

Structure-Immune Response Relationships of Hapten-Modified Collagen II Peptides in a T-Cell Model of Allergic Contact Dermatitis

Meirav Holmdahl,^{†,‡,||} Stefan R. Ahlfors,^{‡,||} Rikard Holmdahl,^{‡,§} and Christer Hansson^{*,†}

Department of Dermatology, Lund University Hospital, SE-221 85 Lund, Sweden, Medical Inflammation Research, Lund University, Lund, Sweden, and Medicity, Turku University, Turku, Finland

Received March 17, 2008

Allergic contact dermatitis (ACD) is mediated by T cells that specifically recognize hapten-modified peptides. T cells are known to recognize antigens as short processed peptides bound to major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APC). It has previously been demonstrated that T cells can specifically recognize carbohydrates on the lysine at position 264 of the immunodominant (256–273) sequence from type II collagen (CII) and that such recognition is critical for the development of arthritis in mice and may play a role in rheumatoid arthritis in humans. In the present study, we have used this approach in modeling ACD, but instead of the carbohydrate, the strong sensitizer 2,4-dinitrofluorobenzene (DNFB) is bound to the ϵ -amine of the lysine at position 264. Specific T-cell hybridomas of this antigenic peptide, with dinitrophenyl (Dnp) on the ϵ -amine of lysine at position 264 (CII_{Lys}Dnp **3**), were established from mice immunized with CII_{Lys}Dnp **3**. In an immune response assay, these T-cell hybridomas were tested with a series of new synthetic hapten-modified peptides, all chemically identical except for the stereochemistry (D,L) and the length of the position-264 amino acid side chain bonding the hapten. The T-cell hybridomas recognized the CII_{Lys}Dnp **3** peptide used for immunization; interestingly, they also recognized the CII peptide with a one-carbon-longer side chain (homolysine), CII_{Lys}Dnp **6**, and CII_{Ala}PipDnp **11**, having a ring structure analogous to that of lysine with the same number of carbons in the bonding chain as in the CII_{Lys}Dnp **3** peptide used for immunization. Dnp-modified CII peptides with a shorter bonding chain produced no immune response. These data demonstrate that the T-cell recognition of the Dnp-modified peptides is highly specific and moreover dependent on the length of the amino acid side chain that bonds the Dnp.

Introduction

Allergic contact dermatitis (ACD¹) is one of the most common allergies, affecting over 25% of the general population in western and northern Europe (1). ACD is a T-cell-mediated immune response resulting from low-molecular-weight xenobiotics (haptens), which are able to penetrate the skin and bind to endogenous proteins or peptides (2). If these modified proteins are captured and processed by antigen-presenting cells (APC), leading to the presentation of hapten-modified peptides to T cells, they may trigger and perpetuate ACD. The immunological mechanism of ACD is not well established at the molecular

level (3–6). For many years, it was believed that CD₄⁺ cells were the main immunological targets of ACD. Over the last 10 years, CD₈⁺ cells have been given a more prominent role, mainly on the basis of animal studies (7–11). In a recent human study of ACD, Pickard et al. demonstrated the importance of both CD₄⁺ and CD₈⁺ cells, in agreement with a study of knockout mice (12, 13). Our investigation is focused on the mechanisms of major histocompatibility complex (MHC) class II-restricted (usually CD₄⁺) T cells in ACD. In this model, haptens bind to endogenous skin proteins, which are taken up and processed by epidermal or dermal antigen-presenting cells (APC). The antigen-loaded APC migrate to the draining lymph node where they present the hapten-modified peptide, in the context of MHC class II molecules, to T cells. A subset of T cells bearing unique T-cell receptors (TCR) specific to the antigen is formed. On subsequent skin exposure to the same antigen, an allergic reaction can be initiated at the place of exposure. Thus, the classic contact allergic reaction involves highly specific mono or polyclonal T cells recognizing newly formed modifications of self-proteins conjugated with foreign molecules. A similar type of antigen recognition is today believed to occur in autoimmune diseases in which self-proteins may be modified post-translationally and therefore poorly tolerated. An example of such recognition is the T-cell recognition of type II collagen (CII). CII is the major protein component of cartilage, and immune recognition of CII is critical for the development of collagen-induced arthritis (CIA), the most common animal model of rheumatoid arthritis (RA (14, 15). In

* To whom correspondence should be addressed. Tel: +46-46-173151. Fax: +46-46-172114. E-mail: Christer.Hansson@med.lu.se.

[†] Lund University Hospital.

[‡] Lund University.

[§] Turku University.

^{||} MH and SA contributed equally to this work.

¹ Abbreviations: ACD, allergic contact dermatitis; APC, antigen-presenting cells; Boc, *tert*-butoxycarbonyl; tBu, *tert*-butyl; CII, type II collagen; CIA, collagen-induced arthritis; COSY, homonuclear correlation spectroscopy; CTLL, cytotoxic T lymphocyte lines; DMEM, Dulbecco's modified Eagle's medium; DNCB, 2,4-dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; Dnp, dinitrophenyl; FCS, fetal calf serum; Fmoc, 9-fluorenylmethoxycarbonyl; HMBC, heteronuclear multiple-bond connectivity; HMQC, ¹H-detected heteronuclear multiple-quantum coherence; HRMS, high-resolution mass spectra; IL-2, interleukin-2; LNC, lymph node cells; MHC, major histocompatibility complex; NOESY, nuclear Overhauser enhancement spectroscopy; Pmc, 2,2,5,7,8-pentamethyl-chromane-6-sulfonyl; RA, rheumatoid arthritis; SPPS, solid phase peptide synthesis; TCR, T-cell receptor; TFA, trifluoroacetic acid; TIS, triisopropyl silane; Tnp, Trinitrophenyl; TOCSY, total correlation spectroscopy; Trt, triphenylmethyl.

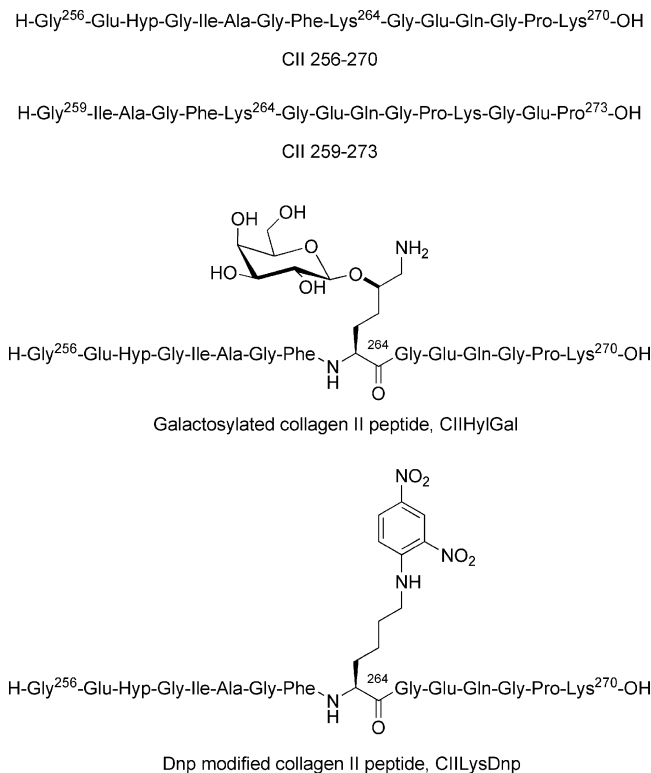


Figure 1. Immunodominant CII sequences used in this and previous (20, 30) studies, together with the 5-*O*-(β -D-galactopyranosyl)-5-hydroxy-modified-lysine 264 in the CII 256–270 peptide that has been found to be involved in CIA and RA. The complete Dnp-modified antigen CIIlys-Dnp **3** is also shown.

the present study, we have used an immune-dominant peptide sequence from collagen II as the carrier peptide binding the hapten to give a complete antigen (Figure 1). Presentation of peptides from region 256–273 of collagen II is critical for triggering CII-specific reactivity in both CIA and RA (14, 16). This epitope contains lysine residues at position 264, which can become post-translationally modified by hydroxylation and then galactosylated (Figure 1), creating many different epitopes with immunogenic properties in both CIA and RA (17–19). When the peptide is bound to MHC H-2A^q molecules, the dominant T-cell epitope is the side chain of lysine at position 264 (19, 20). The lysine side chain can be post-translationally modified by hydroxylation and subsequently glycosylated with galactose or glucose. The predominant T cell recognition in mice with the H2-A^q MHC haplotype is the galactosylated form. The peptide also binds human DR4 class II molecules, and in this context, two different lysines at position 264 and 270 can be recognized by T cells, although the predominant position is the lysine at position 264. An additional similarity with the murine system is that post-translational modifications can also be recognized. T cell reactivities have been studied in both DR4 transgenic mice and in humans with RA, and in both cases, there is a significant response to the post-translational modified peptide (14). Interestingly, the DR4 transgenic mice are susceptible to CIA (21). In both mice and humans, the susceptibility to arthritis is MHC class II associated. The minimal peptide backbone for binding to MHC class II H-2A^q molecules is CII260–267 (22), with the MHC anchor points Ile 260 and Phe 263. The presentation of peptides 260–267 is enhanced by stabilizing the MHC/peptide complex with additional flanking amino acids; the complex seems, however, to be immunodominant, as this is the only peptide sequence in the CII molecule that gives rise to an immune response in H-2A^q-expressing mouse strains. The

variability of the T-cell response is dependent on the post-translational modifications of the lysine side chain, such as hydroxylation and glycosylation. Thus, this is a well-characterized self-protein in which a lysine side chain is recognized by the immune system and in which T-cell recognition and activation is dependent on side chain modifications. This post-translational modification of the immunodominant amino acid lysine mimics the process in the development of ACD.

