T Cells Recognize a Glycopeptide Derived from Type II Collagen in a Model for Rheumatoid Arthritis

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Abstract: Even though most eucaryotic proteins are glycosylated, very little is known on if, or how, the glycans influence essential immunological events such as antigen processing, major histocompatibility complex (MHC) restricted presentation, and recognition by T cells. We have used synthetic glycopeptides to elucidate the specificity of T cell hybridomas, obtained by immunization with the glycoprotein type II collagen in a mouse model for rheumatoid arthritis. To enable these studies, glycosylated and suitably protected derivatives of (*5R*)-5-hydroxy-L-lysine, and the similar 5-hydroxy-L-norvaline, were prepared and then used in Fmoc solid-phase synthesis of glycopeptides related to the immunodominant fragment from type II collagen, CII(256−270). Evaluation of the synthetic glycopeptides provided evidence that antigen-presenting cells can indeed process glycoproteins to glycopeptides, which elicit a T cell response when presented by class II MHC molecules. A glycopeptide carrying a single *β*-D-galactosyl residue attached to hydroxylysine at position 264 in the center of the CII(256−270) peptide was recognized by most of the hybridomas in a way involving specific contacts between the carbohydrate and the T cell receptor. The results suggest an explanation for the recent observation that glycosylated type II collagen induces more severe forms of arthritis in the mouse than deglycosylated type II collagen and provide additional knowledge on how rheumatoid arthritis may occur also in humans.

Introduction

Processing of protein antigens into short peptides by antigen-presenting cells is critical for proper function of the immune system of higher vertebrates.[1] In higher organisms most cells are able to process foreign or altered protein antigens produced intracellularily, for instance due to viral infections or malignant transformations. Peptides resulting from this degradation are bound by class I major histocompatibility complex (MHC) molecules, and the complexes are then transported to the cell surface where they are displayed to circulating T cells. Recognition of peptide−class I MHC complexes by receptors on "cytotoxic" ($CD8^+$) T cells triggers release of compounds which selectively kill the presenting cell, thereby eliminating the viral infection or the malignant transformation. In contrast, specialized antigen-presenting cells, such as macrophages, take up and process extracellular protein material, e.g., bacterial proteins. Recognition of complexes between peptides and class II MHC molecules on the surface of such antigen-presenting cells by "helper" ($CD4^+$) T cells elicits release of immunomodulating cytokines, such as interleukines. These cytokines are essential for inducing isotype switching from production of IgM to high-affinity IgG antibodies in B cells, as well as for production of memory B cells and activation of phagocytic cells; events that all are essential in elimination of the infection. Peptides derived by processing of endogenous proteins are also presented to T cells, but various mechanisms normally ensure that they do not elicit an immune response, thereby preventing occurrence of autoimmune disease.

Although most eucaryotic proteins and many viral proteins are glycosylated, very little is known concerning if, or how, the glycans influence antigen processing, MHC-restricted presentation, and T cell recognition.[2] In fact, prior to the beginning of the 1990s carbohydrates were regarded only as T cell independent antigens; i.e., it was assumed that T cells were unable to recognize carbohydrates. Recent model studies with synthetic neoglycopeptides have, however, revealed that mono- or small oligosaccharides can be attached to T cell immunogenic peptides without loss of MHC binding, provided that the position for the glycan is chosen carefully.[3] These studies have also

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(1) (a) Grey, H. M.; Sette, A.; Buus, S. *Sci. Am.* **1989**, *Nov*, 38−46. (b) Engelhard, V. H. *Sci. Am.* **1994**, *Aug*, 44−51. (c) Monaco, J. J. *J. Leukocyte Biol.* **1995**, *57*, 543−547. (d) Janeway, C. A., Jr.; Travers, P. *Immunobiology: the immune system in health and disease*, 2nd ed.; Current Biology Ltd./Garland Publishing Inc.: London/New York, 1996.

(2) Reviewed in (a) Kihlberg, J.; Elofsson, M. *Curr. Med. Chem.* **1997**, *4*, 79−110. (b) Carbone, F. R.; Gleeson, P. A. *Glycobiology* **1997**, *7*, 725−730.

(3) (a) Ishioka, G. Y.; Lamont, A. G.; Thomson, D.; Bulbow, N.; Gaeta, F. C. A.; Sette, A.; Grey, H. M. *J. Immunol.* **1992**, *148*, 2446−2451. (b) Haurum, J. S.; Arsequell, G.; Lellouch, A. C.; Wong, S. Y. C.; Dwek, R. A.; McMichael, A. J.; Elliot, T. *J. Exp. Med.* **1994**, *180*, 739−744. (c) Otvos Jr., L.; Urge, L.; Xiang, Z. Q.; Krivulka, G. R.; Nagy, L.; Szendrei, G. I.; Ertl, H. C. J. *Biochim. Biophys. Acta* **1994**, *1224*, 68−76. (d) Deck, B.; Elofsson, M.; Kihlberg, J.; Unanue, E. R. *J. Immunol.* **1995**, *155*, 1074−1078. (e) Abdel-Motal, U. M.; Berg, L.; Rosén, A.; Bengtsson, M.; Thorpe, C. J.; Kihlberg, J.; Dahmén, J.; Magnusson, G.; Karlsson, K.-A.; Jondal, M. *Eur. J. Immunol.* **1996**, *26*, 544−551. (f) Jensen, T.; Galli-Stampino, L.; Mouritsen, S.; Frische, K.; Peters, S.; Meldal, M.; Werdelin, O. *Eur. J. Immunol.* **1996**, *26*, 1342−1349. (g) Jensen, T.; Hansen, P.; Galli-Stampino, L.; Mouritsen, S.; Frische, K.; Meinjohanns, E.; Meldal, M.; Werdelin, O. *J. Immunol.* **1997**, *158*, 3769−3778.

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demonstrated that immunization of mice with neoglycopeptides can elicit T cells which specifically recognize the carbohydrate moiety if this is located in the center of the peptide. For glycoproteins, such as HIV gp120 and influenza virus hemagglutinin which carry large N-linked carbohydrate moieties, indirect evidence has been obtained suggesting that the glycans influence uptake in antigen-presenting cells, as well as processing and MHC restricted presentation of peptide epitopes derived from the glycoproteins.[4] In addition, desialylated ovine submaxillary mucin (A-OSM), which carries multiple copies of the O-linked tumor-associated Tn antigen (GalNAcα-Ser/Thr), was found to elicit a carbohydrate dependent cellular immune response.[5] However, as for the N-linked glycoproteins, the role of the carbohydrate in influencing uptake in or processing by antigen-presenting cells, or as part of the antigenic determinant being recognized by the T cell receptor, was not established.

We now present a study of the response and carbohydrate specificity of helper T cell hybridomas obtained in mice after immunization with the glycoprotein type II collagen. Immunization of mice with type II collagen induces an inflammatory response, termed collagen-induced arthritis (CIA).[6] CIA is accompanied by erythema and severe, painful swelling of peripheral joints, i.e., with symptoms and histopathology similar to those displayed by patients suffering from rheumatoid arthritis. It should be emphasized that type II collagen is found mainly in joint cartilage, in contrast to type I collagen which is the major constituent in connective tissue, and that CIA is the most frequently used model for rheumatoid arthritis. In mice the immunization leads to a type II collagen specific and class II MHC (A^q) restricted T cell response. Since rheumatoid arthritis in humans is correlated to the presence of certain class II MHC molecules, it is possible that a T cell response to type II collagen plays a role also in humans for development of this autoimmune disease. We previously showed that a panel of T cell hybridomas,[7] obtained from mice immunized with type II collagen, recognized an immunodominant T cell epitope located within a polypeptide fragment of type II collagen which contained residues 256−270 [CII(256−270)].[7a] Surprisingly, even though all of the T cell hybridomas recognized type II collagen and proteolytic fragments containing CII(256−270) after antigen processing, only a few of them responded to the synthetic peptide CII(256−270) (cf. **12**, Figure 1) or to related synthetic peptides.[7b] This observation could be due to the fact that lysine residues in collagen, e.g., those at positions 264 and 270 of CII(256−270), can undergo posttranslational hydroxylation and subsequent glycosylation by *β*-D-galactopyranosyl or α-D-glucopyranosyl-(1−2)-*β*-D-galactopyranosyl moieties.[8] The importance of posttranslational modifications was suggested

