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Identification of the minimal glycopeptide core recognized by T cells in a model for rheumatoid arthritis

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Abstract—Collagen induced arthritis (CIA) is a common mouse model for rheumatoid arthritis. Two sets of truncated peptides derived from type II collagen have been prepared and tested for binding to A^q, a MHC-II molecule associated with development of CIA. Binding to A^q correlated well with predictions from a computer-based model. T-cell hybridomas, obtained in CIA, were also used to study the ability of A^q bound peptides to trigger a T-cell response. The minimal peptide epitope required for binding, as well as for giving a T-cell response, was determined to be CII260–267. In collagen this epitope is often glycosylated at hydroxylysine 264 and glycosylation has been shown to be an immunodominant feature in CIA. Synthesis and evaluation of CII260–267 carrying a β-D-galactosyl moiety at position 264 revealed that this glycopeptide stimulated representative members from a panel of carbohydrate-specific T-cell hybridomas obtained in CIA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory disease characterized by a tissue-specific attack on peripheral, cartilageous joints. RA affects 0.5–1% of the population in the industrialized world and is generally considered to be of autoimmune origin. Symptoms include swelling, stiffness and pain in the joints, and subsequent erosion of the underlying bone.[1] A large number of therapeutics are available for treatment of RA, but no cure is available and most treatments do not retard disease progression sufficiently in a majority of patients.[2] Current RA therapies are directed towards several targets, and more are under investigation, but as long as the causative agent(s) in RA remains unknown development of significantly improved therapies will be difficult.

The finding that RA is associated with the class II molecules DR4 and DR1, encoded from the major histocompatibility complex class II region (MHC-II) in man[3,4] suggests that the disease is associated with binding of an auto-antigenic peptide to these MHC-II molecules. Presentation of peptide—MHC-II complexes to $CD4^+$ T cells would then cause the T cells to initiate the inaccurate, autoimmune response seen in RA. The initiating agent is still unknown but collagen type II (CII) is a potential candidate.[5] CII is abundant in cartilage, that is, the tissue attacked in RA, and even though CII not necessarily initiates RA it is definitely a component of the pathology. For instance, collagen specific antibodies have been found in serum and synovial fluid of RA patients,[6,7] and CII reactive T-cell clones have been isolated from a group of severely affected RA patients.[8,9] Following injection of CII in rat[5] or mice,[10] the animal develops collagen induced arthritis (CIA), with symptoms and histopathology very similar to those of patients suffering from RA. Today, murine CIA is a frequently used animal model for RA where susceptibility is linked to the mouse MHC-II molecule A^q.[11] The closely related A^p molecule, which only differs in four amino acids compared to A^q, does not confer susceptibility to RA.[12,13] The crystal structure of the structurally related DR1 molecule has been solved,[14] and based on this a three-dimensional computer model of the A^q molecule has been developed.[15]

In mice, high incidence as well as development of severe forms of CIA is linked to presentation of an immunodominant peptide epitope, located between residues

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256 and 270 of type II collagen (CII256–270).[16] Synthetic peptides with subsequent alanine substitution of the residues in CII256–270 identified the side chains of Ile 260 and Phe 263 as the major anchor points in binding to A^q (Fig. 1).[17,15] Several of the side chains in the CII256–270 peptide (positions 261, 262, 264, 266 and 267) have been found to be of variable importance as interaction sites for different T-cell clones.[18,17,19] In addition, studies based on presentation of synthetic peptides to polyclonal T cells by antigen presenting cells have suggested CII260–267 to be the immunodominant core.[18] Collagen II contains a large number of lysine residues that are often post-translationally hydroxylated and subsequently glycosylated with a β-D-galactopyranosyl- or an α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl unit.[20] The immunodominant CII256–270 epitope contains two such glycosylation sites, lysines 264 and 270, which are involved in T-cell recognition. Glycosylation at these sites has been proposed as an explanation for the unresponsiveness of the majority of T-cell hybridomas obtained in CIA to non-glycosylated peptides.[21] By using synthetic glycopeptides this hypothesis was confirmed since the unresponsive T-cell hybridomas were found to recognize different stages of glycosylation of the central hydroxylysine 264 of CII256–270. In fact most hybridomas (20 out of 29) specifically recognized a β-D-galactopyranose unit located on Hyl 264 (Fig. 1).[19,22,23] The different hydroxyl groups of the galactose unit were found to have varying roles in interactions with different T cells, but HO-4 was most important and is probably involved in hydrogen bonding with the T-cell receptor.[24,25]

Since most T-cell hybridomas obtained in CIA recognize the immunodominant CII256–270 peptide carrying a galactosyl moiety at Hyl 264, it is important to determine the minimal glycopeptide epitope required for binding to A^q and subsequent stimulation of T cells. When this minimal epitope has been defined it may be used for designing of altered peptide ligands (APL) or mimetics of interest in attempts to interfere with the autoimmune T-cell response in RA. In the present paper we have therefore studied the requirements for type II collagen derived peptides and glycopeptides in binding to the A^q molecule and also for stimulation of autoimmune T cells obtained in CIA.