In the present work, we have studied the requirements for CII-derived dinitrophenyl- (Dnp)-conjugated peptides to stimulate reactive T cells obtained by immunization with Dnp on the lysine at position 264 of the CII peptide (Figure 1). Dnp derivatives are formed from DNFB or DNCB, classic and very potent sensitizers used in many studies of ACD (12, 23). We have established T-cell hybridoma clones specific to this hapten-modified peptide and have demonstrated that this T-cell receptor responds to antigens with hapten-binding side chains of lysine length or one carbon atom longer. Shorter side chains do not stimulate the T-cell receptor. When the D isomer of lysine was incorporated into the antigen, only a weak response was observed. We observed no cross-reactivities with the naturally occurring galactosylated hydroxylysine of this peptide nor with other hapten-modified peptides, such as the hydroquinone-modified peptide, CIIcysHQ **18**.

Materials and Methods

Caution: Skin contact with DNFB or DNCB must be avoided. DNFB and DNCB are strong sensitizing substances, and they must be handled with care.

Chemicals and General Methods. The chemicals used were of commercial reagent grade and were used without further purification. TLC was performed on silica gel 60 F254 (Merck, Darmstadt, Germany) using UV light detection and ninhydrin solution. Flash column chromatography was performed on silica gel (60 Å, 35–70 µm, Merck).

NMR. ¹H NMR spectra were recorded at 400 or 500 MHz and ¹³C NMR spectra at 100 MHz using Bruker DRX-400 or Bruker ARX-500 spectrometers (Bruker BioSpin, Rheinstetten, Germany). Chloroform-*d* (δ_H 7.27 or δ_C 77.23 ppm), acetic acid-*d*₄ (δ_H 2.04 or δ_C 20.0 ppm), and water-*d*₂ were used as solvents, and the solvent peaks were used as internal standards. First-order chemical shifts and coupling constants were obtained from one-dimensional (1D) spectra, and the proton and carbon resonances were assigned on the basis of the results of 2D homonuclear correlation spectroscopy (COSY), ¹H-detected heteronuclear multiple-quantum coherence (HMQC), heteronuclear multiple-bond connectivity (HMBC), nuclear Overhauser enhancement spectroscopy (NOESY), and total correlation spectroscopy (TOCSY) experiments, all using solvent (H₂O) suppression with presaturation during the relaxation delay (24).

MS and LC-MS. Fast atom bombardment (FAB) high-resolution mass spectra (HRMS) were recorded on a Jeol SX 102A mass spectrometer (Jeol, Tokyo, Japan). Ions were produced by a beam of xenon atoms (6 keV) from a matrix of glycerol and thioglycerol. The spectra of intermediate products during the synthesis of substance **10** were recorded by means of LC-MS on a Waters Alliance 2695 HPLC system using a Waters Micromass Q-ToF micromass spectrometer with EID detection (Waters, Milford, MA).

Analytical and Preparative HPLC. Analytical HPLC was performed using a Waters 600 pump (Waters Chromatography Division, Milford, MA) equipped with a 20-µL Rheodyne Model 7125 loop injector (Rheodyne, Rohnert Park, CA) and a 1100

Table 1. ¹H NMR Data (δ, ppm) for Peptide 6 (CIIhLysDnp) in Water with 10% D₂O^a

6						
residue	NH	H-α	H-β	H-γ	H-δ	others
Gly ²⁵⁶		3.90				
Glu ²⁵⁷	8.66	3.87	2.12	1.92	2.49	
Hyp ²⁵⁸		4.49	3.32	1.05	3.85	
Gly ²⁵⁹	8.57	4.00				3.91
Ile ²⁶⁰	7.94	4.19		1.89	1.45	1.20 0.85 0.92(β-CH ₃)
Ala ²⁶¹	8.25	4.30	1.39			
Gly ²⁶²	7.92	3.94				
Phe ²⁶³	8.02	8.63	3.08	3.04		Ar 7.35 7.24
hLys ²⁶⁴	8.41	4.31	2.04	1.80	1.52	1.73 8.80 (NHζ), 3.51 (Hζ), 1.73 (Hε) Dnp ^b
Gly ²⁶⁵	8.19	3.79				
Glu ²⁶⁶	8.45	4.41	2.13	1.93	2.14	
Gln ²⁶⁷	8.19	4.39	2.11	2.02	2.17	7.52 6.84 (CONH ₂)
Gly ²⁶⁸	8.25	3.98				
Pro ²⁶⁹		4.47	2.31	2.02	2.04	3.52
Lys ²⁷⁰	8.19	4.07	1.90	1.78	1.47	1.71 3.05 (Hε)

^a Obtained at 500 MHz, 293 K, and pH 5.4 using H₂O as the internal standard (δ_H 4.80 ppm). ^b The chemical shifts from the Dnp part of the structure (δ, ppm): 8.98 (H-3), 8.23 (H-5), and 7.12 (H-6).

series diode-array detector (HP 1050; Hewlett-Packard, Palo Alto, CA). The UV/vis spectra (190–900 nm) of every peak were recorded and evaluated. We used a linear gradient of 5–80% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) over 60 min at a flow rate of 1.5 mL/min; an EC Nucleosil 100–5C₁₈ column (5 μm, 4.6 mm i.d. × 150 mm; Macherey-Nagel, Düren, Germany) equipped with two 100–5C₁₈ guard columns coupled in series (Phenomenex, Torrance, CA), was used. Preparative HPLC was performed using a Shimadzu LC8A system (Shimadzu, Kyoto, Japan) equipped with a Rheodyne Model 7125 loop injector (1.0 or 5.0 mL) and a Spectromonitor D UV-detector (LDC/Milton Roy, Riviera Beach, FL) operating at 214 nm. Separations were performed on an Apex Prepsil C₈ (8 μm, 22 mm i.d. × 250 mm) column (Jones Chromatography, Lakewood, CA). The products were separated using a linear gradient of 5–80% acetonitrile in water containing 0.1% TFA over 60 min at a flow rate of 9.0 mL/min.

Solid-Phase Peptide Synthesis (SPPS). All peptides were synthesized using preloaded Fmoc-Lys(Boc) or the corresponding Fmoc-amino acid peptide resin (25-μmol scale) with 75 μmol of each building block, according to standard Fmoc protocols, using an ABI Synergy 432 Peptide Synthesizer (Applied Biosystems, Foster City, CA) unless otherwise indicated. Fmoc amino acids with Boc-, Dnp-, Pmc-, tBu-, or Trt-protected side chains were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA), Bachem (Bubendorf, Switzerland), RSP Amino Acids LLC (Shirley, MA), or synthesized in our laboratory (FmocLysDnp 1). The general procedure for cleaving crude resin-bound peptides and deprotecting acid labile protecting groups was performed with TFA/thioanisole/ethanedithiol/water (90:5:2.5:2.5; vol %). The Dnp-peptide resins were cleaved and deprotected with TFA/triisopropylsilane (TIS)/water (95:2.5:2.5; vol %). The peptide content was approximately 80% for all of the isolated pure lyophilized peptides (HPLC).

N^α-(9-Fluorenylmethoxycarbonyl)-7-N-(2,4-dinitrophenyl)-L-homolysine (1). Building block 1 for the synthesis of peptide 6 was prepared according to a modified method of Sanger (25). The Fmoc- (26) and Boc-protecting groups were cleaved by reacting a solution of N^α-(9-fluorenylmethoxycarbonyl)-7-N-(*t*-butoxycarbonyl)-L-homolysine (435 mg, 0.901 mmol) in dichloromethane (18 mL) and piperidine (2 mL), under nitrogen and stirring at room temperature for 24 h. The precipitated dibenzofulvene piperidine adduct was removed by filtration and