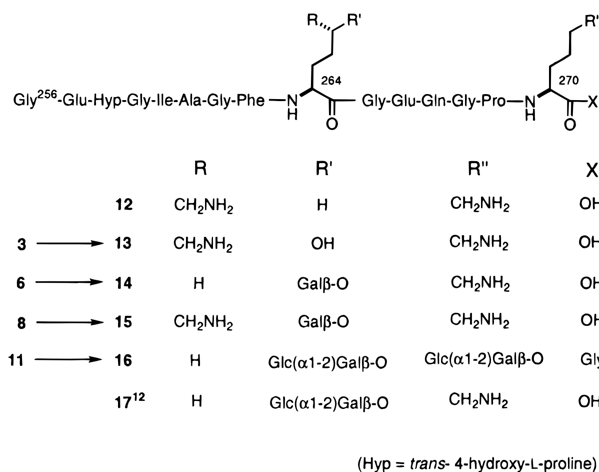


Figure 1. Peptides **12** and **13** and glycopeptides **14**−**17**, which correspond to residues 256−270 of type II collagen, were used to delineate the response and specificity of T cell hybridomas obtained from mice immunized with this O-linked glycoprotein.

by the observation that chemical removal of carbohydrates from type II collagen resulted in loss of recognition by most of the T cell hybridomas which did not respond to the synthetic CII-(256−270) peptide.[7b] In addition, deglycosylated type II collagen was found to induce less severe arthritis in the mouse than the native form of the glycoprotein. However, as in previous studies of the cellular immune response elicited by glycoproteins,[4,5] it was not established if the carbohydrate moieties influenced antigen processing, MHC restricted presentation, T cell recognition, or other factors.

Results and Discussion

To delineate the role(s) of the posttranslational hydroxylation and glycosylation of lysine in type II collagen for the T cell immune response elicited by this O-linked glycoprotein, we have prepared protected (5*R*)-5-hydroxy-L-lysine **3** and the corresponding *β*-galactoside **8**, as well as the glycosylated derivatives **6** and **11** of the structurally related amino acid 5-hydroxy-L-norvaline (cf. Scheme 1). These building blocks were then used for synthesis of peptides and glycopeptides related to type II collagen (cf. **12**−**17**, Figure 1). (5*R*)-5-Hydroxy-L-lysine was protected by transformation into a cupric chelate which allowed regioselective *t*Boc protection of the ϵ-amino group.[9] After dissociation of the chelate the α-amino and carboxyl groups were protected with Fmoc and benzyl groups,[10] respectively, to give **2**. Silylation of the hydroxyl group of **2** followed by selective[11] cleavage of the benzyl ester gave hydroxylysine building block **3**, which was used for synthesis of peptide **13** having a hydroxylysine residue at position 264 of type II collagen. As reported recently by us, *β*-galactosylation of protected 5-hydroxy-L-norvaline **1** failed using galactosyl donors having participating groups at O-2.[12] However, epoxidation of

(4) (a) Doe, B.; Steimer, K. S.; Walker, C. M. *Eur. J. Immunol.* **1994**, *24*, 2369−2376. (b) Botarelli, P.; Houlden, B. A.; Haigwood, N. L.; Servis, C.; Montagna, D.; Abrignani, S. *J. Immunol.* **1991**, *147*, 3128−3132. (c) Barnett, B. C.; Graham, C. M.; Burt, D. S.; Skehel, J. J.; Thomas, D. B. *Eur. J. Immunol.* **1989**, *19*, 515−521. (d) Thomas, D. B.; Hodgson, J.; Riska, P. F.; Graham, C. M. *J. Immunol.* **1990**, *144*, 2789−2794. (e) Drummer, H. D.; Jackson, D. C.; Brown, L. E. *Virology* **1993**, *192*, 282−289. (f) Brown, L. E.; White, D. O.; Jackson, D. C. *J. Virol.* **1993**, *67*, 2887−2893. (g) Jackson, D. C.; Drummer, H. E.; Urge, L.; Otvos Jr., L.; Brown, L. E. *Virology* **1994**, *199*, 422−430.

(5) Singhal, A.; Fohn, M.; Hakomori, S.-i. *Cancer Res.* **1991**, *51*, 1406−1411.

(6) (a) Trentham, D. E.; Townes, A. S.; Kang, A. H. *J. Exp. Med.* **1977**, *146*, 857−868. (b) Holmdahl, R.; Andersson, M.; Goldschmidt, T. J.; Gustafsson, K.; Jansson, L.; Mo, J. A. *Immunol. Rev.* **1990**, *118*, 193−232.

(7) (a) Michaëlsson, E.; Andersson, M.; Engström, Å.; Holmdahl, R. *Eur. J. Immunol.* **1992**, *22*, 1819−1825. (b) Michaëlsson, E.; Malmström, V.; Reis, S.; Engström, Å.; Burkhardt, H.; Holmdahl, R. *J. Exp. Med.* **1994**, *180*, 745−749.

(8) Spiro, R. G. *J. Biol. Chem.* **1967**, *242*, 4813−4823.

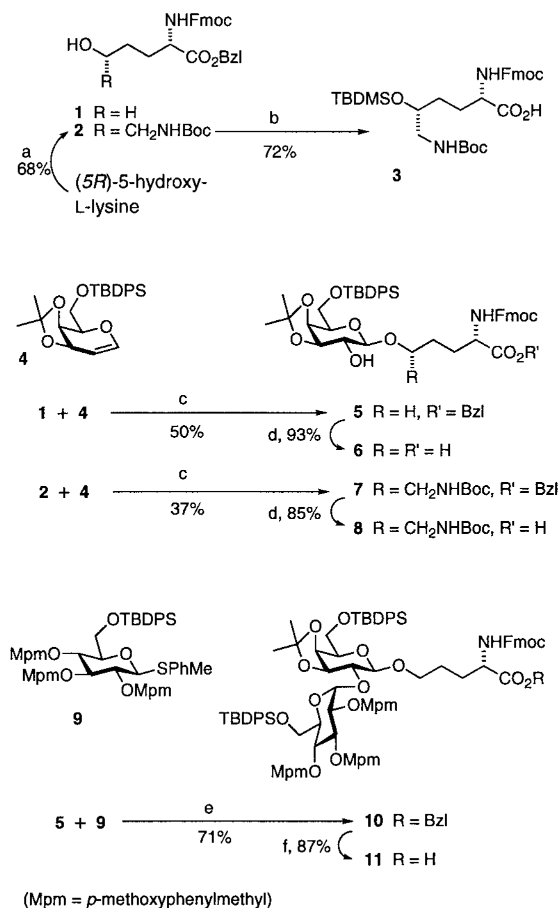
(9) Scott, J. W.; Parker, D.; Parrish, D. R. *Synth. Commun.* **1981**, *11*, 303−314.

(10) Wang, S.-S.; Gisin, B. F.; Winter, D. P.; Makofske, R.; Kulesha, I. D.; Tzougraki, C.; Meienhofer, J. *J. Org. Chem.* **1977**, *42*, 1286−1290.

(11) (a) Carpino, L. A.; Tunga, A. *J. Org. Chem.* **1986**, *51*, 1930−1932. (b) Cleavage of the Fmoc group may occur during hydrogenolysis, but use of ethyl acetate as solvent and adjustment of the amount of Pd/C allowed selective removal of the benzyl ester to give **3**. Selective debenzylation was also possible in preparation of the glycosylated amino acids **6**, **8**, and **11**.

(12) (a) Broddefalk, J.; Bergquist, K.-E.; Kihlberg, J. *Tetrahedron Lett.* **1996**, *37*, 3011−3014. (b) Broddefalk, J.; Bergquist, K.-E.; Kihlberg, J. *Tetrahedron*, submitted for publication.

