Brief Definitive Report

T Cell Recognition of Carbohydrates on Type II Collagen

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Summary

A critical event in an immune response is the T cell recognition of peptides bound to major histocompatibility complex (MHC) molecules on the surface of an antigen presenting cell (APC). Although the majority of eukaryotic proteins are glycosylated, it has not yet been shown that T cell recognition of such proteins involves recognition of the bound carbohydrates. Type II collagen (CII), the major protein constituent of joint cartilage, is posttranslationally modified by hydroxylation and glycosylation of lysines. In this report we show that posttranslational modifications of the immunodominant peptide CII(256-270) generate a structural determinant that is distinct from the determinant represented by the corresponding synthetic peptide. Elimination of carbohydrates, present on CII, by two different biochemical methods revealed that the carbohydrates, O-linked to the hydroxylysines within the CII(256-270) determinant, were crucial for the reactivity towards the posttranslationally modified peptide. Furthermore, a T cell hybridoma specific for the glycosylated determinant was stimulated by tryptic CII-peptides presented by fixed APCs, thus showing that the carbohydrates are involved in the trimolecular complex T cell receptor/peptide/MHC. Finally, the importance of the bound carbohydrates for the arthritogenicity of CII was investigated by comparing the development of arthritis after immunization with carbohydrate-depleted and glycosylated CII, respectively. Incidence, time of onset, and severity of the disease were significantly affected by the elimination of carbohydrates, whereas no significant difference in anti-CII antibody titers was seen.

In autoimmune disease, T cells recognize peptides from proteins that are located to specific target tissues. An important question is whether the development of autoimmune disease is associated with T cell recognition of posttranslationally altered peptides, i.e., peptides that the T cells have not yet recognized as self. One such posttranslational modification could be glycosylation, although T cell recognition of naturally glycosylated peptides has not yet been described. One well-defined tissue-specific protein which is glycosylated is type II collagen (CII). Immunization of mice with rat CII leads to an antigen-specific and MHC class II (A^q)–restricted immune response (1). This eventually results in arthritis due to a cross-reactive autoimmune response to CII in cartilage, the so-called collagen-induced arthritis (CIA) (1, 2). The CNBr-cleaved fragment CB11 of CII harbors determinants that elicit immunodominant T cell responses (3). Furthermore, vaccination with CB11 suppresses CIA (4). Using CII-primed lymph node cells and CII-reactive T cell hybridomas, we have previously identified an immunodominant peptide (positions 256-270 of CII), present on CB11 (5). However, several T cell hybridomas, which did respond to CB11, did not respond to the synthetic peptide CII(256-270) or to overlapping synthetic peptides covering this amino acid stretch (5). Collagen is posttranslationally modified by hydroxylation of proline and lysine residues, if these are situated at certain positions (G-X-P and G-X-K, respectively). If hydroxylated, lysine residues can also be glycosylated, resulting in either β-D-galactopyranosyl hydroxylysine or α-D-glucopyranosyl-(1 → 2)-β-D-galactopyranosyl hydroxylysine. Since two lysine residues susceptible to hydroxylation and glycosylation are located in the immunodominant CII(256-270) peptide (see Fig. 3), we speculated that such posttranslational modifications of CII could explain the failure of synthetic peptides to stimulate T cells and that they might be of critical importance for the T cell reactivity towards this immunodominant peptide and possibly also for the development of CIA.

Materials and Methods

Rat CII Amino Acid Sequence. The rat CII sequence was obtained from mRNA prepared from limbs of newborn DA rats.

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cDNA was prepared using the cDNA kit (Pharmacia, Uppsala, Sweden). PCR reactions were performed using CII-specific primers of human, mouse, and rat origin and direct sequencing was performed using Dynabeads™ M-280 Streptavidin (Dynal, Oslo, Norway) as earlier described (6).

Establishment of T Cell Hybridomas and Measurement of T Cell Hybridoma Response. A^q-restricted T cell hybridomas were established from rat CII-primed C3H.Q lymph node cells by fusion with BW 5147 $\alpha^-\beta^-$ (5, 7). The reactivity of the T cell hybridomas were determined using cytotoxic T lymphocyte line (CTLL) (8) as evaluation of IL-2 produced (5). CTLL were harvested in a Filtermate™ cell harvester (Packard Instruments, Meriden, CT). The incorporation of [^{3}H]TdR was determined in a matrix 96™ Direct Beta Counter (Packard). All results are mean values of triplicate cultures, except for the results in Fig. 4 which are duplicates.