the residue concentrated at reduced pressure. The resulting crude product was treated with a cold solution of TFA/TIS/water (95:2.5:2.5) and purified using preparative TLC; SiO₂; isopropanol/conc. NH₃ 7/3 (vol/vol) to give L-homolysine (235 mg). Copper (II) carbonate (420 mg, 1.90 mmol) was added gradually at 85–90 °C to a solution of L-homolysine (380 mg, 2.37 mmol) in water (6.5 mL). The mixture was refluxed for 30 min and filtered while hot through celite; the filter pad was washed with small amounts of hot water until the filtrate was colorless. The volume was adjusted to 15 mL, and sodium hydrogen carbonate (398 mg, 4.74 mmol) was added to the solution at room temperature; then DNFB (420 μL, 3.32 mmol) in 1,4-dioxane (6.5 mL) was added slowly over 1 h and 30 min, and the suspension was stirred overnight. The green precipitate was filtered off and washed with small amounts of cold water; the combined filtrates were then concentrated and dissolved in 1,4-dioxane/water (1:1; 10 mL) and cooled to 5 °C. After standing overnight, the cold solution yielded yet more precipitate. The combined precipitates were stirred with methanol (15 mL) overnight. Na⁺ Chelex 100 (50 mL), which had been converted to its H⁺ form (27), and water (15 mL) were added, and the mixture was gently shaken for 3 days. The resulting yellow resin was filtered and gently shaken with acetic acid (100%) until colorless. The yellow filtrate was concentrated and the crude N^ε-(2,4-dinitrophenyl)-L-homolysine (200 mg) was identified using NMR spectroscopy: ¹H NMR (400 MHz, CD₃COOD) δ 9.03 (d, 1 H, *J* = 2.6 Hz, H-3), 8.30 (dd, 1 H, *J* = 2.6, 9.6 Hz, H-5), 7.12 (d, 1 H, *J* = 9.6 Hz, H-6), 4.10–3.98 (m, 1 H, Hα), 3.51 (t, 2 H, *J* = 7.1 Hz, Hζ), 2.05–1.95 (m, 2 H, Hβ), 1.81 (bt, 2 H, *J* = 6.4 Hz, Hε), 1.65–1.45 (m, 4 H, Hγ; Hδ). ¹³C NMR (100 MHz, CD₃COOD) δ 149.15, 136.59, 130.99, 130.79, 124.54, 115.03, 43.65, 30.64, 28.79, 26.95, 25.27.

To a solution of N^ε-(2,4-dinitrophenyl)-L-homolysine (140 mg, 429 μmol) in 1,4-dioxane/10% Na₂CO₃ 1/2 (10 mL) at 0–5 °C, 9-fluorenylmethyl chloroformate (110 mg, 429 μmol) in 1,4-dioxane (5 mL) was added dropwise; the mixture was stirred at room temperature overnight. The mixture was filtered, cold water (20 mL) was added, and the solution was carefully acidified to pH 3 by adding 0.1 M of aqueous KHSO₄ and then extracting with ethylacetate (3 × 30 mL). The organic solution was dried (Na₂SO₄) and concentrated at reduced pressure. The resulting yellow oil (280 mg) was purified in portions using preparative HPLC (analytical HPLC of Fmoc-homoLys(Dnp) had a retention time of 47 min) to give pure 1 (80 mg, 34%) as a yellow powder after lyophilization: ¹H NMR (400 MHz,

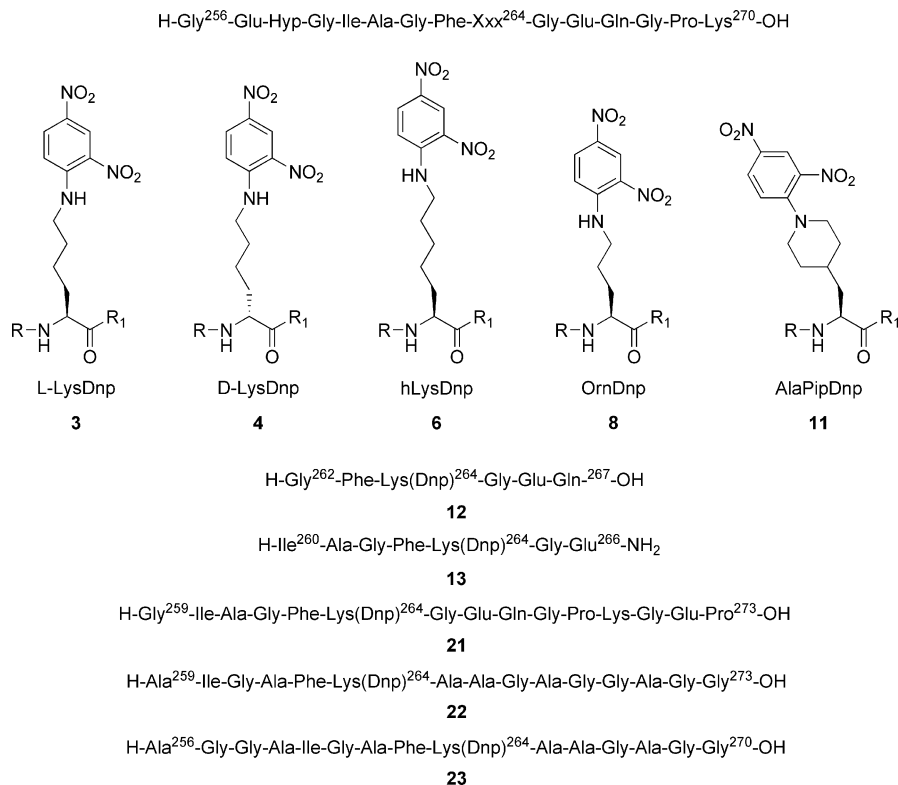


Figure 2. Structures of selected Dnp peptides **3**, **4**, **6**, **8**, and **11**, with Dnp bound to the side chain amino group at position 264 (Xxx) of the shown sequence (top), together with truncated peptides **12** and **13** derived from this CII sequence (256–270). In the lower half are peptides **21**, **22**, and **23** with other amino acid sequences, but with LysDnp and the MHC binding Ile and Phe unchanged.

CDCl₃) δ 9.12 (d, 1 H, J = 2.6 Hz, H-3), 8.53 (bs, 1 H, NH ζ), 8.23 (dd, 1 H, J = 2.5, 9.5 Hz, H-5), 7.76 (d, 2 H, J = 7.5 Hz, Ar), 7.62–7.56 (m, 2 H, Ar), 7.42–7.38 (m, 2 H, Ar), 7.33–7.30 (m, 2 H, Ar), 6.87 (d, 1 H, J = 9.5 Hz, H-6), 5.35 (bd, 1 H, J = 8.1 Hz, NH α), 4.42–4.38 (m, 3 H, H α ; ArCHCH₂), 4.22 (t, 1 H, J = 6.9 Hz, OCOCH₂CH), 3.41–3.36 (m, 2 H, H ζ), 2.02–1.96 (m, 1 H, H β), 1.79–1.66 (m, 3 H, H β ;H ϵ), 1.50–1.34 (m, 4 H, H γ ;H δ). ¹³C NMR (100 MHz, CDCl₃) δ 177.48, 156.51, 148.42, 143.84, 143.77, 141.40, 136.07, 130.45, 130.36, 127.95, 127.22, 125.45, 120.17, 113.99, 67.28, 67.19, 47.23, 43.46, 28.55, 26.49, 25.05, 21.03. HRMS (FAB) calculated for C₂₈H₂₉N₄O₈ 549.1985 (M + H⁺); found, 549.1982.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglcyl-L-phenylalanyl-L-lysylglcyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (2, CII256–270). SPPS gave 32 mg of pure peptide (86% yield) after preparative HPLC with a retention time of 21.3 min on analytical HPLC. HRMS (FAB) calculated for C₆₅H₁₀₃N₁₈O₂₂ [M + H]⁺ 1487.7494; found, 1487.7495.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglcyl-L-phenylalanyl-6-N-(2,4-dinitrophenyl)-L-lysylglcyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (3, CIIhLysDnp). SPPS gave 66 mg of peptide resin and 13 mg of pure peptide (32% yield) with a retention time of 25.1 min on analytical HPLC after preparative HPLC. HRMS (FAB) calculated for C₇₁H₁₀₅N₂₀O₂₆ [M + H]⁺ 1653.7509; found, 1653.7489.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglcyl-L-phenylalanyl-6-N-(2,4-dinitrophenyl)-D-lysylglcyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (4, CII-D-LysDnp). SPPS gave 77 mg of peptide resin and 9 mg of pure peptide (20% yield) with a retention time of

26.1 min on analytical HPLC after preparative HPLC. HRMS (FAB) calculated for C₇₁H₁₀₅N₂₀O₂₆ [M + H]⁺ 1653.7509; found, 1653.7504.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglcyl-L-phenylalanyl-L-homolysylglcyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (5, CIIhLys). SPPS gave 16 mg of pure peptide (43% yield) with a retention time of 15.1 min on analytical HPLC after preparative HPLC. HRMS (FAB) calculated for C₆₆H₁₀₅N₁₈O₂₂ [M + H]⁺ 1501.7651; found, 1501.7660.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglcyl-L-phenylalanyl-7-N-(2,4-dinitrophenyl)-L-homolysylglcyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (6, CIIhLysDnp). SPPS with building block **1** gave 83 mg of peptide resin and 4 mg of pure peptide (10% yield) after preparative HPLC with a retention time of 26.7 min on analytical HPLC. HRMS (FAB) calculated for C₇₂H₁₀₇N₂₀O₂₆ [M + H]⁺ 1667.7666; found, 1667.7673. NMR data are presented in Table 1.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglcyl-L-phenylalanyl-L-ornithylglcyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (7, CIIOrn). SPPS gave 18 mg of pure peptide (49% yield) after preparative HPLC with a retention time of 14.5 min on analytical HPLC. HRMS (FAB) calculated for C₆₄H₁₀₁N₁₈O₂₂ [M + H]⁺ 1473.7338; found, 1473.7341.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglcyl-L-phenylalanyl-5-N-(2,4-dinitrophenyl)-L-ornithylglcyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (8, CIIOrnDnp). SPPS gave 76 mg of peptide resin and 6 mg of pure peptide (15% yield) after preparative HPLC with a retention time of 24.4 min on analytical HPLC. HRMS