Scheme 1[a]



[a] Reagents and conditions: (a) (i) $CuCO_3 \cdot Cu(OH)_2 \cdot H_2O$, $t$$Boc_2O$, H_2O−dioxane then Chelex 100 (H^+-form), (ii) FmocCl, Na_2CO_3, H_2O−dioxane 1:1, (iii) Cs_2CO_3, benzyl bromide, DMF. (b) (i) TBDMSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C, (ii) H_2, 10% Pd/C, EtOAc. (c) (i) dimethyldioxirane, acetone, CH_2Cl_2, 0 °C, (ii) $ZnCl_2$, THF, AW-300, −50 °C → room temp. (d) H_2, 10% Pd/C, EtOAc. (e) NIS, AgOTf, CH_2Cl_2, 4 Å MS, −45 → −15 °C. (f) H_2, 10% Pd/C, NH_4OAc, EtOAc.

galactal **4**[13] (1.2 equiv) with dimethyldioxirane gave the corresponding α-1,2-anhydrosugar,[14] which under zinc chloride promotion gave *β*-galactoside **5** (50%) on reaction with **1**. Galactosylated hydroxylysine **7** was prepared from **2** in the same manner by glycosylation with **4** (37% yield).[15] Regioselective[11] removal of the benzyl ester in **5** and **7** then gave the glycosylated amino acids **6** and **8**. These were subsequently used for synthesis of glycopeptides **14** and **15** which have a *β*-D-galactopyranosyl residue at position 264 of CII(256−270). Glucosyl donor **9** allowed a highly stereoselective glycosylation of the hydroxynorvaline galactoside **5** when using *N*-iodosuccinimide and silver triflate as promoters,[16] and the α-glucoside **10** was obtained in a high yield (71%).[12b] Regrettably, all attempts to attach an α-D-glucosyl moiety to the galactosylated hydroxylysine **7** by this, or other procedures, have so far been unsuccessful. Regioselective hydrogenolysis of the benzyl ester in **10** was performed over Pd/C in the presence of ammonium acetate,[17] without simultaneous cleavage of the Fmoc or *p*-methoxybenzyl groups. This gave building block **11**, used for synthesis of glycopeptide **16** which carries disaccharide moieties both at position 264 and at position 270 of CII(256−270).

Synthesis of peptide **13** and glycopeptide **15** was performed on a polystyrene resin grafted with poly(ethylene glycol) spacers (TentaGel resin) in an automatic peptide synthesizer. Synthesis was performed according to the Fmoc strategy[2a,18] under conditions identical to those reported[12] for synthesis of glycopeptides **14**, **16**, and **17**, as well as other glycopeptides[19] prepared recently by us. Use of acid labile protective groups for the carbohydrate residues of the target glycopeptides had the advantage that both the glycan and the peptide moieties were deprotected simultaneously with acid-catalyzed cleavage from the resin, allowing glycopeptides **14**−**17** to be isolated in 26−45% yields after a single purification by reversed-phase HPLC. The glycopeptides were characterized by ^{1}H NMR spectroscopy, fast atom bombardment mass spectroscopy, and amino acid analysis (cf. ^{1}H NMR data for glycopeptide **15** in Table 1). Previously, dipeptides containing hydroxylysine have been glycosylated and then used for solution synthesis of short glycopeptides,[20] but the present work respresents the first use of a glycosylated building block of hydroxylysine in Fmoc solid-phase synthesis.

Use of peptides **12** and **13** together with glycopeptides **14**−**17** allowed a detailed investigation of the specificity of the MHC class II restricted T cell hybridomas[7] obtained after immunization of mice with type II collagen. To this end each of the 29 hybridomas was incubated with spleen cells as antigen-presenting cells and dilution series of peptides **12** and **13**, glycopeptides **14**−**17**, as well as type II collagen as control. After 24 h the response of each hybridoma, i.e., interleukin 2 (IL-2) secreted into the supernatant as a result of recognition of a (glyco)-peptide−MHC complex, was determined in a standard radioassay[21] based on proliferation of the IL-2 sensitive T cell clone CTLL. Seven of the hybridomas raised to type II collagen did not respond to any of the glycopeptides, but six of these instead recognized peptide **12** which has lysine at position 264, whereas one responded only to peptide **13** having hydroxylysine at this position (cf. hybridoma HDBR.1, Figure 2a). The response of the remaining 22 hybridomas appeared to depend on carbohydrate since they all recognized type II collagen but not peptides **12** and **13**. Interestingly, as many as 20 of the 22 hybridomas responded to glycopeptide **15**, in which hydroxylysine carrying a *β*-D-galactopyranosyl residue has been incorporated at position 264 of CII(256−270) (cf. hybridomas

(13) Alonso, R. A.; Vite, G. D.; McDevitt, R. E.; Fraser-Reid, B. *J. Org. Chem.* **1992**, *57*, 573−584.

(14) (a) Halcomb, R. L.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1989**, *111*, 6661−6666. (b) Danishefsky, S. J.; Bilodeau, M. T. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1380−1419.

(15) In the synthesis of both **5** and **7**, the excess of galactal **4** had to be adjusted carefully so as to avoid galactosylation of the secondary hydroxyl group in the products. The decreased reactivity of the secondary hydroxyl group in hydroxylysine **2**, as compared to the primary hydroxyl group of hydroxynorvaline **1**, resulted in formation of larger amounts of higher order glycosides in the synthesis of **7**, thus lowering the yield of **7**.

(16) Konradsson, P.; Udodong, U. E.; Fraser-Reid, B. *Tetrahedron Lett.* **1990**, *31*, 4313−4316.

(17) Sajiki, H. *Tetrahedron Lett.* **1995**, *36*, 3465−3468.

(18) Reviewed in: (a) Kunz, H. *Angew. Chem., Int. Ed. Engl.* **1987**, *26*, 294−308. (b) Paulsen, H. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 823−839. (c) Garg, H. G.; von dem Bruch, K.; Kunz, H. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 277−310. (d) Meldal, M. In *Glycopeptide synthesis*; Lee, Y. C., Lee, R. T., Eds.; Academic Press: San Diego, 1994; pp 145−198. (e) Meldal, M. *Curr. Opin. Struct. Biol.* **1994**, *4*, 710−718. (f) Norberg, T.; Lüning, B.; Tejbrant, J. *Methods Enzymol.* **1994**, *247*, 87−106. (g) Nakahara, Y.; Iijima, H.; Ogawa, T. In *Stereocontrolled approaches to O-glycopeptide synthesis*; Kovác, P., Ed.; American Chemical Society: Washington, DC, 1994; pp 249−266. (h) Schultz, M.; Kunz, H. In *Chemical and enzymatic synthesis of glycopeptides*; Jollès, P., Jörnvall, H., Eds.; Birkhäuser Verlag: Basel, 1995; pp 201−228. (i) Kihlberg, J.; Elofsson, M.; Salvador, L. A. *Methods Enzymol.* **1997**, *289*, 221−245. (j) Arsequell, G.; Valencia, G. *Tetrahedron: Asymmetry* **1997**, *8*, 2839−2876.

(19) Elofsson, M.; Salvador, L. A.; Kihlberg, J. *Tetrahedron* **1997**, *53*, 369−390.

(20) Koeners, H. J.; Schattenkerk, C.; Verhoeven, J. J.; van Boom, J. H. *Tetrahedron* **1981**, *37*, 1763−1771.

(21) Gillis, S.; Smith, K. A. *Nature* **1977**, *268*, 154−156.