Antigens. For construction of the mutant Col1A1 gene (pWTCk8 kol7.11) a derivative (pWTC1) of the murine Col1A1 genomic clone 10D (9, 10) was kindly provided by Dr. Hong Wu (Whitehead Institute, Boston, MA). For cassette mutagenesis of the Col1A1 gene, a 3-kb fragment of pWTC1 was excised and cloned in a modified pGEM 11zf$^+$ vector (Promega, Madison, WI). Between a single SacI and BstEII site, the wild-type sequence containing the complete exon 43 of Col1A1 (encoding aa 856-891 of CI) was excised. Two complementary oligonucleotides, encoding CII (aa 256-270), were synthesized (122 and 113 bp, respectively) and annealed, giving rise to a fragment with SacI and BstEII compatible ends. After insertion of the mutated construct, the 3-kb fragment was reintroduced into the genomic Col1A1 clone. The mutated Col1A1 gene was transfected along with the selection marker pNEO (neomycin resistance gene; Pharmacia) into NIH 3T3 fibroblasts at a molar ratio of 5:1. After selection in 0.4 mg/ml G418, individual clones of the transfected NIH 3T3 cells were isolated and screened for expression of the mutant Col1A1 gene by PCR analysis of the reversed transcribed RNA. Wild-type I collagen (from fibroblasts transfected with pNEO only) and collagen containing mutant α1(I) chains (pWTCk8 kol7.11) were prepared from the NIH 3T3 cells after limited digestion of proteins with pepsin. T cell reactivity towards pWTCk8 kol7.11 required that the transfected fibroblasts had been cultured in the presence of ascorbic acid (cofactor for lysylhydroxylase) (data not shown).

Rat CII was prepared from the Swarm chondrosarcoma (11) by pepsin digestion (12). Sodium periodate oxidation of CII was performed by incubating CII, 5 mg/ml, in 0.05 M sodium acetate, pH 4.5, containing 0.02 M sodium periodate ($NaIO_4$, Sigma Chemical Co., St. Louis, MO) for 24 h at 4°C in darkness (13), after which a molar excess of glycol was added and the material dialyzed against 0.1 M acetic acid. Trifluoromethanesulphonic acid (TFMSA) cleavage was performed by incubating CII in TFMSA and anisole (2:1 ratio) for 3 h on ice, or for 3 h on ice followed by 1 h at room temperature (14). The two different TFMSA cleavage protocols were used because they have been shown to result in different degrees of carbohydrates elimination (14, 15). All collagens used in cell cultures were denatured at 50°C for 30 min before use. The synthetic peptide CII(256-270) was assembled as previously described (5).

Preparation and Presentation of Tryptic CB11-Peptides. CB11 was prepared by fractioning CNBr-treated rat CII by ion exchange chromatography on a Mono S column (Pharmacia), equilibrated with 20 mM sodium citrate, pH 3.4, and eluted with a gradient of sodium chloride. CB11-containing fractions were further purified by gel filtration on a Superdex 75 column eluted with 0.1 M NH_4HCO_3. CB11 was digested with trypsin (1:50 wt/wt, 0.1 M NH_4HCO_3, for 4 h at 37°C) and the digest separated by reversed phase chromatography (model 218TP; Vydac, Hesperia, CA). The material was eluted with a gradient of acetonitril in 0.1% TFA/water, 0–40% acetonitril in 60 min, and fractions collected every 0.5 min. The fractions were dried and redissolved in 0.1 M acetic acid. Equal volumes of each fraction were added to 5×10^5 (B10.QxDBA/1)F_1 NH_4Cl-treated spleen cells, fixed with 0.15% formaldehyde, in duplicate cultures. T cell hybridoma response was determined as described above. Amino acid sequence analysis (ABI 477A) of T cell stimulatory fractions revealed the presence of NH_2-terminal parts of T1 in fractions 66–70 and of peptides corresponding to cleavage after K_{252} in T1 in fractions 61–63. Fractions 65–71 were analyzed by laser desorption on a mass spectrometer (model MALDI II; Kratos Analytical Instruments, Ramsey, NJ) using 3,5-Dimethoxy-4-hydroxy-cinnamic acid as matrix. In fractions 67–69, masses corresponding to Na-adduct ions of T1 with different states of glycosylation were found.