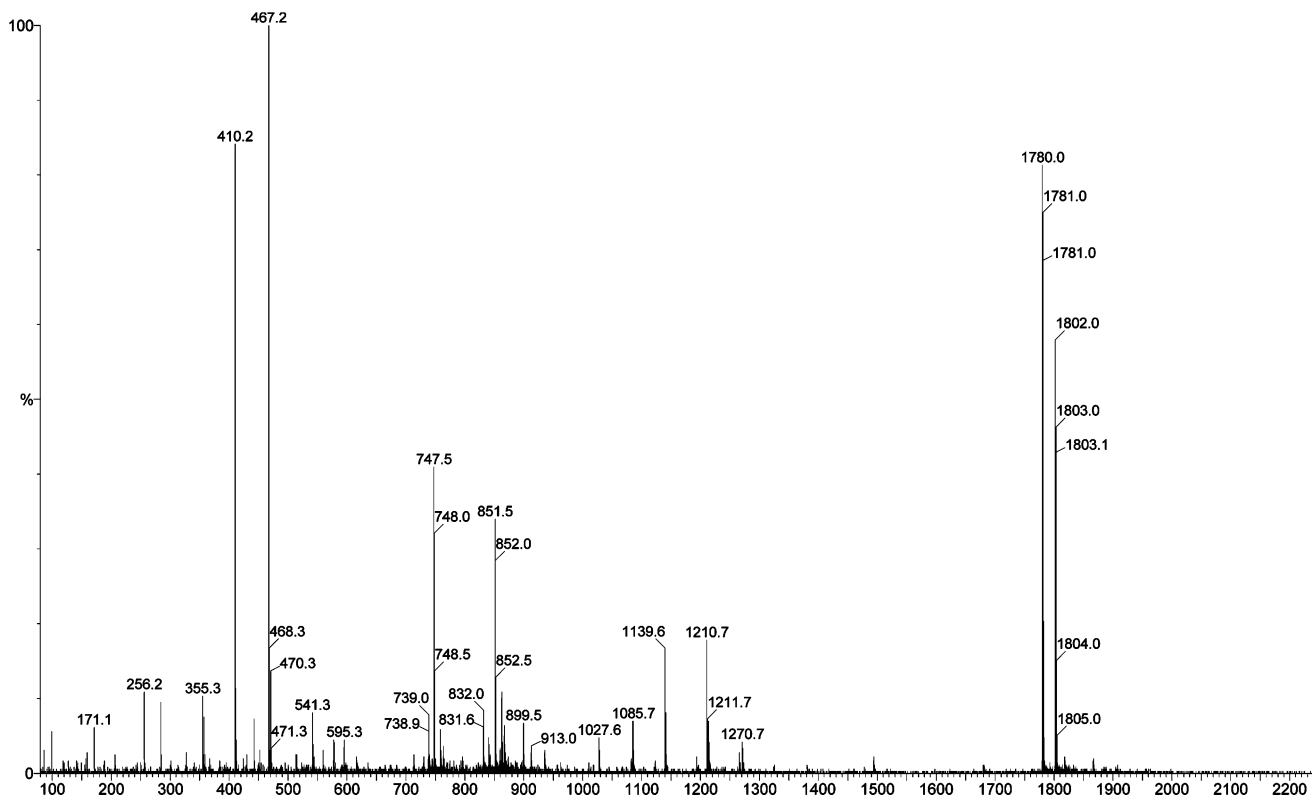


Figure 3. TOF MS spectra of the HPLC peak at 29.9 min. The ions at 1781 indicate the CIIAlaPip **10** peptide with one Dnp and one Boc-group present, while the abundant ions at 1803 indicate the CIIAlaPip **10** peptide with one Dnp, one Boc-group, and one Na present. This is in agreement with the expected values for the intermediate CIIAlaPipDnp peptide with the Boc-group bound either to the N-terminal amino group of glycine or to the side chain of the C-terminal lysine ϵ -amino group.

(FAB) calculated for $C_{70}H_{103}N_{20}O_{26}$ $[M + H]^+$ 1639.7352; found, 1639.7378.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-L-arginylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (9, CIIArg). SPPS gave 5.5 mg of pure peptide (15% yield) after preparative HPLC with a retention time of 14.9 min on analytical HPLC. HRMS (FAB) calculated for $C_{65}H_{103}N_{20}O_{22}$ $[M + H]^+$ 1515.7556; found, 1515.7565.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-3-(4-piperidyl)-L-alanylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (10, CIIAlaPip). SPPS gave 85 mg of peptide resin and 26 mg of pure peptide (69% yield) after preparative HPLC with a retention time of 15.5 min on analytical HPLC. HRMS (FAB) calculated for $C_{67}H_{105}N_{18}O_{22}$ $[M + H]^+$ 1513.7651; found, 1513.7644.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-3-(4-(N-(2,4-dinitrophenyl)-piperidyl)-L-alanylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (11, CIIAlaPipDnp). To a solution of peptide **10** (9.0 mg, 5.90 μ mol) in water (0.5 mL) and saturated $NaHCO_3$ (0.5 mL), a solution of di-*tert*-butyl dicarbonate in dioxane (10 mg/mL, 250 μ L, 12.0 μ mol) was added slowly over 2 h while stirring at ambient temperature. The reaction was monitored using analytical HPLC, as described previously, but using a VYDAC 259VHP575 (5 μ m, 4.6 mm i.d. $\times$ 150 mm) column (Grace, Deerfield, IL). The reaction was measured as the consumption of the starting peptide and as the new peaks formed with retention times of 19.8 and 24.6 min. The reaction mixture was then diluted with water (1 mL); Na_2CO_3 (4.4 mg) and then DNFB (2.2 mg, 11.9 μ mol) in 1,4-dioxane (85 μ L) were added, after which the mixture was stirred for 24 h. Analytical HPLC of the reaction mixture revealed new peaks with UV spectra

characteristic of Dnp-peptides, and the peaks at 29.9 and 32.4 min were identified using LC/TOF/MS (Figure 3). The retention time of the peaks at 29.9 and 32.4 min are in agreement with that expected for the intermediate CIIAlaPipDnp peptides with one Boc-group bound either to the N-terminal amino group of glycine or to the ϵ -amino group of the C-terminal lysine, as determined by LC/TOF/MS. However, it was not possible to isolate the pure intermediates by preparative HPLC. The solvent was removed, and the crude product mixture was treated with TFA/TIS/ H_2O to give 1 mg of peptide **11**.

Glycyl-L-phenylalanyl-6-N-(2,4-dinitrophenyl)-L-lysylglycyl-L-glutam-1-yl-L-glutamin (12, CII262–267Dnp). SPPS gave 52 mg of peptide resin and 6 mg of pure peptide with a retention time of 25.1 min on analytical HPLC after preparative HPLC. HRMS (FAB) calculated for $C_{35}H_{47}N_{10}O_{14}$ $[M + H]^+$ 831.3273; found, 831.3275.

L-Isoleucyl-L-alanylglycyl-L-phenylalanyl-6-N-(2,4-dinitrophenyl)-L-lysylglycyl-L-glutamic amide (13, CII260–266Dnp). SPPS gave 60 mg of peptide resin and 4 mg of pure peptide with a retention time of 27.3 min on analytical HPLC after preparative HPLC. HRMS (FAB) calculated for $C_{39}H_{56}N_{11}O_{13}$ $[M + H]^+$ 886.4059; found, 886.4103.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-6-N,N-dimethyl-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (14, CIIlysMe₂). SPPS gave 5.0 mg of pure peptide (15% yield) after preparative HPLC with a retention time of 15.0 min on analytical HPLC. HRMS (FAB) calculated for $C_{67}H_{107}N_{18}O_{22}$ $[M + H]^+$ 1515.7807; found, 1515.7823.

Glycyl-L-glutam-1-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-L-homocysteinylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (15, CIIhCys). SPPS gave 87 mg of peptide resin and 6.5 mg of pure peptide

(18% yield) after preparative HPLC with a retention time of 18.1 min on analytical HPLC. HRMS (FAB) calculated for $C_{63}H_{98}N_{17}O_{22}S$ $[M + H]^+$ 1476.6793; found, 1476.6807.

Glycyl-L-glutamyl-L-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-4-S-(2,4-dinitrophenyl)-L-homocysteinyglycyl-L-glutamyl-L-yl-L-glutaminyglycyl-L-prolyl-L-lysine (16, CIIhCysDnp). To a solution of **15** (17 mg, 11.5 μ mol) in sodium borate buffer (0.1 M, 5 mL) at pH 8.5, a solution of DNFB (1 mg, 5.37 μ mol) in dry acetonitrile (3 $\text{\AA}$, 0.5 mL) was added while stirring under argon at room temperature. The reaction was monitored using analytical HPLC as the consumption of **15** and a new peak was observed at 25.1 min. After 45 min of reaction time, the reaction mixture was frozen in a CO_2 /ethanol cooling bath and lyophilized. The product was purified using preparative HPLC, and 5.0 mg (26% yield) of pure substance was obtained. MS (FAB) calculated for $C_{69}H_{101}N_{19}O_{26}S$ $[M + H]^+$ 1643.6886; found, 1643.71.

