54±6	82±3	65±9	87±4
Ek							
20 μM PGCC 81-104	100	50±17	39±7	66±10	55±6	113±7	83±11
10 μM	100	60±8	17±5	60±7	42±6	128±5	101±4
Ek							
0.4 μM THMCC 82-103	100	104±5	89±7	94±4	91±12	104±3	72±24
0.2 μM	100	105±19	121±22	98±14	74±15	84±15	87±19

a) The cpm of the T cell response to antigenic peptide-prepulsed APC in the absence of effector peptide is set as 100 % response in each system for each concentration of antigenic peptide.
b) Effector peptides were tested at 64 μM.
c) The concentration of antigenic peptide used in each prepulse within each system.
d) The % of response = [(cpm of T + Ag-prepulsed APC + effector peptide)/(cpm of T + Ag-prepulsed APC)] × 100 %. The values are the mean ± SEM of two to four independent experiments.

Table 4. Effector peptide prepulse assays

Allele/Antigenic Peptide	PBS a)	mIi-4 b)	hIi-4	mIi 80-103	hIi 81-104	PH-1.0
Ad/HEL 11-25 c)	100	88 d)	96	36	38	80
		±8	±10	±8	±9	±6
Ed/HEL 106-116	100	590	927	145	208	129
		±184	±342	±9	±19	±4

a) The cpm of the T cell response to APC prepulsed with PBS is set as 100 % response in each system.
b) The concentration of each effector peptide was 64 μM in the prepulse.
c) The concentrations of antigenic peptides used were: 0.3 μM of HE 11–25 and 1.5 μM of HEL 106–116.
d) The % of response = [(cpm of T + effector peptide prepulsed APC + Ag peptide)/(cpm of T + PBS-prepulsed APC)] × 100 %. The values are the mean ± SEM of two to four independent experiments.

neous addition of antigenic and effector peptides, had no significant effect in assays with the initial sole addition of either antigenic peptide or effector peptide.

These results (and those of others [41–46] led to the working hypothesis that Ii-4 peptide disrupts preformed antigenic peptide/MHC class II complexes in a manner permitting peptide exchange to take place when a second peptide is available. Since mIi80–103 and hIi81–104 blocked T cell responses regardless of the order of addition of antigenic and effector peptides, it was not clear whether antigenic peptide and hIi81–104/mIi80–103 bound simultaneously to individual MHC class molecules. Mechanisms for the contrasting effects of the Ii-4 peptide *vs.* the mIi80–103 and hIi81–104 peptides were explored further by analyzing binding of certain biotinylated peptides alone and in the presence of various unlabeled peptides.

3.5 Binding of mIi80–103, mIi-4 and antigenic peptides to MHC class II alleles

The binding of biotinylated murine Ii peptides to A^d, A^k, E^d and E^k murine MHC class II alleles on the respective class II gene-transfected L cell lines was determined using PE-avidin staining and cytofluorometry (Fig. 2). Biotinylated mIi80–103 peptide had the highest relative affinity for A^d (the allele on which it is the most potent inhibitor of antigenic peptide presentation), where 4 μM was saturating, yielding a mean channel shift of 267 on a fluorescence scale of 0–1000. In addition, biotinylated mIi80–103 bound to A^k, E^d, and E^k, but with lower affinity for each of these alleles than for A^d. Biotinylated mIi-4 peptide also bound to these four murine MHC class II alleles, but with smaller differences in apparent affinities among the alleles than seen with mIi80–103.