Table 1. ^{1}H NMR Data (δ, ppm) for Glycopeptide **15** in Water Containing 10% D_2O^a

residue	NH	H-α	H-β	H-γ	H-δ	others
Gly[256]		3.78, 3.63				
Glu[257]	8.73	4.62	1.99, 1.79	2.24^b		
Hyp[258]		4.48	2.30, 1.03	4.59	3.81^b	
Gly[259]	8.69	3.89^b				
Ile[260]	8.08	4.14	1.83	1.38, 1.14	0.78	0.87 (β-CH_3)
Ala[261]	8.64	4.24	1.33			
Gly[262]	7.79	3.84^b				
Phe[263]	8.17	4.53	3.04^b			7.31, 7.20 (arom)
Hyl[264]	8.48	4.24	1.96, 1.66	1.54^b	3.97	3.12, 2.91 (Hϵ), Galβ^c
Gly[265]	8.44	3.84^b				
Glu[266]	8.48	4.24	2.02, 1.87	2.24^b		
Gln[267]	8.64	4.31	2.11, 1.93	2.33^b		7.61, 6.92 ($CONH_2$)
Gly[268]	8.41	4.12, 3.92				
Pro[269]		4.37	2.21, 1.92	1.97^b	3.55^b	
Lys[270]	8.15	4.10	1.78, 1.65	1.37^b	1.61^b	2.93^b(Hϵ), 7.55 (ϵ-NH_2)

^{a} Obtained at 500 MHz, 278 K, and pH 5.4 with H_2O as internal standard (δ_H 4.98 ppm). ^{b} Degeneracy has been assumed. ^{c} Chemical shifts (δ, ppm) for the galactose moiety: 4.38 (H-1), 3.86 (H-4), 3.71^b (H-6), 3.66 (H-5), 3.61 (H-3), 3.46 (H-2).

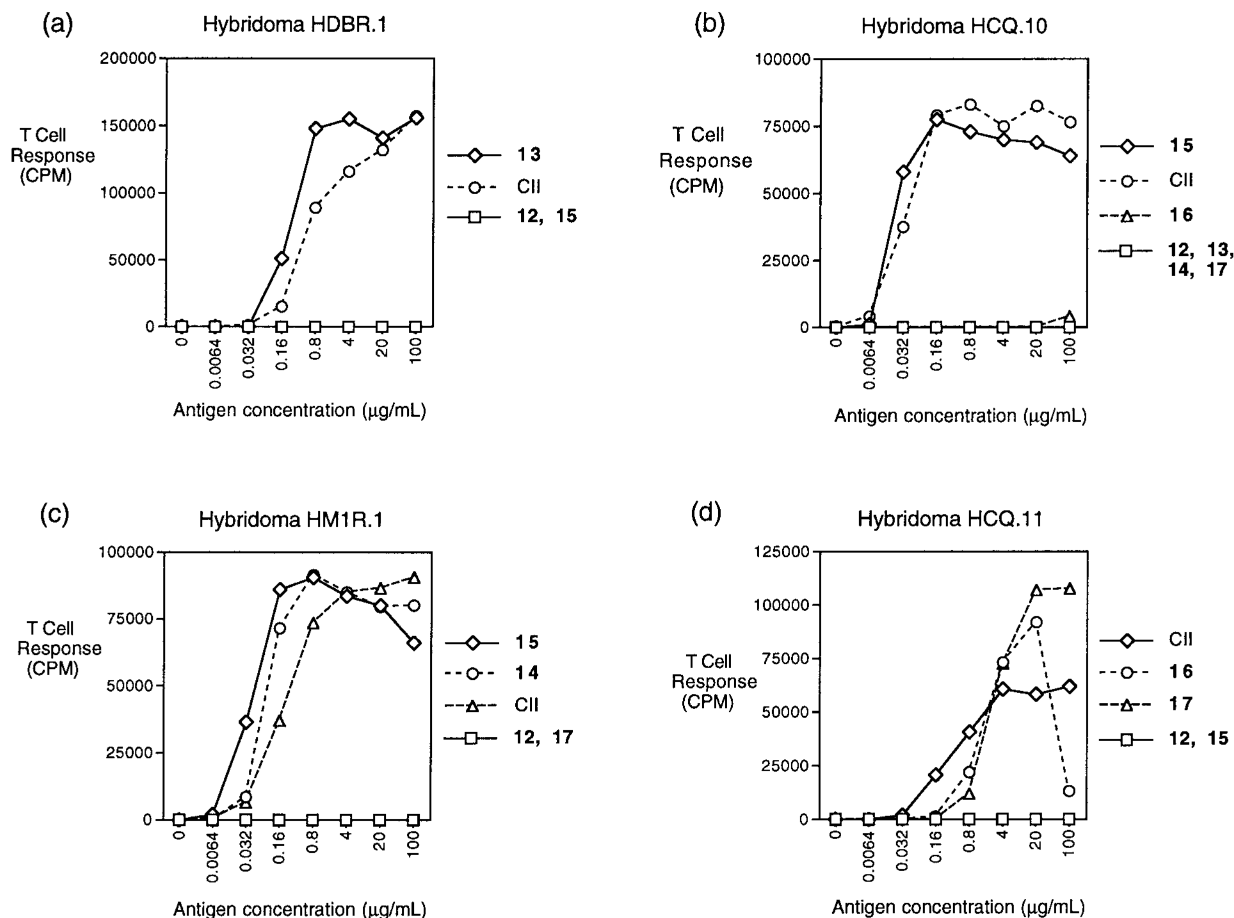


Figure 2. Response of selected T cell hybridomas on incubation with antigen-presenting spleen cells and increasing concentrations of the antigens type II collagen (CII), peptides **12** and **13**, and glycopeptides **14**−**17**. Recognition of (glyco)peptide−MHC complexes on the surface of an antigen-presenting cell by a T cell hybridoma results in secretion of the cytokine interleukin 2 (IL-2) in a dose dependent manner. This T cell response is subsequently determined in a radioassay based on proliferation of the IL-2 sensitive T cell clone CTLL.[21] (a) Hybridoma HDBR.1 responds to peptide **13** which has hydroxylysine at position 264 of CII(256−270) but not when lysine or β-D-galactosylated hydroxylysine is found at this position (cf. **12** and **15**). (b) Hybridoma HCQ.10 responds when β-D-galactosylated hydroxylysine is found at position 264 of CII(256−270) (cf. **15**), but it does not respond to peptides **12** and **13** or to glycopeptides **14** and **17**. This hybridoma represents 17 of the 22 hybridomas which require CII to be glycosylated. (c) Hybridoma HM1R.1 requires a β-D-galactopyranosyl residue to be attached to either hydroxylysine or hydroxynorvaline at position 264 (cf. **15** and **14**). Two other hybridomas respond in the same manner. (d) Hybridoma HCQ.11 is specific for an α-D-glucopyranosyl-β-D-galactopyranosyl residue attached to hydroxynorvaline at position 264 (cf. **16** and **17**).

HCQ.10 and HM1R.1 (Figure 2b,c). These 20 hybridomas did not respond to glycopeptides **16** and **17** which carry an α-D-glucopyranosyl-(1−2)-β-D-galactopyranosyl residue attached to hydroxynorvaline 264, but three of them did respond to glycopeptide **14**, which has a galactosyl residue linked to hydroxynorvaline 264 (cf. hybridoma HM1R.1, Figure 2c). Hybridoma HCQ.11, one of the two hybridomas which did not respond to the galactosylated glycopeptides **14** and **15**, was instead stimulated by glycopeptides **16** and **17** having α-D-glucopyranosyl-(1−2)-β-D-galactopyranosyl residues attached

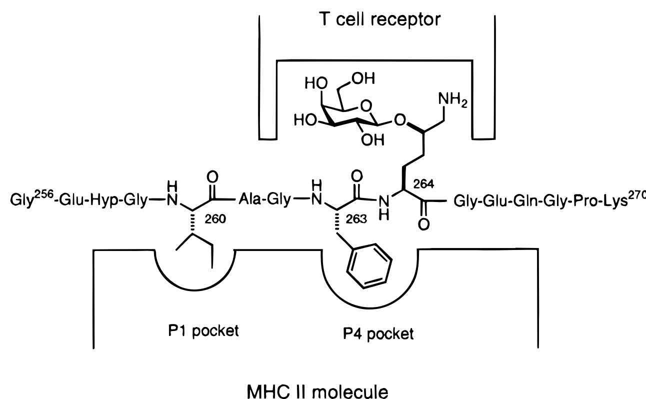


Figure 3. A schematic description of the interactions formed in the complex of glycopeptide **15**, which corresponds to residues 256−270 of type II collagen, with the class II MHC molecule, and the T cell receptor. According to peptide binding studies and computer modeling Ile^{260} and Phe^{263} anchor the glycopeptide in the P1 and P4 pockets of the MHC molecule, respectively.[22] Galactosylated hydroxylysine 264 is then located optimally in the center of the glycopeptide for specific interactions with the T cell receptor. This agrees well with the present finding that galactosylation of hydroxylysine 264 is critical for stimulation of the majority of the T cell hybridomas obtained by immunization of mice with type II collagen in a model for rheumatoid arthritis.

to hydroxynorvaline at positions 264 and 270, or only at 264 (Figure 2d). It can be assumed that this hybridoma, as well as the one remaining nonresponding hybridoma, would have recognized a glycopeptide having α-D-glucopyranosyl-(1−2)-β-D-galactopyranose at hydroxylysine 264 if this had been available through synthesis.