Glycyl-L-glutamyl-L-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-L-cysteinyglycyl-L-glutamyl-L-yl-L-glutaminyglycyl-L-prolyl-L-lysine (17, CIIcys). SPSS gave 8.7 mg of pure peptide (24% yield) after preparative HPLC with a retention time of 17.2 min on analytical HPLC. HRMS (FAB) calculated for $C_{62}H_{96}N_{17}O_{22}S$ $[M + H]^+$ 1462.6636; found, 1462.6626.

Glycyl-L-glutamyl-L-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-3-S-(1,4-hydroquinone-2-yl)-L-cysteinyglycyl-L-glutamyl-L-yl-L-glutaminyglycyl-L-prolyl-L-lysine (18, CIIcysHQ). Synthesis was performed at ambient temperature by the dropwise addition of 0.64 mg of 1,4-benzoquinone in 1.0 mL of dry acetonitrile to a stirred solution of 8.7 mg of pure peptide **17** in 10.0 mL of water/acetic acid (1:1) under argon. The reaction was monitored using analytical HPLC, and after 2.5 h, the reaction mixture was frozen in a CO_2 /ethanol cooling bath and lyophilized. Preparative HPLC gave 1.3 mg of **18** with a retention time of 26.3 min on analytical HPLC. MS (FAB) calculated for $C_{68}H_{100}N_{17}O_{24}S$ $[M + H]^+$ 1570; found, 1570.

Glycyl-L-glutamyl-L-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-L-histidylglycyl-L-glutamyl-L-yl-L-glutaminyglycyl-L-prolyl-L-lysine (19, CIIHis). SPSS gave 96 mg of peptide resin and 9.0 mg of pure peptide after preparative HPLC with a retention time of 14.8 min on analytical HPLC. HRMS (FAB) calculated for $C_{65}H_{98}N_{19}O_{22}$ $[M + H]^+$ 1496.7134; found, 1496.7134.

Glycyl-L-glutamyl-L-yl-trans-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-L-tyrosylglycyl-L-glutamyl-L-yl-L-glutaminyglycyl-L-prolyl-L-lysine (20, CII Tyr). SPSS gave 85 mg of peptide resin and 12.0 mg of pure peptide after preparative HPLC with a retention time of 18.1 min on analytical HPLC. HRMS (FAB) calculated for $C_{68}H_{100}N_{17}O_{23}$ $[M + H]^+$ 1522.7178; found, 1522.7178.

L-Glycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-6-N-(2,4-dinitrophenyl)-L-lysylglycyl-L-glutamyl-L-yl-L-glutaminyglycyl-L-prolyl-L-lysylglycyl-L-glutamyl-L-yl-L-proline (21, CII259–273Dnp). SPSS gave 103 mg of peptide resin and 1.5 mg of pure peptide with a retention time of 26.9 min on analytical HPLC after preparative HPLC. HRMS (FAB) calculated for $C_{71}H_{105}N_{20}O_{25}$ $[M + H]^+$ 1637.7560; found, 1637.76.

L-Alanyl-L-isoleucylglycyl-L-alanyl-L-phenylalanyl-6-N-(2,4-dinitrophenyl)-L-lysyl-L-alanylalanylglycyl-L-alanylglycylglycyl-L-alanylglycylglycine (22, CIIAG259–273Dnp). SPSS gave 66 mg of peptide resin and 2 mg of pure peptide with a retention time of 26.3 min on analytical HPLC after

preparative HPLC. HRMS (FAB) calculated for $C_{57}H_{85}N_{18}O_{20}$ $[M + H]^+$ 1341.6187; found, 1341.6190.

L-Alanylglycylglycyl-L-alanyl-L-isoleucylglycyl-L-alanyl-L-phenylalanyl-6-N-(2,4-dinitrophenyl)-L-lysyl-L-alanylalanylglycyl-L-alanylglycylglycine (23, CIIAG256–270Dnp). SPSS gave 67 mg of peptide resin and 4 mg of pure peptide with a retention time of 28.3 min on analytical HPLC after preparative HPLC. HRMS (FAB) calculated for $C_{57}H_{85}N_{18}O_{20}$ $[M + H]^+$ 1341.6187; found, 1341.6190.

Medium. All cells were grown in DMEM supplemented with 10% FCS, 10 μ M β -mercaptoethanol, 10 mM Hepes, penicillin, L-glutamine, and streptomycin in a humidified incubator at 37 $^\circ$ C in 7.5% CO_2 .

Establishment of T-Cell Hybridomas. We made new H-2A^a-restricted T-cell hybridomas by fusing B10.QxDBA/1 F1 lymph node cells (LNCs), primed and challenged with CII_{Lys}Dnp (**3**), with the TCR negative variant of thymoma BW 5147 (28). Eleven hybridomas specific to CII_{Lys}Dnp **3** were identified and cloned several times by limited dilution, until they reached stability, after which they were conserved by freezing, as previously described (29, 30). The hybridomas were tested with the synthesized peptides and found to have similar specificities.

T-Cell Hybridoma Assays. T-cell hybridoma cells (50×10^3) were cocultured with syngeneic splenic antigen-presenting cells (APCs) and antigen in a total volume of 200 μ L in flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) for 24 h. In the experiments, the antigens were titrated, and the APCs (500×10^3) and T-cell hybridomas (50×10^3) were kept constant. We used a sandwich ELISA to determine the IL-2 production in the supernatants using the dissociation-enhanced lanthanide fluoroimmunoassay (DELFA) system (Wallac, Turku, Finland) and recombinant mouse IL-2 as a positive control. All results are the mean values of duplicate cultures. We also measured the content of IL-2 using an indicator murine cytotoxic T-cell line (CTLL). After culturing the T-cell hybridomas, APCs, and antigen for 24 h, 100- μ L aliquots of the supernatants were removed and frozen at -20 $^\circ$ C. To measure the content of IL-2, the supernatants were thawed and cultured with 100×10^3 CTLL cells, dependent on IL-2, in a total volume of 200 μ L for 24 h; the cells were then pulsed with [^{3} H] thymidine for an additional 15–18 h. The cells were harvested in a Micromate 196 cell harvester (Canberra Packard, Meriden, CT) and the radioactivity determined in a Matrix direct β -counter (Canberra Packard). The results are the mean values of duplicate experiments.

Results

Synthesis of Complete Antigens. An initial attempt to synthesize Fmoc-homolysine-Dnp using Fmoc-succinimide at pH 9.5, to avoid the reported risk of racemization at the α -carbon, gave only a low product yield. The Fmoc-homolysine-Dnp **1** derivative used in the SPSS of CII_{Lys}Dnp **6** was obtained by standard synthetic procedure, with Fmoc-Cl used in the synthesis of other lysine derivatives (25), and there was no trace of diastereomeric products according to analytical HPLC or NMR. All SPSS gave the desired pure peptides in reasonable to good yields. Analytical HPLC and NMR revealed that the peptides were pure, and it was even possible to separate peptide **4** with the introduced D-lysine in position 264 from the L-form in peptide **3**. By carefully adjusting the pH conditions and Boc protection of primary amino groups, it was even possible to use the deprotected peptide **10** to synthesize CIIAlaPipDnp **11**. The yield of this Dnp-modified peptide,

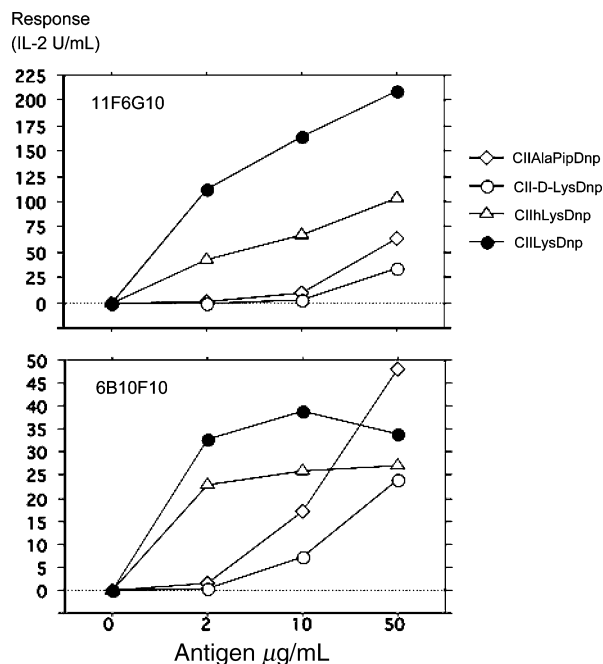


Figure 4. T-cell response as IL-2 production from the CIILysDnp (3)-specific T-cell hybridomas 11F6G10 and 6B10F10 displaying similar, but not identical, specificity for tested peptides. T-cell hybridoma cells (5×10^4 /well) were cocultured with syngeneic spleen cells (5×10^5 /well) as APCs in the presence of titrated concentrations of the modified peptides for 24 h. IL-2 production in the supernatant was assayed using sandwich ELISA with the DELFIA system. Data are represented as the mean values of duplicates.

however, was rather low, though the identity was confirmed by LC-MS and by a characteristic ^1H shift of the methylene signals in the piperidine ring (determined by ^1H NMR; data not shown). A similar approach was used in synthesizing CIILysDnp **16**, and this reaction proceeded very selectively, producing a higher yield (26%). We have previously reported the influence of pH on the reaction outcome, with the addition of electrophilic haptens to nucleophilic amino acids (31, 32). Thus, by selecting the appropriate reaction conditions, selective modifications of amines or thiols can be achieved. In this way, we also prepared the hydroquinone-modified peptide as an example of another hapten capable of inducing ACD.