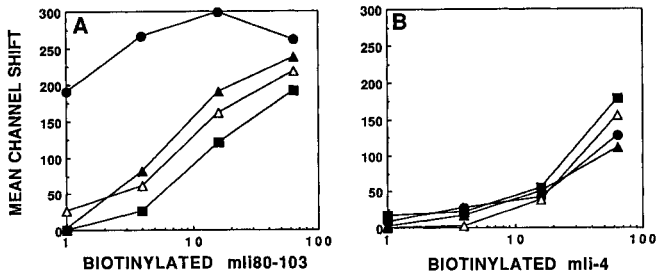

Figure 2. Biotinylated mIi80–103 and mIi-4 binding to MHC class II-positive L cells. The class II-positive cells were incubated for 16 h with the biotinylated peptides, followed by surface-staining with streptavidin-R-phycoerythrin and cytofluorometric analysis. (A) biotinylated mIi80–103 at 1, 4, 16 and 64 μM; (B) biotinylated mIi-4 at 1, 4, 16 and 64 μM. The four murine MHC class II alleles studied were: A^d (solid circle), A^k (open triangle), E^d (solid triangle), and E^k (solid square). Mean channel shift = (mean fluorescence channel shift of the experimental) – (mean fluorescence channel shift of a sham-incubated control). Class II-expression was normalized by staining separate aliquots of cells with anti-murine Ia monoclonal antibodies, followed by biotinylated second-step reagents and then PE-avidin.

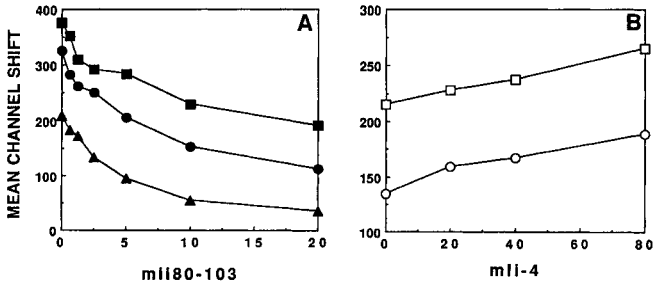

Figure 3. Biotinylated antigenic peptide binding to MHC class II-positive L cells is inhibited by mIi80–103 and enhanced by mIi-4. The cells were incubated with peptides, surface-stained and analyzed as described in the legend of Fig. 2, without normalization. (A) A^d-positive L cells incubated with biotinylated HEL11–25: 10 μM (solid triangle), 40 μM (solid circle), and 100 μM (solid square); without and with serial twofold dilutions of mIi80–103 from 20 μM to 0.625 μM. (B) E^d-positive L cells incubated with biotinylated HEL106–116: 10 μM (open circle) and 20 μM (open square), without and with serial twofold dilutions of mIi-4 from 80 μM to 20 μM.

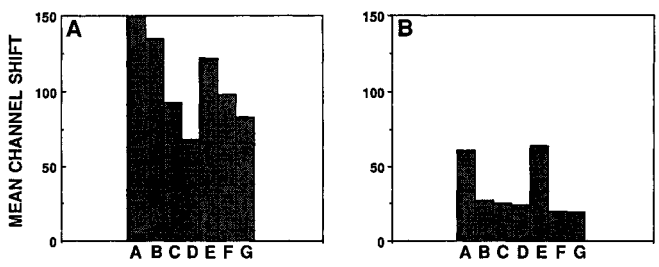

Figure 4. Biotinylated mIi80–103 and mIi-4 binding to E^k-positive L cells is inhibited by the PH-1.0 E^k-competitor peptide and the THMCC82–103 E^k-restricted antigenic peptide. Cells were incubated with peptides, surface-stained and analyzed as described in the legend of Fig. 2, without normalization. (A) biotinylated mIi80–103 at 16 μM, (B) biotinylated mIi-4 at 16 μM. *Subheadings:* A: mIi80–103 or mIi-4 alone, B: A + 4 μM PH-1.0, C: A+16 μM PH-1.0, D: A+64μM PH-1.0, E: A + 4 μM THMCC82–103, F: A + 16 μM THMCC82–103, and G: A + 64 μM THMCC82–103.

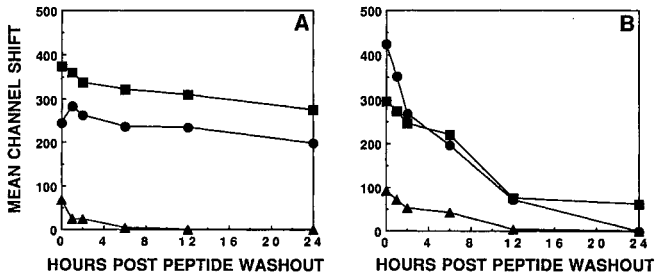

Figure 5. The relative dissociation rates of biotinylated mIi80–103 (square), mIi-4 (triangle) and antigenic peptide (circle) from A^d-positive L cells (A) and from E^d-positive L cells (B) were determined by incubating L cells with 40 μM of each peptide, as described in the legend of Fig. 2 (without normalization), in duplicate samples per peptide for each time point. Antigenic peptides used: HEL11–25 for A^d and HEL106–116 for E^d. After incubation and washing, the cells were incubated again at 37 °C for 0, 1, 2, 6, 12 and 24 h. Following incubation, the cells were surface-stained and analyzed as described in the legend of Fig. 2. The mean channel shift = the average of [(the experimental mean fluorescence channel shift) – (the mean fluorescence channel shift of the sham-incubated control)].