By performing binding studies using alanine-substituted derivatives of peptide **12** and glycopeptide **14**, we recently found that Ile^{260} and Phe^{263} are the most important residues for anchoring CII(256−270) into the binding groove of the A^{q} molecule, i.e., the class II MHC molecule important in eliciting an immune response to type II collagen in mice.[22] In addition it was found that glycosylation did not influence binding of CII-(256−270) to the MHC molecule. Modeling based on the peptide binding studies and the crystal structure[23] of the human class II MHC protein HLA-DR1 placed the MHC anchors, Ile^{260} and Phe^{263}, in the P1 and P4 pockets of the MHC molecule, respectively.[22] As a consequence, the glycosylated residue 264 is located in the center of the peptide MHC−complex recognized by the T cell receptor (Figure 3). Studies with synthetic neoglycopeptides have shown such a location to be ideal for direct contacts between the carbohydrate moiety and the T cell receptor.[2,3] Moreover, the recent crystal structure between a class I MHC molecule, a peptide, and a T cell receptor revealed that the side chain of a residue in the center of the peptide formed critical contacts with the highly variable CD3 loops of the T cell receptor.[24] The importance of residue 264 in CII(256−270) for the response of most of the T cell hybridomas therefore appears to originate from contacts formed by the carbohydrate moiety, and the side chain amino group, of this residue and the CD3 loops of the T cell receptor. Specificity for the carbohydrate moiety is revealed by the ability of glycopeptide **15**, galactosylated on hydroxylysine, to elicit a response from most of the carbohydrate dependent T cell hybridomas, in contrast to the nonglycosylated peptides **12** and **13** (Figure 2b). The fact that only 3 of the 20 hybridomas which responded to **15** also recognized glycopeptide **14** (β-D-galactosylated on hydroxynorvaline) reveals the additional importance of the ε-amino group of hydroxylysine in contacting the T cell receptor (Figure 2c). Specific recognition of the carbohydrate moiety is further confirmed by the inability of these three hybridomas to respond when an α-D-glucopyranosyl-(1−2)-β-D-galactopyranosyl residue was attached instead of β-D-galactose to hydroxynorvaline 264 (glycopeptide **17**). The equal response displayed by clone HCQ.11 to glycopeptides **16** and **17** indicates that glycosylation of hydroxylysine at position 270 has no, or limited, importance for the T cell response (Figure 2d). Residue 270 is therefore most likely outside the determinant recognized by the T cell receptor.

It is interesting to note that the majority of the carbohydrate dependent T cell hybridomas (20 out of 22) respond when galactose is attached to hydroxylysine 264 of CII(256−270) whereas, so far, only one hybridoma recognizing an α-D-glucopyranosyl-(1−2)-β-D-galactopyranosyl moiety at this position has been identified. This T cell selectivity for recognition of a mono- over a disaccharide at position 264 may have different explanations. The glycosylation pattern of type II collagen is not known, but a galactosyl residue might predominate at hydroxylysine 264. Alternatively, enzymatic removal of the glucosyl moiety to give a galactosylated hydroxylysine may occur during processing in the antigen-presenting cell. If saccharide processing does not occur, another explanation could be that the T cell receptor is less willing to accommodate the larger disaccharide when forming the ternary complex with the glycopeptide and the class II MHC molecule. As a result T cells recognizing galactosylated glycopeptides would be selected even if hydroxylysine 264 in collagen predominantly carried a disaccharide moiety. Future studies with a synthetic CII(256−270) glycopeptide having a disaccharide at hydroxylysine 264 should provide answers to some of these questions.

In conclusion, glycosylated derivatives of (*5R*)-5-hydroxy-L-lysine, and the related 5-hydroxy-L-norvaline, have been synthesized and used for preparation of glycopeptide fragments from type II collagen by the Fmoc strategy. The glycopeptides were used for evaluation of a panel of helper T cell hybridomas obtained previously in collagen-induced arthritis, a mouse model for rheumatoid arthritis. In this model disease is elicited by immunization with type II collagen, the major protein of joint cartilage. It was thus, for the first time, established that immunization with a naturally occurring glycoprotein can elicit carbohydrate specific T cells. In the present study, a glycopeptide carrying a single β-D-galactosyl residue attached to hydroxylysine was recognized by most of the T cell hybridomas obtained toward type II collagen, in a way involving direct contacts between the carbohydrate and the T cell receptor. The fact that most of the hybridomas responded to a glycopeptide, but not to the corresponding peptide, suggests an explanation for the recent observation[7b] that glycosylated type II collagen induces more severe forms of arthritis in the mouse than deglycosylated collagen. Finally, the possibility that T cells directed to glycopeptide fragments from joint cartilage participate in development of autoimmune rheumatoid arthritis in humans should not be overlooked.

Experimental Section

General Methods and Materials. All reactions were carried out under an inert atmosphere with dry solvents under anhydrous conditions, unless otherwise stated. CH_2Cl_2 and THF were distilled from calcium

(22) Kjellén, P.; Brunsberg, U.; Broddefalk, J.; Hansen, B.; Vestberg, M.; Ivarsson, I.; Engström, Å.; Svejgaard, A.; Kihlberg, J.; Fugger, L.; Holmdahl, R. *Eur. J. Immunol.* **1998**, *28*, 755−767.
(23) Stern, L. J.; Brown, J. H.; Jardetzky, T. S.; Gorga, J. C.; Urban, R. G.; Strominger, J. L.; Wiley: D. C. *Nature* **1994**, *368*, 215−221.
(24) Garboczi, D. N.; Ghosh, P.; Utz, U.; Fan, Q. R.; Biddison, W. E.; Wiley: D. C. *Nature* **1996**, *384*, 134−141.

hydride and sodium–benzophenone, respectively. Pyridine was dried over 4 Å molecular sieves; DMF was distilled and then sequentially dried over two portions of 3 Å molecular sieves. TLC was performed on silica gel 60 F_{254} (Merck) with detection by UV light and charring with aqueous sulfuric acid or phosphomolybdic acid/ceric sulfate/aqueous sulfuric acid. Flash column chromatography was performed on silica gel (Matrex, 60 Å, 35–70 μm, Grace Amicon). Centrifugal TLC was performed using rotors coated with silica gel 60 PF_{254} containing gypsum (Merck). The moving bands were visualized using UV light. Organic solutions were dried over Na_2SO_4 before being concentrated.

The ^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-360, Bruker DRX-400, or Bruker ARX-500 spectrometer for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.27 ppm) or $CDCl_3$ (δ_C 77.0 ppm) as internal standard] or CD_3OD [residual CD_2HOD (δ_H 3.35 ppm) or CD_3OD (δ_C 49.0 ppm) as internal standard] at 300 K. The ^{1}H NMR spectrum of glycopeptide **15** was recorded with a Bruker ARX-500 spectrometer in a 9:1 mixture of H_2O and D_2O [H_2O (δ_H 4.98 ppm) as internal standard] at 278 K. First-order chemical shifts and coupling constants were obtained from one-dimensional spectra, and proton resonances were assigned from COSY,[25] TOCSY,[26] and ROESY[27] experiments. Resonances for aromatic protons and resonances that could not be assigned are not reported. IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer. Optical rotations were measured with a Perkin-Elmer 343 polarimeter. Ions for positive fast atom bombardment mass spectra were produced by a beam of xenon atoms (6 keV) from a matrix of glycerol and thioglycerol. In the amino acid analyses, 5-hydroxynorvaline was not determined, and glutamine was determined as glutamic acid.