Specificity of CIILysDnp (3)-Reactive T-Cell Hybridomas. Eleven T-cell hybridomas were isolated, cloned, and conserved after immunization of F1 mice (B10.Q $\times$ DBA/1) with CIILysDnp **3** (28). These T-cell hybridomas were selected according to their reactivity toward CIILysDnp in culture, by measuring their IL-2 production when cocultured with spleen cells as APCs. All of the tested hybridomas displayed a similar strong reactivity toward the immunized peptide. We found only small differences in specificity among the 11 hybridomas tested with the Dnp-modified peptides. The responses of two selected hybridomas, 11F6G10 and 6B10F10, are presented in Figure 4, and the data of all 11 clones are given in Supporting Information.

The structures of selected CII peptides and modifications are shown in Figures 1 and 2. None of the hybridomas cross-reacted with the unmodified CII peptides or with peptides having a lysine side chain at position 264 modified by hydroxylation or galactosylation (25). We found a strong specificity for the L-form of CIILysDnp **3** used at the sensitization and only a weak response to the corresponding D-form **4** (Figure 4). The response to the Dnp-containing peptides correlated well with the Dnp bound to amino acid residues having a minimum length of lysine

Table 2. CII Peptides Used to Study the Side Chain Requirements of T-Cell Hybridomas Obtained from CIILysDnp (3)-Immunized Mice^a

peptide	immune response	
	CIILysDnp-specific T-cell hybridomas	CIILysDnp-specific T-cell hybridomas (cross-reactivity)
CII256–270 (2)	–	–
CIILysDnp (3)	+	–
CII-D-LysDnp (4)	+	–
CIILys (5)	–	–
CIILysDnp (6)	+	–
CIIOrn (7)	–	–
CIIOrnDnp (8)	–	–
CIIArg (9)	–	–
CIIAlaPip (10)	–	–
CIIAlaPipDnp (11)	+	–
CII262–267Dnp (12)	–	–
CII260–266Dnp (13)	–	–
CIILysMe ₂ (14)	–	–
CIILysCys (15)	–	–
CIILysCysDnp (16)	–	–
CIIHis (17)	–	–
CIIHisHQ (18)	–	–
CIIHis (19)	–	–
CIIHis (20)	–	–
CII259–273Dnp (21)	+	–
CIIAG259–273Dnp (22)	–	–
CIIAG256–270Dnp (23)	–	–
CIILysGal ^b	–	+

^a T-cell hybridoma cells (5×10^4 /well) were co-cultured with syngeneic spleen cells (5×10^5 /well) as APCs in the presence of titrated concentrations of the peptides listed in Table 2 for 24 h. The IL-2 produced by the stimulated T-cell hybridoma cells was determined as the proliferation of CTLL cells, and cpm were counted. We also tested the possibility of cross-reactivity between the CII-glyco-specific T-cell hybridomas and Dnp-modified CII peptides. The T-cell hybridomas, HCQ.10, HDBR 1, HM1R1, HCQ.11, and HM2.2b, were obtained in CIA and chosen to represent five different subgroups having different fine specificities for the galactose moiety. In total, 5×10^4 /well were co-cultured with syngeneic spleen cells and 5×10^5 /well with titrated concentrations of antigen. The IL-2 produced by the stimulated T-cell hybridomas was determined as the proliferation of CTLL cells. The results are the means of duplicate cultures. ^b CIILysGal prepared in a previous work (25).

in the side chain at position 264. The one-carbon-shorter chain at position 264 was achieved by using ornithine-Dnp. This antigen did not stimulate the T-cell receptor at all. However, by using a one-carbon-longer chain, as in homolysine, stimulation close to that observed for the original antigen (CIILysDnp **3**) was registered (Table 2). The piperidine analogue CIIAlaPipDnp **11**, with a side chain of length similar to that of lysine, also gave a similar response compared to **3** as shown in Figure 4. The CII260–266Dnp **13** and CII262–267Dnp **12** peptides with shorter amino acid sequences produced no T-cell response, nor did the alanine- and glycine-substituted CIIAG256–270Dnp **23**, or the CIIAG259–273Dnp **22** antigen. However, the CII259–273Dnp **21** peptide gave a T-cell response comparable with that of the CIILysDnp **3** peptide used at immunization.

Are Glycospecific T-Cell Hybridomas Cross-Reacting to Haptenized Peptides? To test whether the T cells recognizing the nonmodified CII peptides are specific to naturally post-translationally modified peptides (i.e., hydroxylated and galactosylated at the position 264 lysine side chain), we selected five previously characterized T-cell hybridomas. These hybridomas, obtained from CII immunized mice, represent different types of specificities (19) of the various post-translational modifications of lysine 264. In particular, the galactose is the immunodominant structure, and this monosaccharide can be recognized differently by different T-cell hybridomas depending on their

T-cell receptor. Table 2 shows that the glyco-specific T-cell hybridomas are strictly antigen specific, with no cross-reactivity toward the CII peptide with the lysine 264-Dnp-modified side chains.

MHC Restriction of the CIILysDnp (3) Hybridoma Cells.

The CIILysDnp 3-reactive T-cell hybridoma cells were stimulated with spleen cells from H-2A^q, -A^r, or -A^p-expressing mice, together with the CIILysDnp 3 peptide. As shown in Figure 5, the H-2A^q-expressing spleen cells efficiently present the peptide and stimulate the T-cell hybridomas. The H-2A^r-expressing spleen cells could not present the peptide, thus indicating MHC restriction. However, even though the T-cell hybridomas were made in H-2A^q-restricted mice, the CIILysDnp 3 peptide with spleen cells from H-2A^p mice produced a response, thus overriding MHC restriction.

Discussion

This study was designed to investigate how T cells recognize haptens in an antigen-specific and MHC-restricted way. Although nearly 4000 haptens have been chemically completely defined, the various steps in the mechanism of contact hypersensitivity, on a chemical basis, are essentially unknown or based on hypotheses (2, 11, 23). The reactive proteins and the amino acid sequences of the carrier peptides binding the haptens are unknown, and the defined chemical structures of the complete antigens formed are also unknown. In an elegant series of works, Weltzien et al. used trinitrophenyl (Tnp)-modified carrier peptides as a tool to study the T-cell responses to synthetic antigens (33–35). They found it was essential that the Tnp-modified lysine residue occupy a central position, though the amino acid sequence and length of the carrier peptide could vary without loss of T-cell response. However, they used Tnp-modified lysine in all of their synthetic antigens and did not replace the hapten-binding amino acid residue with other structures or study cross-reactivity toward other structurally related haptens. In our study, we have used chemically well-defined antigens with the Dnp bound to the ϵ -amine of lysine at position 264 in the central position of immunodominant CII peptides to stimulate the T-cell receptors of hybridoma cells. We synthesized CIILysDnp 3 as a complete antigen with which to immunize mice and produce hybridoma cells with a T-cell receptor specific to this antigen. These T-cell hybridomas were used to investigate how small changes in the chemical structure, for example, changes in the length of the hapten-binding amino acid side chain in position 264, influenced the T-cell response. We synthesized the new building block 1 and an array of 22 new synthetic complete antigens to evaluate the immune responses to these antigens. We changed the stereochemical structure by replacing L-lysine with its D-isomer and by altering the hapten-binding side chain of the amino acid, making them one-carbon-shorter and one-carbon-longer structures. We also studied the results when the Dnp group was bound to the amine of a less flexible piperidine ring structure. We found a high specificity and a strong response for the CIILysDnp 3 antigen used for sensitization in all of the studied T-cell hybridomas, but only a weak response to the corresponding D-form. Only the piperidine analogue, i.e., CIIAlaPipDnp 11, and the peptide with a one-carbon-longer Dnp-binding chain CIIhLysDnp 6, i.e., homolysine, produced stimulation similar to that of the CIILysDnp 3 antigen in the T-cell hybridomas. We also replaced lysine with homocysteine since most electrophilic haptens, such as DNFB, have a stronger reactivity toward thiols than toward amines (31, 32). However, homocysteine is two carbons shorter than lysine, and the CIIhCysDnp 16 peptide did not stimulate