The binding of biotinylated, respectively restricted antigenic peptide to MHC class II-positive L cells was inhibited by mIi80–103 and enhanced by mIi-4 when Ii-effector and biotinylated antigenic peptides were combined (Fig. 3). The A^d and E^d MHC class II-positive L cells were incubated for 16 h with biotinylated HEL11–25 and HEL106–116 antigenic peptides, respectively, without and with different concentrations of mIi80–103 or mIi-4, to determine whether mIi80–103-mediated inhibition and mIi-4-mediated enhancement of antigenic peptide presentation to T cells indeed resulted from inhibition or augmentation of antigenic peptide binding to MHC class II. Biotinylated HEL11–25 binding to A^d was inhibited in a dose-dependent fashion by mIi80–103 (Fig. 3A). Likewise, biotinylated HEL106–116 binding to E^d was enhanced in a dose-dependent fashion by mIi-4 (Fig. 3B). These findings correlated with the results of the assays for competition *in vitro* among simultaneously added peptides for presentation by MHC class II^+, Ii^+ cell lines (Tables 1 and 2).

To test directly whether mIi80–103 bound in the same site (or subsite) of the MHC class II molecule as the antigenic peptide, biotinylated mIi80–103 was incubated with E^k-positive L cells without and with different concentrations of PH-1.0, another competitive inhibitor on E^k, or the THMCC82–103 E^k-restricted antigenic peptide (Fig. 4A). Both the PH-1.0 peptide and the THMCC 82–103 antigenic peptide competed with mIi80–103 for binding to E^k MHC class II molecules. Thus, these peptides appear to share some binding positions in the antigenic peptide binding groove. Similarly, the PH-1.0 and THMCC82–103 peptides also competed the binding of biotinylated mIi-4 by MHC class II E^k (Fig. 4B).

The relative dissociation rates of biotinylated mIi80–103, mIi-4 and antigenic peptide were determined from preformed peptide/class II complexes on the surfaces of A^d-positive and E^d-positive L cells (Fig. 5). In the A^d system, more mIi80–103 was bound per cell than HEL11–25 at all time points after peptide washout, (Fig. 5A), which corre-

lates with the relatively high potency of the mIi80–103 peptide as a competitive inhibitor in the A^d system as defined by T cell functional assays (Table 1). In the A^d system, the relative half-lives of biotinylated mIi80–103 and HEL11–25 were both > 24 h, while that of biotinylated mIi-4 was < 2 h (Fig. 5A). In the E^d system, there was initially more HEL106–116 than mIi80–103 bound per cell (Fig. 5B). The mIi80–103 peptide also inhibited antigenic peptide binding in the E^d system, although with a lesser potency than in the A^d system (Table 1). In the E^d system, the relative half-lives of biotinylated mIi80–103 and HEL106–116 were 9 and 6 h, respectively, while that of biotinylated mIi-4 was 2–6 h (Fig. 5B). Finally, these dissociation experiments indicated that considerably less mIi-4 was bound to the cell surface following an overnight incubation than was mIi80–103 or either of the antigenic peptides. These findings suggest either that the Ii-4/I-E complex is relatively short-lived, or that the Ii-4 peptide is relatively unstable.