Induction and Evaluation of Arthritis. (B10.QxDBA/1)F_1 male mice were immunized with 50 μg rat CII or periodate-treated rat CII in CFA (Difco Laboratories, Inc., Detroit, MI) intradermally at day 0, a procedure previously described (16). The results from two experiments using identical protocols and with balanced groups are shown. Development of arthritis was followed for 70 d. Sera from day 35 were analyzed with usage of an earlier described quantitative ELISA (16). No significant difference in anti-CII antibody serum levels was seen. Statistical significances are calculated with the Mann-Whitney U test (onset, severity, and antibody levels) or with the Chi Square test using Yates correction (incidence).

Results and Discussion

To address the role of posttranslational modifications for the T cell reactivity towards the CII(256-270) peptide, an oligonucleotide coding for CII(256-270) was inserted into the gene encoding the α1 chain of mouse type I collagen,

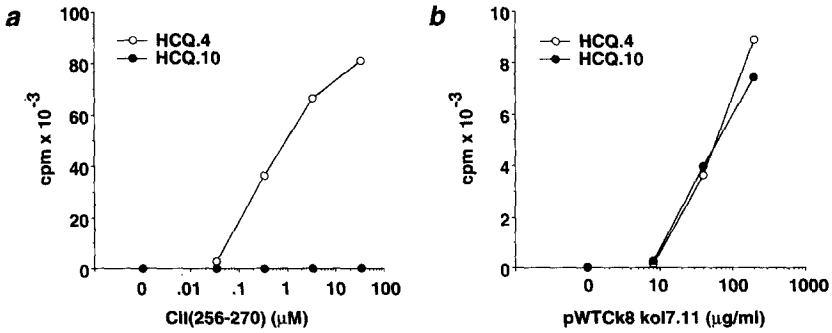

Figure 1. T cell recognition of the immunodominant peptide CII(256-270). (*a*) Response of HCQ.4 (○) and HCQ.10 (●) towards the synthetic peptide CII(256-270); (*b*) response of HCQ.4 and HCQ.10 to the mutated type I collagen pWTCk8 kol7.11 (containing the nucleotides encoding CII[256-270]). The response towards 200 μg/ml pNEO (collagen prepared from 3T3 fibroblasts transfected with geneticin resistance gene only) was <50 cpm for both T cell hybridomas (data not shown).

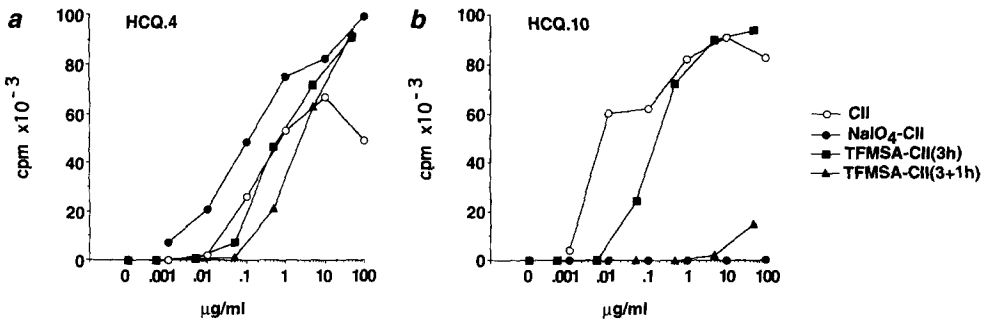

Figure 2. T cell recognition of carbohydrate-depleted CII. The response of HCQ.4 (*a*) and HCQ.10 (*b*) towards rat CII (○), rat CII cleaved with TFMSA for 3 h on ice (■), rat CII cleaved with TFMSA for 3 h on ice plus 1 h in room temperature (▲) or rat CII cleaved with periodate (●). The response towards periodate-treated pWTCk8 kol7.11 displayed similar results, i.e., HCQ.4 responded to periodate-treated pWTCk8 kol7.11, whereas HCQ.10 did not (data not shown).

which lacks antigenicity in this system. The resulting mutated gene construct was transfected into NIH 3T3 fibroblasts. The mutated type I collagen, termed pWTCk8 kol7.11, was prepared from these fibroblasts and tested for antigenicity, using CB11-reactive T cell hybridomas. As shown in Fig. 1, the T cell hybridoma HCQ.4 (reactive with the synthetic peptide CII[256-270]), as well as HCQ.10 (reactive with CB11, but not with the synthetic peptide CII[256-270]), responded to pWTCk8 kol7.11. Accordingly, the amino acid stretch CII(256-270) contains two distinct T cell determinants, one of which is represented by the synthetic peptide and the other which seems to be dependent on posttranslational modifications of CII.