Analytical normal phase HPLC was performed on a Kromasil silica column (100 Å, 5 μm, 4.6 × 250 mm) with a flow rate of 2 mL/min and detection at 254 nm. Preparative purifications were performed on a Kromasil silica column (100 Å, 5 μm, 20 × 250 mm) with a flow rate of 20 mL/min.

Compounds **1**,[12] **4**,[13] **5**,[12] **6**,[12b] **9**,[12b] **10**,[12b] and **11**[12b] were prepared as described in the cited references. Synthesis of glycopeptides **14**, **16**, and **17** has been described elsewhere.[12]

(5*R*)-*N*$^\alpha$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^\epsilon$-(*tert*-butoxycarbonyl)-5-hydroxy-L-lysine Benzyl Ester (2). Copper(II) carbonate (658 mg, 2.98 mmol) was added gradually at 85–90 °C to a solution of (5*R*)-5-hydroxy-L-lysine dihydrochloride monohydrate (945 mg, 3.8 mmol) in water (10 mL). The mixture was refluxed for 20 min and then filtered (Hyflo-Supercel). The filter pad was washed with several small portions of hot water until the filtrate was colorless. The volume of the combined filtrate was adjusted to 25 mL with water, and then $NaHCO_3$ (662 mg, 7.9 mmol) was added at 20 °C. A solution of di-*tert*-butyl dicarbonate (1.14 g, 5.2 mmol) in dioxane (10 mL) was added over 1 h, and the reaction mixture was then stirred overnight at 20 °C. The resulting suspension was filtered, and the filter cake was washed with small portions of water. The combined filtrates were concentrated, diluted with water/THF (10 mL, 1:1), and cooled to 5 °C. After 2 h another portion of precipitate was filtered off, and the combined precipitates were suspended in MeOH (15 mL) and stirred overnight. Na^+ Chelex 100 (25 mL) which had been converted to its H^+ form[9] was added together with water (20 mL). The resulting mixture was gently swirled for 4 h and then filtered. The resin was washed with several portions of 50% aqueous MeOH, and the combined filtrates were concentrated at high vacuum to give the *N*$^\epsilon$-*tert*-butoxycarbonyl-protected derivative (764 mg, ~78%) which was used in the next step without purification. A solution of 9-fluorenylmethyl chloroformate (588 mg, 2.28 mmol) in dioxane (3.5 mL) was added dropwise to a solution of the *N*$^\epsilon$-*tert*-butoxycarbonyl-protected derivative from above (600 mg, 2.28 mmol) in dioxane/10% aqueous Na_2CO_3 (7 mL, 1:2) at 0 °C. The solution was stirred for 4 h at room temperature, cold water (120 mL) was added, and the reaction mixture was carefully acidified to pH 3 with cold 1 M aqueous $KHSO_4$, then extracted with EtOAc, dried, and concentrated at reduced pressure. The resulting oil (1050 mg) was dissolved in 80% aqueous EtOH (15 mL), and the pH was carefully adjusted to pH 7 with 30% aqueous Cs_2CO_3, concentrated at reduced pressure, then diluted with EtOH (absolute), and concentrated at high vacuum. The resulting powder was dissolved in DMF (6.5 mL) and cooled to 0 °C, after which benzyl bromide (301 μL, 2.5 mmol) was added dropwise. After 12 h at room temperature the reaction mixture was diluted with water and extracted with Et_2O. The organic phase was dried, and after concentration at reduced pressure the residue was purified by centrifugal TLC fractionation (heptane–ethyl acetate, 1:1) to give **2** (1153 mg, 68% overall): $[\alpha]^{20}_D$ −6° (*c* 1.3, $CHCl_3$); IR (neat) 3360, 1743, 1688, 1534 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.61 (d, J = 7.2 Hz, 1 H, NHα), 5.21 (ABd, 1 H, J = 12.0 Hz, PhC*H*$_2$O), 5.16 (ABd, 1 H, J = 12.1 Hz, PhC*H*$_2$O), 4.90 (br s, 1 H, NHϵ), 4.49–4.41 (m, 1 H, Hα), 4.41 (ABdd, 1 H, J = 7.2, 10.6 Hz, OCOC*H*$_2$CH), 4.36 (ABdd, 1 H, J = 7.3, 10.5 Hz, OCOC*H*$_2$CH), 4.21 (t, J = 7.1 Hz, 1 H, OCOCH$_2$C*H*), 3.67 (br s, 1 H, Hδ), 3.26–3.19 (m, 1 H, Hϵ), 3.01–2.90 (m, 1 H, Hϵ), 2.08–2.00 (m, 1 H, Hβ), 1.83–1.74 (m, 1 H, Hβ), 1.47–1.40 (m, 2 H, Hγ), 1.44 (s, 9 H, *t*Bu); ^{13}C NMR (91 MHz, $CDCl_3$) δ 172.2, 156.9, 156.1, 143.8, 143.7, 141.2, 135.2, 128.6, 128.5, 128.3, 127.6, 127.0, 125.0, 119.9, 79.7, 71.0, 67.2, 67.0, 53.7, 47.1, 46.5, 30.1, 28.9, 28.3; HRMS (FAB) calcd for $C_{33}H_{39}O_7N_2$ 575.2757 (M + H$^+$), found 575.2756. Anal. Calcd for $C_{33}H_{38}O_7N_2$: C, 69.0; H, 6.7; N, 4.9. Found: C, 68.9; H, 6.8; N, 4.9.

(5*R*)-*N*$^\alpha$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^\epsilon$-(*tert*-butoxycarbonyl)-5-*O*-(*tert*-butyldimethylsilyl)-5-hydroxy-L-lysine (3). 2,6-Lutidine (40 μL, 0.34 mmol) followed by *tert*-butyldimethylsilyl trifluoromethanesulfonate (58 μL, 0.26 mmol) was added to a solution of **2** (100 mg, 0.17 mmol) in CH_2Cl_2 (1 mL) at 0 °C, and the solution was stirred for 2 h. Et_2O was added, and the solution was washed with water followed by saturated aqueous NaCl. The organic phase was dried, concentrated, and filtered through silica gel (heptane–ethyl acetate, 1:1) to give the desired silyl ether (115 mg): ^{1}H NMR (360 MHz, $CDCl_3$) δ 5.47 (d, 1 H, J = 6.3 Hz, NHα), 5.21 (ABd, 1 H, J = 12.2 Hz, PhC*H*$_2$O), 5.16 (ABd, 1 H, J = 12.2 Hz, PhC*H*$_2$O), 4.73 (br s, 1H, NHϵ), 4.44–4.32 (m, 3 H, OCOC*H*$_2$CH, Hα), 4.22 (t, 1 H, J = 7.0 Hz, OCOCH$_2$C*H*), 3.74–3.68 (m, 1 H, Hδ), 3.19–3.02 (m, 2 H, Hϵ), 2.03–1.93 (m, 1 H, Hβ), 1.76–1.64 (m, 1 H, Hβ), 0.88 (s, 9 H, *t*Bu), 0.07 and 0.03 (2 s, each 3 H, SiCH$_3$). The silyl ether from above (100 mg, 0.14 mmol) in EtOAc (5 mL) was subjected to hydrogenolysis (1 atm) over 10% Pd–C (40 mg) at room temperature for 1.5 h. The catalyst was then filtered off (Hyflo-Supercel) and washed with EtOAc, EtOH, and MeOH. The combined filtrates were concentrated, and centrifugal TLC fractionation (EtOAc–EtOH, 10:1 → 0:1) of the residue gave **3** (61 mg, 72%): $[\alpha]^{20}_D$ +3° (*c* 1, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD) δ 6.41 (t, 1 H, J = 5.7 Hz, NHϵ), 4.34 (ABdd, 1 H, J = 7.4, 10.4 Hz, OCOC*H*$_2$CH), 4.30 (ABdd, 1 H, J = 6.7, 10.5 Hz, OCOC*H*$_2$CH), 4.20 (t, 1 H, J = 6.9 Hz, OCOCH$_2$C*H*), 4.05 (br s, 1 H, Hα), 3.78–3.73 (m, 1 H, Hδ), 3.06–2.99 (m, 2 H, Hϵ), 2.03–1.94 (m, 1 H, Hβ), 1.70–1.43 (m, 3 H, Hβ',γ,γ'), 0.88 (s, 9 H, *t*Bu), 0.08 and 0.06 (2 s, each 3 H, SiCH$_3$); ^{13}C NMR (91 MHz, CD_3OD) δ 177.7, 158.5, 158.4, 145.3, 145.2, 142.6, 128.8, 128.2, 126.3, 120.9, 80.0, 72.2, 67.9, 56.3, 48.4, 47.1, 32.5, 29.1, 28.8, 26.4, 18.9, −4.2, −4.4; HRMS (FAB) calcd for $C_{32}H_{46}N_2O_7SiNa$ 621.2972 (M + Na$^+$), found 621.2966.