4 Discussion

These experiments address the behavior of two Ii peptides which partially overlap in sequence and have sharply contrasting biological activities. In functional assays for the stimulation of T cell hybridomas by respective antigenic peptides, hIi81–104 and mIi80–103 (the longest forms of the CLIP series of Ii-derived peptides) are potent inhibitors whose kinetics, in these types of assays, are consistent with competitive inhibition. In contrast, the hIi-4 and mIi-4 peptides greatly augmented (up to as much as 10–12 times) T cell responses to limiting doses of antigenic peptides in such assays. Both the hIi81–104/mIi80–103 and the hIi-4/mIi-4 peptides inhibited antigenic peptide-specific T cell responses to APC which had been pre-incubated with limiting doses of antigenic peptide, following the removal of unbound antigenic peptide by extensive washing. While APC pre-incubated with the hIi81–104/mIi80–103 peptides (followed by washing) demonstrated decreased presentation of antigenic peptide, APC similarly pre-incubated with the hIi-4/mIi-4 peptides displayed augmented presentation of antigenic peptide. The results with the Ii-4 peptides are consistent with the view that the hIi-4/mIi-4 peptides induce structural changes about the antigenic peptide binding site of cell-surface MHC class II molecules, leading to the release of previously bound peptides and to the binding of a second peptide (peptide exchange). It has also been shown that competitive inhibitor peptides accelerate the dissociation rate of preformed MHC class II-antigenic peptide complexes in certain antigenic systems [41], which correlates with the mIi80–103/hIi81–104-mediated inhibition of T cell responses to antigenic peptide-prepulsed APC seen here.

In correlation with the results from T cell functional assays, mIi80–103 peptide was found to inhibit biotinylated antigenic peptide binding to MHC class II-positive cells, while the mIi-4 peptide enhanced biotinylated antigenic peptide binding. In addition, a second competitive inhibitor peptide, PH-1.0 and an E^k-restricted antigenic peptide, THMCC82–103, both competed with the biotinylated forms of mIi80–103 and mIi-4 for binding to E^k-positive cells. Biotinylated mIi80–103 and mIi-4 bound to all four murine MHC class II alleles studied. While mIi80–103 had a higher affinity for A^d than for the other three murine MHC class II alleles in this study, the affinities of mIi-4 were less variable over the four alleles. Fewer mIi-4/MHC class II complexes were formed per cell than with mIi80–103 or with antigenic peptide, and the half-life of cell surface MHC class II/mIi-4 complexes was shorter than that of those containing mIi80–103 or antigenic peptide.

Variations in the dissociation rates of hCLIP-region peptides from HLA-DR3, HLA-DR4, and HLA-DR11 have been observed by Avva and Cresswell [31]; and Chicz et al. [25] have shown that HLA-DR1, HLA-DQ1, and HLA-DQ8 differ in hCLIP-region peptide binding capacity. Sette et al. [47] have shown that allelic polymorphisms in MHC class II structure regulate CLIP-region peptide binding to MHC class II molecules. Malcherek et al. [48] have also found that naturally produced CLIP-region peptides contain motifs which allow these Ii peptides to interact with many MHC class II molecules. Variations in the affinities of certain mCLIP-region peptides have been observed for A^d and E^d using purified MHC class II molecules [26]. The variations in the effects on antigenic peptide presentation and, to a lesser extent, in MHC class II binding of the CLIP-region and Ii-4 peptides, imply local allelic differences in the structure of the MHC class II receptor sites for these peptides. These results support the view that the binding sites of hIi81–104/mIi80–103 and hIi-4/mIi-4 on MHC class II molecules both overlap, at least in part, with the antigenic peptide binding groove.

Others have demonstrated requirements for Ii residues in a segment including the CLIP region for binding of Ii to MHC class II molecules [49–51] and the release of CLIP peptides (including hIi81–104 or mIi80–103) to permit the binding of antigenic peptides [13, 52]. The presence of Ii blocks the binding of antigenic peptides to MHC molecules [1]. The Ii-CLIP series of peptides has been identified after acid elution from MHC class II molecules from various human and murine cell lines [21, 22, 24–27], and in greater amounts from the T2 cell line [23]. T2 cells are deficient in the HLA-DM molecule which might catalyze both the removal of Ii-CLIP peptides [31, 52–55] and the charging of MHC class II molecules with antigenic peptides [55]. Through the analysis of the binding of truncated forms of Ii, the requirement for a region of Ii terminating about hIi104 and mIi107 in binding, respectively, to human and murine MHC class II α,β chains, has been established [49–51]. While such experiments establish the C-terminal extent of Ii segment binding to MHC class II molecules, Bijlmakers et al. [50] observed that such truncation experiments indicate only that the Ii residues responsible for binding to MHC class II α,β chains and for inhibiting peptide binding are either co-expressed in the CLIP region sequences, or that the domain inhibiting peptide binding might actually be more N-terminal to the domain required for MHC class II association.