The importance of the hydroxylysine-linked carbohydrates for the reactivity towards CII(256-270) was determined by treating CII with either sodium periodate ($NaIO_4$) or TFMSA. Periodate efficiently cleaves carbohydrates, but can also cleave the side chain of nonglycosylated hydroxylysine residues (13). The use of TFMSA, on the other hand, makes it possible to cleave O-linked carbohydrates without affecting the amino acid side groups (14, 15). This could be confirmed by amino acid analysis of the periodate- and TFMSA-treated CII; periodate treatment resulted in a loss of ~50% of the hydroxylysines, whereas TFMSA treatment did not affect the hydroxylysine content (data not shown). As shown in Fig. 2, HCQ.4 recognized periodate- and TFMSA-treated CII whereas the response of HCQ.10 was abolished by periodate treatment and significantly reduced by TFMSA treatment of CII.

These experiments showed that the presence of carbohydrates on CII was essential for the T cell reactivity towards the posttranslationally modified CII(256-270) peptide. Nevertheless, the role of the bound carbohydrates for this T cell reactivity could be either to serve as structural determinants within the CII(256-270) peptide, or to direct the antigen processing, leading to protection against proteolytic destruction of the T cell determinant. To clarify this, we examined the response of HCQ.4 and HCQ.10 towards RP-HPLC-separated tryptic fragments of CB11, presented by formaldehyde-fixed APC. Trypsin cleavage of CB11 generates a fragment consisting of CII(238-290), termed T1 (17), which contains three lysines in susceptible position for hydroxylation and glycosylation (Fig. 3). Tryptic CB11-fragments presented by fixed APC did stimulate both HCQ.4 and HCQ.10, showing that naturally glycosylated peptides can bind directly to the MHC class II molecule and stimulate antigen-specific T cells (see Fig. 4). It is interesting to note that mass spectrometry analysis of T cell stimulatory fractions revealed that the glycosy-

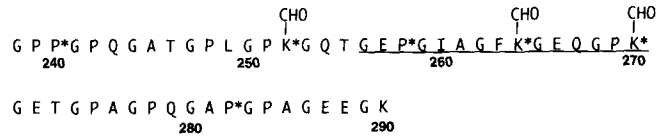

Figure 3. The amino acid sequence of rat CII(238-290) (T1 fragment). Indicated in the figure are the locations for the possible posttranslational modifications. (*) Hydroxyl groups and -CHO the hydroxylysine-linked carbohydrates β-D-galactopyranose or α-D-glucopyranosyl-(1 → 2)-β-D-galacto-pyranose. The immunodominant peptide CII(256-270) is underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number X79816.