(5*R*)-*N*$^\alpha$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^\epsilon$-(*tert*-butoxycarbonyl)-5-*O*-(6-*O*-(*tert*-butyldiphenylsilyl)-3,4-*O*-isopropylidene-β-D-galactopyranosyl)-5-hydroxy-L-lysine Benzyl Ester (7). A solution of dimethyldioxirane[28] in acetone (15.5 mL, ~0.07 M, 0.78 mmol) was added to 6-*O*-(*tert*-butyldiphenylsilyl)-3,4-*O*-isopropylidene-D-galactal[13] (**4**; 274 mg, 0.646 mmol) in CH_2Cl_2 (7 mL) at 0 °C, and the solution was protected from light and stirred at 0 °C for 60 min. Concentration of the solution under reduced pressure yielded the corresponding α-1,2-anhydrosugar. A solution of **2** (743 mg, 1.29 mmol) in THF (8 mL) containing crushed molecular sieves (AW-300, 500 mg) was added to the α-1,2-anhydrosugar, and the mixture was stirred for 30 min at room temperature and then cooled to −50 °C. Zinc chloride (970 μL, 1.0 M in Et_2O, 0.970 mmol) was added, and the reaction mixture was allowed to attain room temperature over 19 h. The mixture was then diluted with EtOAc, filtered (Hyflo-Supercel), and washed with water. The aqueous phase was twice extracted with EtOAc, and the combined

(25) Derome, A. E.; Williamson, M. P. *J. Magn. Reson.* **1990**, *88*, 177–185.
(26) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355–360.
(27) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207–213.
(28) Adam, W.; Bialas, J.; Hadjiarapoglou, L. *Chem. Ber.* **1991**, *124*, 2377.

organic phases were dried and concentrated. Flash column chromatography (toluene−MeCN, 6:1 → 4:1) followed by purification by normal-phase HPLC (linear gradient 0 → 20% *tert*-butyl methyl ether in CH_2Cl_2 during 160 min) gave **7** (242 mg, 37%), the corresponding α anomer (30 mg, 5%), and unreacted acceptor **2** (421 mg, 0.733 mmol). Data for Compound **7**: $[\alpha]^{20}_D$ +8° (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.59 (d, *J* = 7.4 Hz, 1 H, NHα), 5.36 (br s, 1 H, NHε), 5.20 (ABd, 1 H, *J* = 12.1 Hz, PhC*H₂*O), 5.16 (ABd, 1 H, *J* = 12.2 Hz, PhC*H₂*O), 4.44−4.38 (m, 1 H, Hα), 4.42 (ABdd, 1 H, *J* = 7.0, 10.6 Hz, OCOC*H₂*CH), 4.35 (ABdd, 1 H, *J* = 7.2, 10.7 Hz, OCOC*H₂*CH), 4.23 (br d, 1 H, *J* = 4.1 Hz, H-4), 4.19 (t, *J* = 6.9 Hz, 1 H, OCOCH₂C*H*), 4.15 (d, 1 H, *J* = 8.1 Hz, H-1), 4.02 (br t, 1 H, *J* = 6.3 Hz, H-3), 3.95−3.91 (m, 2 H, H-6), 3.90−3.86 (m, 1 H, H-5), 3.63 (br s, 1 H, Hδ), 3.51 (t, 1 H, *J* = 7.7 Hz, H-2), 3.29−3.23 (m, 1 H, Hε), 3.18 (br s, 1 H, OH), 3.10 (dt, 1 H, *J* = 5.7, 14.4 Hz, Hε), 2.01−1.93 (m, 1 H, Hβ), 1.92−1.84 (m, 1 H, Hβ), 1.66−1.58 (m, 2 H, Hγ), 1.49 and 1.33 (2 s, each 3 H, CH_3), 1.28 and 1.04 (2 s, each 9 H, *t*Bu); ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.0, 156.2, 156.0, 143.8, 143.7, 141.2, 135.6, 135.5, 135.1, 133.2, 133.0, 129.8, 128.6, 128.5, 128.4, 127.8, 127.7, 127.1, 125.1, 119.9, 110.0, 102.8, 80.2, 79.0, 78.9, 73.6, 73.1, 67.4, 67.1, 62.5, 53.7, 47.2, 44.2, 28.4, 28.2, 28.1, 26.8, 26.3, 19.2; HRMS (FAB) calcd for $C_{58}H_{70}N_2O_{12}SiNa$ 1037.4596 (M + Na$^+$), found 1037.4608. Anal. Calcd for $C_{58}H_{70}O_{12}N_2Si$: C, 68.6; H, 7.0; N, 2.8. Found: C, 68.7; H, 7.0; N, 2.9.

(5*R*)-*N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*N*$^{\epsilon}$-(*tert*-butoxycarbonyl)-5-*O*-(6-*O*-(*tert*-butyldiphenylsilyl)-3,4-*O*-isopropylidene-β-D-galactopyranosyl)-5-hydroxy-L-lysine (8). A solution of **7** (198 mg, 0.195 mmol) in EtOAc (12 mL) was treated with 10% Pd−C (198 mg) under hydrogen (1 atm) at room temperature for 4 h. The catalyst was then filtered off (Hyflo-Supercel) and washed with EtOAc and EtOH. The combined filtrates were concentrated, and flash column chromatography (toluene−EtOH, 20:1 → 3:1) of the residue gave **8** (154 mg, 85%): $[\alpha]^{20}_D$ +10° (*c* 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD) δ 4.37 (d, 1 H, *J* = 7.4 Hz, OCOC*H₂*CH), 4.36 (d, 1 H, *J* = 6.8 Hz, OCOC*H₂*-CH), 4.32−4.30 (m, 1 H, H-4), 4.27 (d, 1 H, *J* = 8.2 Hz, H-1), 4.23 (br t, 1 H, *J* = 7.0 Hz, OCOCH₂C*H*), 4.15−4.11 (m, 1 H, Hα), 4.02 (br t, 2 H, *J* = 6.2 Hz, H-3, H-5), 3.89 (d, 2 H, *J* = 6.9 Hz, H-6), 3.71−3.66 (m, 1 H, Hδ), 3.42 (dt, 1 H, *J* = 7.7 Hz, H-2), 3.21 (ABdd, 1 H, *J* = 3.9, 13.6 Hz, Hε), 3.08 (ABdd, 1 H, *J* = 7.1, 13.7 Hz, Hε), 2.10−2.01 (m, 1 H, Hβ), 1.80−1.71 (m, 1 H, Hβ), 1.66−1.60 (m, 2 H, Hγ), 1.48 and 1.33 (2 s, each 3 H, CH_3), 1.25 and 1.02 (2 s, each 9 H, *t*Bu); ^{13}C NMR (126 MHz, CD_3OD) δ 178.0, 158.6, 158.2, 145.4, 145.2, 142.6, 136.8, 136.7, 134.4, 134.2, 131.0, 128.9, 128.8, 128.2, 126.3, 120.9, 110.8, 103.7, 80.7, 80.6, 80.0, 74.7, 74.6, 74.3, 67.9, 63.8, 56.4, 45.5, 30.2, 28.8 28.5, 27.3, 26.7, 20.0; HRMS (FAB) calcd for $C_{51}H_{64}N_2O_{12}SiNa$ 947.4126 (M + Na$^+$), found 947.4142.