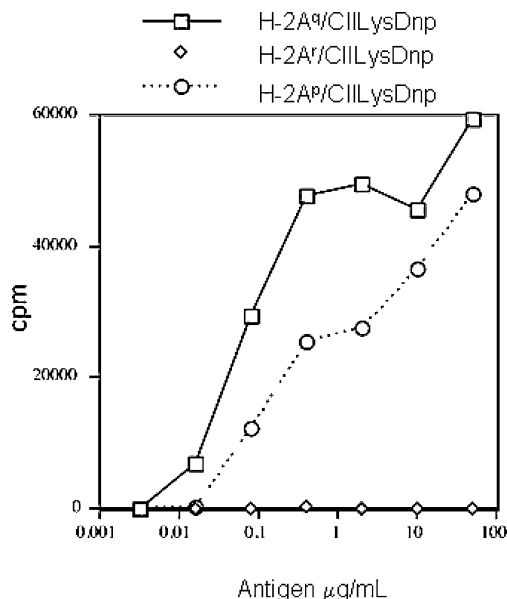


Figure 5. MHC class II specificity of the CIILysDnp (3)-restricted T-cell hybridoma. T-cell hybridoma cells (5×10^4 /well) were cocultured with spleen cells as APCs from (□) H-2A^q-restricted mice, (◇) H-2A^r -, or (○) H-2A^p-restricted mice for 24 h. The level of the IL-2 production of the T-cell hybridomas was determined as proliferation of CTLL cells, and cpm were counted. Data are represented as the mean values of duplicates.

the T-cell hybridomas at all. By binding the Dnp to a piperidine-type ring structure, the mobility of the bound hapten will be decreased while the length of the carbon chain is comparable to that of the lysine side chain. Furthermore, the amine will be a tertiary rather than a secondary amine, which is the case with lysine haptenization. The availability of the free electron pair of the nitrogen atom differs between the two types of amines, which can also be of importance in stimulating the T-cell receptor. This antigen with Dnp bound to the more rigid piperidine ring produced a stronger response in one T-cell hybridoma than did the homolysine-Dnp antigen with the one-carbon-longer side chain, though at a higher initial antigen concentration.

We can now also confirm that the carrier peptide CII 256–270 is important in anchoring the peptide on MHC class II molecules since the peptides CII260–266Dnp 13 and CII262–267Dnp 12, which have shorter amino acid sequences, do not bind MHC properly or cannot elicit a sufficient T-cell response (22); this was also the result obtained with the alanine- and glycine-substituted antigens, CIIAG256–270Dnp 23 and CIIAG259–273Dnp 22. The positive T-cell response from CII259–273Dnp 21 also confirms our earlier studies that the backbone of this peptide sequence binds H-2A^q (20, 30). The observation that T-cell recognition requires a specific length of the lysine 264 side chain indicates that the carrier peptide is deeply buried in the MHC molecule and that the side chain must be long enough to reach the TCR. This confirms earlier studies using the glycosylated or unmodified peptides bound to the H-2A^q molecule using mutations in the peptide and is also supported by a quantitative structure–activity relationship analysis on how the glycopeptides bind to H-2A^q (20, 30).

The strong affinity of the CIILysDnp 3 antigen for the MHC class II H-2A^q molecule has been noted. In experiments with CIILysDnp-reactive T-cell hybridoma cells, we also stimulated these cells with spleen cells from H-2A^r or -A^p-expressing mice, together with the CIILysDnp peptide 3. The H-2A^r-expressing spleen cells could not present the peptide, thus demonstrating

MHC restriction. However, even though the T-cell hybridomas were from H-2A^q-restricted mice, the CII_{Lys}Dnp peptide **3** and spleen cells from H-2A^p mice produced a T-cell response, thus overriding MHC restriction (Figure 5). Similar phenomena have earlier been noted for other CII peptides (**20**) and could be explained by the α/β chain differing at only four amino acids between H-2A^q and H-2A^p; this causes binding of the CII peptide by both class II molecules, though the binding is poorer with H-2A^p than with H-2A^q.

Importantly, glycosylated CII peptides are endogenously processed, and the post-translationally modified peptides are then selected for presentation; thus, the modifications on the lysine side chain is not removed (**36**, **37**). We have earlier demonstrated that these are the major recognition structures for the CII autoantigen in CIA and in RA (**14**). The question arises whether T cells recognizing Dnp-modified collagen could cross-react to glycosylated collagen (CIA and RA autoantigen, Figure 1) and vice versa or whether the fine specificity is more discriminative than promiscuous. We were able to demonstrate that T cells discriminate between these structures, i.e., Dnp-specific T cells do not cross-react with glycopeptides, and glyco-specific T cells associated with CIA or RA do not cross-react with Dnp or the hydroquinone-modified peptides. In addition, the hybridomas were strictly Dnp-specific as shown in Table 2, displaying no reactivity toward either the unmodified peptides (**2**, **5**, **7**, **9**, **10**, **15**, **17**, **19**, and **20**) or the CII_{Lys}Me₂ peptide **14** and displaying only a weak response to the CII-D-LysDnp peptide **4** having a side chain in the stereoisomeric D-form.

Our results thus demonstrate the very fine specificity of the antigen recognition process. Clearly, a specific chemical structure must be presented to the T cells in order to elicit an immune response, and a small difference in antigen chemical structure will result in the loss of T-cell response. In this model of ACD, we have access to an immunological synapse with a Dnp-specific T-cell receptor and an antigen-presenting cell having a defined MHC II molecule on its surface with known affinity for the carrier peptide used. We thus have stable and completely defined conditions for every parameter and, by synthesizing other complete antigens with small chemical changes in the displayed hapten-modified peptides, can define the specificity of the T-cell receptor and its capacity to cross-react. Our results will be significant for the general understanding of how other haptens causing ACD are recognized and presented to T-cell receptors. This may contribute to the development of new drugs for treating ACD based on epitope-specific T-cell therapy.

Acknowledgment. This investigation was supported by grants from the Swedish Council for Working Life and Social Research, the Swedish Asthma and Allergy Association's Research Foundation, the Crafoord, Kock, Welander-Finsen, and Österlund foundations, the Swedish Association against Rheumatism, the Swedish Science Research Council, and the Strategic Science Foundation, as part of EU projects LSHB-CT-2006-018661 (AUTOCURE) and LSHG-CT-2005-005203 (MUGEN). We thank Carlos Palestro for technical assistance with the animal experiments, Ola Bergendorff for productive discussions, and Karl-Erik Bergquist for setting up the 500 MHz NMR experiments and discussions.

Supporting Information Available: Test results of 11 T-cell hybridomas. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Thyssen, J. P., Linneberg, A., Menne, T., and Johansen, J. D. (2007) The epidemiology of contact allergy in the general population—prevalence and main findings. *Contact Derm.* **57**, 287–299.
- (2) Lepoittevin, J.-P. (2006) Molecular Aspects of Allergic Contact Dermatitis. In *Contact Dermatitis*, 4th ed. (Frosch, P. J., Menne, T., and Lepoittevin, J.-P., Eds.) pp 45–66, Springer, Berlin/Heidelberg.
- (3) Eisen, H. N. (2001) Specificity and degeneracy in antigen recognition: Yin and Yang in the immune system. *Ann. Rev. Immunol.* **19**, 1–21.
- (4) Smith, C. H., and Hotchkiss, S. A. M. (2001) *Allergic Contact Dermatitis: Chemical and Metabolic Mechanisms*, Taylor and Francis, London/New York.
- (5) Watanabe, H., Unger, M., Tuvel, B., Wang, B., and Sauder, D. N. (2002) Contact hypersensitivity: The mechanism of immune responses and T cell balance. *J. Interferon Cytokine Res.* **22**, 407–412.
- (6) O'Leary, J. G., Goodarzi, M., Drayton, D. L., and von Andrian, U. H. (2006) T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat. Immunol.* **7**, 507–516.
- (7) Kimber, I., and Dearman, R. J. (2002) Allergic contact dermatitis: The cellular effectors. *Contact Derm.* **46**, 1–5.
- (8) Vocanson, M., Hennino, A., Cluzel-Tailhardat, M., Saint-Mezard, P., Benetiere, J., Chavagnac, C., Berard, F., Kaiserlian, D., and Nicolas, J.-F. (2006) CD8+ T cells are effector cells of contact dermatitis to common skin allergens in mice. *J. Invest. Dermatol.* **126**, 815–820.
- (9) Gorbachev, A. V., and Fairchild, R. L. (2004) CD4+ T cells regulate CD8+ T cell-mediated cutaneous immune responses by restricting effector T cell development through a Fas ligand-dependent mechanism. *J. Immunol.* **172**, 2286–2295.
- (10) Cemerski, S., and Shaw, A. (2006) Immune synapses in T-cell activation. *Curr. Opin. Immunol.* **18**, 298–304.
- (11) Rustemeyer, T., van Hoogstraten, I. M. W., von Blomberg, B. M. E., and Scheper, R. J. (2006) Mechanisms in Allergic Contact Dermatitis. In *Contact Dermatitis*, 4th ed. (Frosch, P. J., Menne, T., and Lepoittevin, J.-P., Eds.) pp 11–33, Springer, Berlin/Heidelberg.
- (12) Pickard, C., Smith, A. M., Hywel, C., Strickland, I., Jackson, J., Healy, E., and Friedmann, P. S. (2006) Investigation of mechanisms underlying the T-cell response to the hapten 2,4-dinitrochlorobenzene. *J. Invest. Dermatol.* **137**, 630–637.
- (13) Wang, B., Fujisawa, H., Zhuang, L., Freed, I., Howell, B. G., Shahid, S., Shivji, G. M., Mak, T. W., and Sauder, D. N. (2000) CD4+ Th1 and CD8+ type 1 cytotoxic T cells both play a crucial role in the full development of contact hypersensitivity. *J. Immunol.* **165**, 6783–6790.
- (14) Bäcklund, J., Carlsen, S., Höger, T., Holm, B., Fugger, L., Kihlberg, J., Burkhardt, H. L., and Holmdahl, R. (2002) Predominant selection of T cells specific for the glycosylated collagen type II epitope (263–270) in humanized transgenic mice and in rheumatoid arthritis. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 9960–9965.
- (15) Rosloniec, E. F., Brand, D. D., Myers, L. K., Esaki, Y., Whittington, K. B., Zaller, D. M., Woods, A., Stuart, J. M., and Kang, A. H. (1998) Induction of autoimmune arthritis in HLA-DR4 (DRB1* 0401) transgenic mice by immunization with human and bovine type II collagen. *J. Immunol.* **160**, 2573–2578.
- (16) Michaëlsson, E., Andersson, M., Engström, A., and Holmdahl, R. (1992) Identification of an immunodominant type-II collagen peptide recognized by T-cells in H-2q mice: Self tolerance at the level of determinant selection. *Eur. J. Immunol.* **22**, 1819–1825.
- (17) Michaëlsson, E., Malmström, V., Reis, S., Engström, A., Burkhardt, H., and Holmdahl, R. (1994) T-Cell recognition of carbohydrates on type-II collagen. *J. Exp. Med.* **180**, 745–749.
- (18) Michaëlsson, E., Broddefalk, J., Engström, A., Kihlberg, J., and Holmdahl, R. (1996) Antigen processing and presentation of a naturally glycosylated protein elicits major histocompatibility complex class II-restricted, carbohydrate-specific T cells. *Eur. J. Immunol.* **26**, 1906–1910.
- (19) Corthay, A., Backlund, J., Broddefalk, J., Michaëlsson, E., Goldschmidt, T. J., Kihlberg, J., and Holmdahl, R. (1998) Epitope glycosylation plays a critical role for T cell recognition of type II collagen in collagen-induced arthritis. *Eur. J. Immunol.* **28**, 2580–2590.
- (20) Kjellen, P., Brunsberg, U., Broddefalk, J., Hansen, B., Vestberg, M., Ivarsson, I., Engstrom, A., Svejgaard, A., Kihlberg, J., Fugger, L., and Holmdahl, R. (1998) The structural basis of MHC control of collagen-induced arthritis; binding of the immunodominant type II collagen 256–270 glycopeptide to H-2A(q) and H-2A(p) molecules. *Eur. J. Immunol.* **28**, 755–767.
- (21) Andersson, E. C., Hansen, B. E., Jacobsen, H., Madsen, L. S., Andersen, C. B., Engberg, J., Rothbard, J. B., Sonderstrup McDevitt, G., Malmstrom, V., Holmdahl, R., Svejgaard, A., and Fugger, L. (1998) Definition of MHC and T cell receptor contacts in the HLA-DR4 restricted immunodominant epitope in type II collagen and characterization of collagen-induced arthritis in HLA-DR4 and human CD4 transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7574–7569.