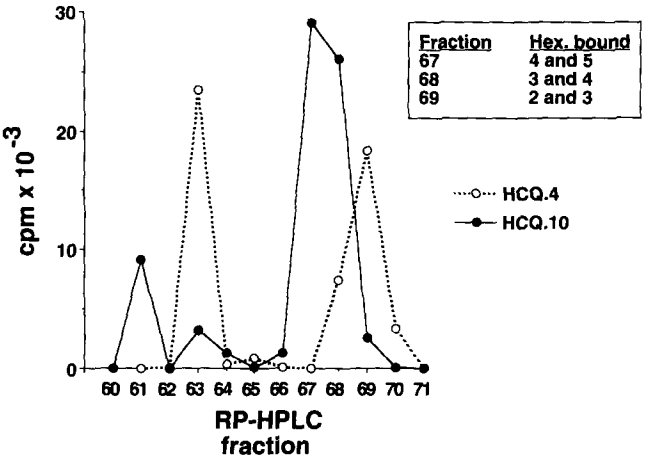

Figure 4. Presentation of glycosylated tryptic CB11-peptides by fixed APC. The response of HCQ.4 (---○---) and HCQ.10 (—●—) towards RP-HPLC fractions of trypsin-cleaved rat CB11, using formaldehyde-fixed spleen cells as APC. An amino acid sequence corresponding to T1 (amino acids 238-290) was found as a major component in fractions 67, 68, and 69 and an amino acid sequence corresponding to a further degraded T1 (amino acids 253-290) was found as a minor component in fractions 61, 62, and 63. T1 and the truncated T1 was identified by the positions of amino acids other than glycine, proline, or hydroxyproline. A separation of T1 with different levels of glycosylation, could be detected in fractions 67-69 by mass spectrometry analysis (see Table 1). (*Inset*) Calculated numbers of hexose equivalents (*Hex.*) bound to T1 within fractions 67-69.

Table 1. *Mass Spectrometry Analysis of T Cell Stimulatory RP-HPLC Fractions Containing the T1 Fragment*

Calculated mass of T1 with different numbers of hexose-equivalents bound		Mass/charge found in fraction		
Hexose equivalents bound to T1	Mass	67	68	69
0	4,912	–	–	–
1	5,074	–	–	–
2	5,236	–	–	5,237
3	5,398	–	5,403	5,400
4	5,560	5,554	5,555	–
5	5,722	5,712	–	–
6	5,884	–	–	–

* See Fig. 4 and Materials and Methods. The masses obtained by mass spectrometry (Na-adduct ions) have been subtracted by 23 to obtain the mass of the peptide.

lation of CII was diverse inasmuch as T1 containing between two (fraction 69) and five (fraction 67) hexose equivalents could be detected (Table 1). Fraction 67, which was stimulatory for HCQ.10 but not for HCQ.4, contained T1 with four and five hexose equivalents bound, respectively. Fraction 69, which stimulated mainly HCQ.4, contained T1 with two and three hexose equivalents bound. Although these data imply that the carbohydrates bound to CII(256-270) are involved in the trimolecular complex TCR/peptide/MHC, the question still remains whether the carbohydrate moieties interact directly with MHC class II or the TCR, or possibly distort the peptide structure as proposed by Harding et al. (18).

It is of obvious interest that CII, a protein which induces arthritis in mice and is believed to play a critical role in the development of rheumatoid arthritis (19), contains an immunodominant epitope which is glycosylated. To examine the importance of the bound carbohydrates for the development of arthritis, we immunize mice with either periodate-treated or glycosylated CII (Table 2). It was found that the incidence, time of onset, and severity of arthritis were

Table 2. *Elimination of Carbohydrates Decreases Arthritogenicity but not Immunogenicity of Type II Collagen**

Immunogen	Incidence	Onset	Max. severity	Anti-CII Ab levels
	Median day		*Median score*	*μg/ml*
Rat CII	36/40	37	6	271
p-values	0.02	<0.0001	<0.0001	>0.05
Periodate-treated rat CII	26/40	56	3	309

* See Materials and Methods.

significantly affected by the elimination of carbohydrates. Still, periodate treatment of CII did not seem to influence the immunogenicity, since no significant difference in anti-CII antibody titers was seen.

Carbohydrates themselves do not bind the MHC class II molecule (20). In addition, there is only one example of naturally processed glycosylated peptides binding to MHC class II molecules (21), although no T cell reactivity towards this glycopeptide has been shown. Recent reports have demonstrated that T cells are able to recognize glycosylated peptides bound to MHC class II (18, 22). However, those experimental systems were based on synthetic analogues of immunodominant peptides which in their natural form are not glycosylated. Therefore, in neither of the studies was it possible to address the question of whether the T cell response towards the protein involves recognition of carbohydrates or not. Here we have demonstrated the existence of a naturally glycosylated immunodominant T cell determinant which is strictly dependent on the presence of carbohydrates. The determinant is present on CII, a protein that induces arthritis in mice. In fact, recognition of carbohydrates also seemed to influence the development of arthritis, since carbohydrate-depleted CII induced arthritis with later onset, lower incidence, and milder symptoms compared to arthritis induced with glycosylated CII. To conclude, we propose that T cell recognition of glycosylated CII-peptides is of importance for CIA, suggesting that carbohydrates on posttranslationally modified tissue-specific proteins might be a critical factor for development of autoimmune disease.

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