General Procedure for Solid-Phase Peptide Synthesis. Peptide **13** and glycopeptide **15** were synthesized in a custom-made, fully automatic continuous flow peptide synthesizer constructed essentially as described.[29] A resin consisting of a cross-linked polystyrene backbone grafted with poly(ethylene glycol) chains was used for the syntheses. The resin carried the C-terminal lysine on a *p*-hydroxymethylphenoxy linker (TentaGel S PHB, Rapp Polymere, Germany). *N*$^{\alpha}$-Fmoc-amino acids (Bachem, Switzerland) with the following protective groups were used: triphenylmethyl (Trt) for glutamine; *tert*-butyl for glutamic acid and hydroxyproline; and *tert*-butoxycarbonyl (Boc) for lysine. DMF was distilled before being used.

In the synthesis of glycopeptide **15**, 60 μmol of resin was used in the peptide synthesizer. The *N*$^{\alpha}$-Fmoc-amino acids were activated as 1-benzotriazolyl esters.[30] These were prepared in situ by reaction of the appropriate *N*$^{\alpha}$-Fmoc-amino acid (0.24 mmol), 1-hydroxybenzotriazole (HOBt) (0.36 mmol), and 1,3-diisopropylcarbodiimide (0.234 mmol) in DMF (1.3 mL). After 45 min bromophenol blue (51 nmol, 0.3 mL of a 0.15 mM solution in DMF) was added to the 1-benzotriazolyl ester solution which was then recirculated through the column containing the resin. The acylation was monitored[31] using the absorbance of bromophenol blue at 600 nm, and the peptide−resin was automatically washed with DMF after 1 h or when monitoring revealed the coupling to be complete. *N*$^{\alpha}$-Fmoc deprotection of the peptide resin was performed by a flow of 20% piperidine in DMF (2 mL/min) through the column for 12.5−27.5 min, and was monitored[32] using the absorbance of the dibenzofulvene−piperidine adduct at 350 nm. After completion of the *N*$^{\alpha}$-Fmoc deprotection, the peptide−resin was again washed automatically with DMF. The glycosylated amino acid **8** (72 μmol) was activated separately in DMF (1.0 mL) at room temperature during 30 min by addition of 1,3-diisopropylcarbodiimide (72 μmol) and 1-hydroxy-7-azabenzotriazole[33] (HOAt, 0.216 mmol). Compound **8** was then coupled manually to the peptide−resin which had been removed from the synthesizer. The coupling of **8** was performed in a mechanically agitated reactor during 24 h, and it was monitored by bromophenol blue as described above. After coupling of **8** the glycopeptide resin was reinserted in the synthesizer, and coupling of the remaining amino acids was performed as outlined above. Peptide **13** was synthesized essentially in the same manner.

After completion of the synthesis, the resins carrying protected peptide **13** and glycopeptide **15** were washed with CH_2Cl_2 and dried under vacuum. A portion of each (glyco)peptide−resin was cleaved (*cf.* details given below for **13** and **15**), the amino acid side chains were deprotected, and acid-labile carbohydrate protective groups were removed by treatment with trifluoroacetic acid/water/thioanisole/ethanedithiol [87.5:5:5:2.5, ∼20 mL/200 mg of (glyco)peptide resin] for 2 h followed by filtration. Acetic acid (5 mL) was added to the filtrate, the solution was concentrated, and acetic acid (2 × 5 mL) was added again followed by concentration after each addition. The residue was triturated with diethyl ether (15 mL) which gave a solid, crude glycopeptide which was dissolved in a mixture of acetic acid and water (10 mL) and freeze-dried. Purification by preparative HPLC gave pure **13** and **15**.

Peptide **13** and glycopeptide **15** were analyzed on a Kromasil C-8 column (100 Å, 5 μm, 4.6 × 250 mm) using a linear gradient of 0 → 100% of B in A over 60 min with a flow rate of 1.5 mL/min and detection at 214 nm (solvent systems: A, 0.1% aqueous trifluoroacetic acid; B, 0.1% trifluoroacetic acid in CH_3CN). Purification of crude **13** and **15** was performed on a Kromasil C-8 column (100 Å, 5 μm, 20 × 250 mm) using the same eluant and a flow rate of 11 mL/min.

The peptide content of the purified glycopeptide, as determined by amino acid analysis, has been taken into account in calculating the final yields for the glycopeptides. For example, 54 mg of **15** with a peptide content of 72% was obtained, and the yield was therefore based on 38.9 mg (54 mg × 0.72).

Glycyl-L-glutam-1-yl-*trans*-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-5-hydroxy-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (13). Synthesis, cleavage of the resin-bound peptide (133 mg, 25 μmol) with simultaneous deprotection, and then purification by reversed-phase HPLC (linear gradient 0 → 100% B in A during 60 min), according to the general procedure, gave **13** (10 mg, 74% peptide content, 20% overall yield): MS (FAB) calcd 1504 (M + H$^+$), found 1503; amino acid analysis, Ala 0.98 (1), Glu 2.96 (3), Gly 5.01 (5), Hyl 1.00 (1), Hyp 1.02 (1), Ile 1.01 (1), Lys 1.01 (1), Phe 0.99 (1), Pro 1.01 (1).

Glycyl-L-glutam-1-yl-*trans*-4-hydroxy-L-prolylglycyl-L-isoleucyl-L-alanylglycyl-L-phenylalanyl-5-*O*-(β-D-galactopyranosyl)-5-hydroxy-L-lysylglycyl-L-glutam-1-yl-L-glutaminylglycyl-L-prolyl-L-lysine (15). Synthesis, cleavage of the resin-bound glycopeptide (368 mg, 60 μmol) with simultaneous deprotection, and then purification by reversed-phase HPLC (linear gradient 0 → 100% B in A during 60 min), according to the general procedure, gave **15** (54 mg, 72% peptide content, 39% overall yield): ^{1}H NMR data, see Table 1; MS (FAB) calcd 1666 (M + H$^+$), found 1666; amino acid analysis, Ala 1.00 (1), Glu 2.93 (3), Gly 5.04 (5), Hyl 0.97 (1), Hyp 1.02 (1), Ile 1.02 (1), Lys 1.00 (1), Phe 1.01 (1), Pro 1.03 (1).

Determination of T Cell Hybridoma Response. The response of each T cell hybridoma, i.e., IL-2 secreted on incubation of the hybridoma with antigen-presenting spleen cells and increasing con-

(29) Cameron, L. R.; Holder, J. L.; Meldal, M.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2895−2901.
(30) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788−798.
(31) Flegel, M.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1990**, 536−538.
(32) Dryland, A.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1986**, 125−137.
(33) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397−4398.

centrations of antigen [type II collagen or (glyco)peptides **12**−**17**], was determined in a standard assay using the T cell clone CTLL.[21] In brief, 5×10^4 T cell hybridomas were cocultured with 5×10^5 syngeneic, irradiated (1500 rad) spleen cells and antigen in a volume of 200 μL in flat-bottom microtiter plate wells. After 24 h, 100 μL aliquots of the supernatants were removed and frozen to kill any transferred T cell hybridomas. To the thawed supernatant, 10^4 IL-2 sensitive CTLL T cells were added. The CTLL cultures were incubated for 24 h, after which they were pulsed with 1 μCi of ^{3}H-TdR for an additional 15−18 h. The cells were harvested on glassfiber sheets in a Filtermate TM cell harvester (Packard Instruments, Meriden, CT), and the amount of radioactivity was determined in a matrix 96 Direct Beta Counter (Packard). All experiments were performed in duplicate.

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Supporting Information Available: Spectra of **3** and **8** (4 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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