

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^g)-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 \rightarrow 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.

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 (Dyna, 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 α - β (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 [³H]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 α 1(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 (NaIO₄, 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 NH₄HCO₃. CB11 was digested with trypsin (1:50 wt/wt, 0.1 M NH₄HCO₃, 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 × 10⁵ (B10.QxDBA/1)F₁ NH₄Cl-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 NH₂-terminal parts of T1 in fractions 66-70 and of peptides corresponding to cleavage after K₂₅₂ 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)F₁ male mice were immunized with 50 μ g 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 α 1 chain of mouse type I collagen,

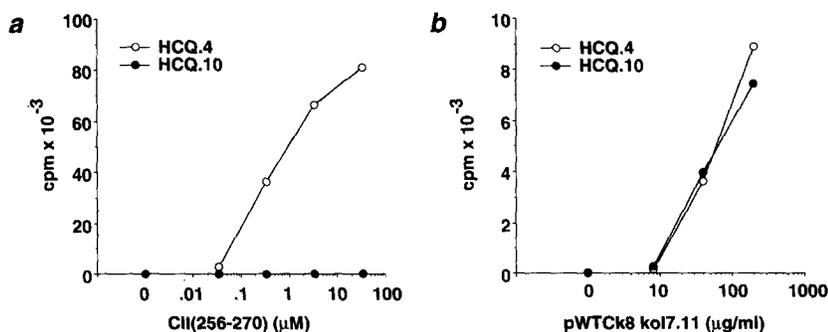


Figure 1. T cell recognition of the immunodominant peptide CII(256-270). (a) Response of HCQ.4 (O) and HCQ.10 (●) towards the synthetic peptide CII(256-270); (b) response of HCQ.4 and HCQ.10 to the mutated type I collagen pWTCk8 kol7.11 (containing the nucleotides encoding CII[256-270]). The response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

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

Hexose equivalents bound to T1	Calculated mass of T1 with different numbers of hexose-equivalents bound Mass	Mass/charge found in fraction		
		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
		<i>Median day</i>	<i>Median score</i>	<i>µg/ml</i>
Rat CII	36/40	37	6	271
<i>p</i> -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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References

1. Wooley, P.H., H.S. Luthra, J.M. Stuart, and C.S. David. 1981. Type II collagen induced arthritis in mice. I. Major histocompatibility complex (I-region) linkage and antibody correlates. *J. Exp. Med.* 154:688.
2. Holmdahl, R., M. Andersson, T.J. Goldschmidt, K. Gustafsson, L. Jansson, and J.A. Mo. 1990. Type II collagen autoimmunity in animals and provocations leading to arthritis. *Immunol. Rev.* 118:193.
3. Andersson, M., M. Cremer, K. Terato, H. Burkhardt, and R. Holmdahl. 1991. Analysis of type II collagen reactive T cells in the mouse. II. Different localization of immunodominant T cell epitopes on heterologous and autologous type II collagen in the DBA/1 mouse. *Scand. J. Immunol.* 33:505.
4. Myers, L.K., J.M. Stuart, J.M. Seyer, and A.H. Kang. 1989. Identification of an immunosuppressive epitope of type II collagen that confers protection against collagen-induced arthritis. *J. Exp. Med.* 170:1999.
5. Michaëlsson, E., M. Andersson, Å. Engström, and R. Holmdahl. 1992. Identification of an immunodominant type II collagen peptide recognized by T-cells in H-2^s mice. Self tolerance at the level of determinant selection. *Eur. J. Immunol.* 22:1819.
6. Holmdahl, R., and M. Karlsson. 1993. A rapid method to determine structural polymorphism of rat class II genes. *Transplant. Proc.* 25:2770.
7. White, J., M. Blackman, J. Bill, J. Kappler, P. Marrack, D.P. Gold, and W. Born. 1989. Two better cell lines for making hybridomas expressing specific T cell receptors. *J. Immunol.* 143:1822.
8. Gillis, S., and K.A. Smith. 1977. Long term culture of tumour-specific cytotoxic T cells. *Nature (Lond.)* 268:154.
9. Schnieke, A., M. Dziadek, J. Bateman, T. Mascara, K. Harbers, R. Gelinas, and R. Jaenisch. 1987. Introduction of the human pro α 1(I) collagen gene into pro α 1(I)-deficient Mov-13 mouse cells leads to formation of functional mouse-human hybrid type I collagen. *Proc. Natl. Acad. Sci. USA.* 84:764.
10. Monson, J.M., J. Friedman, and B.J. McCarthy. 1982. DNA sequence analysis of a mouse Pro α 1(I) procollagen gene: evidence for a mouse B1 element within the gene. *Mol. Cell. Biol.* 2:1362.
11. Smith, B.D., G.R. Martin, A. Dorfman, and R. Swarm. 1975. Nature of the collagen synthesized by a transplanted chondrosarcoma. *Arch. Biochem. Biophys.* 166:181.
12. Miller, E.S. 1972. Structural studies on cartilage employing limited cleavage and solubilization with pepsin. *Biochemistry.* 11:4901.
13. Spiro, R.G. 1967. Studies on the renal glomerular basement membrane. Nature of the carbohydrate units and their attachment to the peptide portion. *J. Biol. Chem.* 242:1923.
14. Edge, A.S.B., C.R. Faltynek, L. Hof, L.E. Reichert, Jr., and P. Weber. 1981. Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal. Biochem.* 118:131.
15. Woodward, H.D., N.J. Ringler, R. Selvakumar, I.M. Simet, V.P. Bhavanandan, and E.A. Davidson. 1987. Deglycosylation studies on tracheal mucin glycoprotein. *Biochemistry.* 26:5315.
16. Holmdahl, R., L. Klareskog, M. Andersson, and C. Hansen. 1986. High antibody response to autologous type II collagen is restricted to H-2q. *Immunogenetics.* 24:84.
17. Seyer, J.M., K.A. Hasty, and A.H. Kang. 1989. Covalent structure of collagen. Amino acid sequence of an arthritogenic cyanogen bromide peptide from type II collagen of bovine cartilage. *Eur. J. Biochem.* 181:159.
18. Harding, C.V., J. Kihlberg, M. Elofsson, G. Magnusson, and E.R. Unanue. 1993. Glycopeptides bind MHC molecules and elicit specific T cell responses. *J. Immunol.* 151:2419.
19. Trentham, D.E., R.A. Dynesius-Trentham, E.J. Orav, D. Combitchi, C. Lorenzo, K.L. Sewell, D.A. Hafler, and H.L. Weiner. 1993. Effects of oral administration of type II collagen on rheumatoid arthritis. *Science (Wash. DC).* 261:1727.
20. Harding, C.V., R.W. Roof, P.M. Allen, and E.R. Unanue. 1991. Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA.* 88:2740.
21. Chicz, R.M., R.G. Urban, J.C. Gorga, D.A.A. Vignali, W.S. Lane, and J.L. Strominger. 1993. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J. Exp. Med.* 178:27.
22. Ishioka, G.Y., A.G. Lamont, D. Thomson, N. Bulbow, F.C.A. Gaeta, A. Sette, and H.M. Grey. 1992. MHC interaction and T cell recognition of carbohydrates and glycopeptides. *J. Immunol.* 148:2446.