Other experiments help to explain the role of Ii-4 in regulating or promoting the process of antigenic peptide charging into the MHC class II antigenic peptide binding site. Urban et al. [56] demonstrated that acidic conditions promoted the loss of the subset of human CLIP-region peptides with N termini extending from residue positions L^{81}, P^{82}, K^{83}, while shorter Ii peptides, with N termini of Lys^{90}, were not lost.

Others have demonstrated that, in addition to certain substitution analogs of antigenic peptides, several other peptides (*e.g.* dynorphin A 1–13 and poly-L-lysine) promoted the exchange of MHC-bound peptides with second peptides [41–46]. The effects of these peptides on antigenic peptide exchange might reflect actions at the Ii-4 binding site on MHC class II molecules.

From our experiments and the studies discussed above, a hypothetical model can be proposed to explain the effects of hIi81–104/mIi80–103 and Ii-4 peptides, and for the roles of these segments of Ii in the staged cleavage and release of Ii, which appears to regulate or promote charging of the MHC class II antigenic peptide binding site [18, 19]. In this model, the MHC class II receptor sites for the hIi81–104/mIi80–103 peptides and for hIi-4/mIi-4 peptides are the same as those occupied by the respective segments in intact Ii as it adheres to the MHC class II α,β dimer. The overlapping region between those two segments in hIi, L^{81} through R^{92}, appears to contain multiple potential cathepsin cleavage sites, as reflected in the N termini of the nested set of Ii-CLIP peptides. Converting the residues R^{78}→A, K^{80}→A, K^{83}→A, and K^{86}→T in this region of hIi yielded a p21 structure which was not cleaved to the p6 intermediate and remained bound to the MHC class II α,β chains [19]. We have shown that the hIi-81–104/mIi80–103 peptides inhibit antigenic peptide binding in a manner which is consistent with competitive inhibition. Others have demonstrated the requirement for the release of Ii-CLIP region peptides prior to antigenic peptide binding [13, 52]. These findings indicate that the Ii-CLIP segment of Ii might either lie in the antigenic peptide binding site, as would an antigenic peptide, or partially overlap that site. This hypothesis is strengthened by the crystallographic characterization of the binding of an Ii-CLIP region peptide in the antigenic peptide binding groove of HLA-DR3 (P. Ghosh, B. Mellins, and D. Wiley, personal communication). The Ii-4 peptide (hIi77–92/mIi76–91) segment of Ii could then lie near one end of the antigenic peptide binding groove.

In the staged cleavage and release of Ii, we propose that the exposed residue positions shared between Ii-4 and hIi81–104 are cleaved, generating the N termini of the Ii-CLIP region-derived peptides (which are protected from proteolysis by lying in the class II groove [31, 57]). The Ii-CLIP peptides are then released, perhaps under the action of HLA-DM [31, 52–55] or acidic pH [23, 31, 52, 56], permitting or promoting the exchange of that Ii segment with antigenic peptides. During this exchange process, a relatively labile conformation of the antigenic peptide binding site could be promoted by a residual Ii-4-containing segment of Ii which is finally released, with subsequent tight closure of the MHC class II molecule on the antigenic peptide. We suggest that dissociation of the Ii-4 fragment permits this final tightening of the secondary structure of the MHC class II molecule on the antigenic peptide, because the Ii-4 peptide alone is able to convert the tight conformation of cell surface MHC class II molecules to a more labile form which releases antigenic peptide and is capable of binding of a second antigenic peptide.

New classes of drugs might be rationally designed based on both the hIi-4 and hIi81–104 peptide structures and their binding sites, ranging from stabile peptide homologs to peptidomimetics and to new organic structures. Peptide-based immunotherapies for vaccination, control of allergy or autoimmune disease, and immunosuppression might be enhanced through the adjuvant action of hIi-4-based drugs. In contrast, allele-specific, hIi81–104-based drugs might be designed to selectively block autoantigenic peptide binding to disease-associated MHC class II alleles.

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