- (22) Holm, L., Kjellen, P., Holmdahl, R., and Kihlberg, J. (2005) Identification of the minimal glycopeptide core recognized by T cells in a model for rheumatoid arthritis. *Bioorg. Med. Chem.* **13**, 473–482.
- (23) Landsteiner, K., and Jacobs, J. (1936) Studies on the sensitization of animals with simple chemical compounds. *J. Exp. Med.* **64**, 629–639.
- (24) Rigby, S. E. J., Burch, A. M., and Moore, G. R. (1991) ^1H NMR determination of the ionization constant of a carboxylic acid group of a haem protein in mixed aqueous-organic solvents using the Super-WEFT sequence. *Magn. Reson. Chem.* **29**, 1036–1039.
- (25) Broddefalk, J., Bäcklund, J., Almqvist, F., Johansson, M., Holmdahl, R., and Kihlberg, J. (1998) T cells recognize a glycopeptide derived from type II collagen in a model for rheumatoid arthritis. *J. Am. Chem. Soc.* **120**, 7676–7683.
- (26) Carpino, L. A., and Han, G. Y. (1972) The 9-fluorenylmethoxycarbonyl amino-protecting group. *J. Org. Chem.* **37**, 3404–3409.
- (27) Scott, J. W., Parker, D., and Parrish, D. R. (1982) Improved syntheses of N ϵ -tert-butyloxycarbonyl-L-lysine and N α -benzyloxycarbonyl-N ϵ -tert-butyloxycarbonyl-L-lysine. *Synth. Commun.* **11**, 303–314.
- (28) White, J., Blackman, M., Bill, J., Kappler, J., Marrack, P., Gold, D. P., and Born, W. (1989) Two better cell lines for making hybridomas expressing specific T-cell receptors. *J. Immunol.* **143**, 1822–1825.
- (29) Michaelsson, E., Holmdahl, M., Engstrom, A., Burkhardt, H., Scheynius, A., and Holmdahl, R. (1995) Macrophages, but not dendritic cells, present collagen to T-cells. *Eur. J. Immunol.* **25**, 2234–2241.
- (30) Holm, L., Frech, K., Dzhabazov, B., Holmdahl, R., Kihlberg, J., and Linusson, A. (2007) Quantitative structure-activity relationship of peptides binding to the class II major histocompatibility complex molecule A(q) associated with autoimmune arthritis. *J. Med. Chem.* **50**, 2049–2059.
- (31) Ahlfors, S. R., Sterner, O., and Hansson, C. (2003) Reactivity of contact allergenic haptens to amino acid residues in a model carrier peptide, and characterization of formed peptide-hapten adducts. *Skin Pharm. Appl. Skin Phys.* **16**, 59–68.
- (32) Ahlfors, S. R., Kristiansson, M. H., Lindh, C. H., Jönsson, B. A. G., and Hansson, C. (2005) Adducts between nucleophilic amino acids and hexahydrophthalic anhydride, a structure inducing both types I and IV allergy. *Biomarkers* **10**, 321–335.
- (33) Martin, S., Ortmann, B., Pflugfelder, U., Birsner, U., and Weltzien, H. U. (1992) Role of hapten-anchoring peptides in defining hapten-epitopes for MHC-restricted cytotoxic T cells. Cross-reactive TNP-determinants on different peptides. *J. Immunol.* **149**, 2569–2575.
- (34) Weltzien, H. U. (1995) How T cells recognize haptens. *Am. J. Contact Dermatitis* **6**, 176–180.
- (35) Martin, S., Delattre, V., Leicht, C., Weltzien, H. U., and Simon, J. C. (2003) A high frequency of allergen-specific CD8 $^{+}$ Tc1 cells is associated with the murine immune response to the contact sensitizer trinitrophenyl. *Exp. Dermatol.* **12**, 78–85.
- (36) Dzhabazov, B., Holmdahl, M., Yamada, H., Lu, S., Vestberg, M., Holm, B., Johnell, O., Kihlberg, J., and Holmdahl, R. (2005) The major T cell epitope on type II collagen is glycosylated in normal cartilage but modified by arthritis in both rats and humans. *Eur. J. Immunol.* **35**, 357–366.
- (37) Holmdahl, M., Grubb, A., and Holmdahl, R. (2004) Cysteine proteases in Langerhans cells limits presentation of cartilage derived type II collagen for autoreactive T cells. *Int. Immunol.* **16**, 717–726.

TX8001077

Peptide	T-cell hybridoma										
	11F6G10	6B10F10	11F6E6	11F6E5	6B10G5	11F6D6	11F6D11	11F6C1	11F3G9	12B4G5	1F3D7
CII (2)	0	0	0	0	0	0	0	0	0	0	0
CII LysDnp (3)	++	++	++	++	++	++	++	++	++	++	++
CII-D-LysDnp (4)	+	+	+	+	nt ^a	nt	nt	nt	nt	nt	nt
CIIhLys (5)	0	0	0	0	0	0	0	0	0	0	0
CIIhLysDnp (6)	++	++	++	++	nt	nt	nt	nt	nt	nt	nt
CIIOrn (7)	0	0	0	0	0	0	0	0	0	0	0
CIIOrnDnp (8)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CIIArg (9)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CIIAlaPip (10)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CIIAlaPipDnp (11)	++	++	++	++	nt	nt	nt	nt	nt	nt	nt
CII262-267Dnp (12)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CII260-267Dnp (13)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CII LysMe ₂ (14)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CIIhCys (15)	0	0	0	0	0	0	0	0	0	0	0
CIIhCysDnp (16)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CII Cys (17)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CII CysHQ (18)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CIIHis (19)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CII Tyr (20)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CII259-273Dnp (21)	++	++	++	++	nt	nt	nt	nt	nt	nt	nt
CIIAG259-273Dnp (22)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CIIAG256-270Dnp (23)	0	0	0	0	nt	nt	nt	nt	nt	nt	nt
CIIHylGal	0	0	0	0	0	0	0	0	0	0	0

Supplement Table.

In total 11 T cell hybridomas were isolated and tested. They were all screened with a standard set of peptides and all were found to respond similarly. Four of the hybridomas (11F6G10, 6B10F10, 11F6E6, 11F6E5) were subsequently tested with all available peptides. ^ant=not tested.