2. Results and discussion

In order to determine the requirements for binding of the immunodominant type II collagen fragment CII256–270 to the MHC-II A^q molecule, as well as the T-cell response, two sets of truncated peptides (N-, respectively, C-terminally truncated) were synthesized. First a series of eight peptides obtained by N-terminal truncation of the rat collagen sequence CII257–275, was prepared using solid-phase peptide synthesis (Table 1). To avoid interference between a charge at the N-terminus of the peptides and A^q and/or the T-cell receptor the N-terminus was acetylated in all peptides. For the same reason the C-terminal was protected as an amide by use of the Rink linker during synthesis. The peptides, henceforth referred to as **N257**, **N258**, …, **N264**, were all purified by reverse-phase HPLC and **N257** and **N264** were characterized by mass spectrometry.

Screening of binding of the N-terminal truncation series to class II MHC was preformed by flow cytometry analysis using whole cells (M12Q 14-7) rich in A^q molecules. The peptides were set to compete for binding to A^q with a fixed concentration of biotinylated CLIP peptide, and the inhibition of the biotin signal was calculated (Fig. 2A). The three longest peptides **N258**, **N259** and **N260** were all able to inhibit the binding of the biotinylated CLIP peptide to A^q to a significant level (>60%).

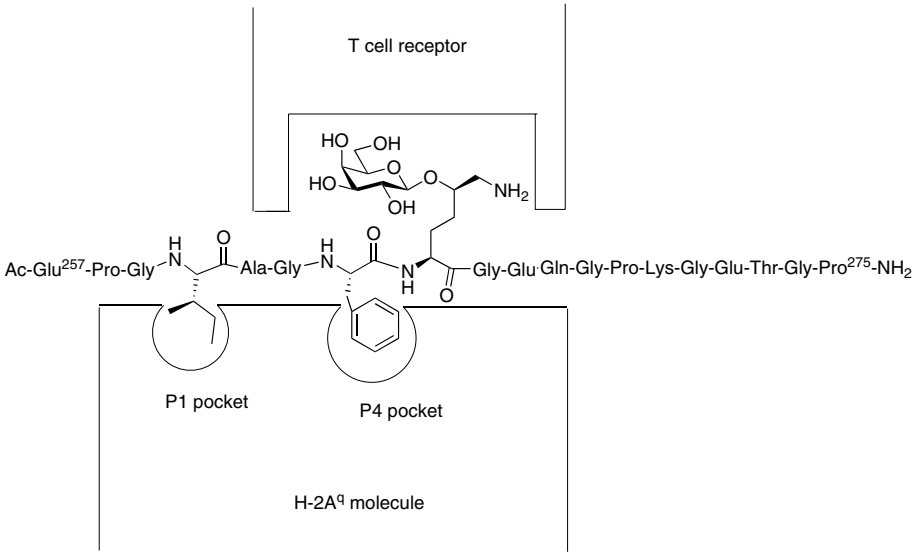


Figure 1. An epitope found within residues CII257–275 of type II collagen is presented by class II MHC (H-$2A^q$) molecules on antigen presenting cells to T cells and their receptors. Most T-cell hybridomas recognize a post-translational modification on hydroxylysine 264, that is, a β-D-galactopyranose unit.

Table 1. Truncated peptide series and reference peptides used to probe the interaction between peptide–MHC complexes and T-cell receptor

N-terminally truncated peptides		
N257	Ac-Glu^{257}-Pro-Gly-Ile-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr-Gly-Pro^{275}-NH_2	rCII 257-275
N258	Ac-Pro^{258}-Gly-Ile-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr-Gly-Pro^{275}-NH_2	rCII 258-275
N259, N260 N261, N262 N263	5 peptides	
N264	Ac-Lys^{264}-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr-Gly-Pro^{275}-NH_2	rCII 264-275
C-terminally truncated peptides		
C275	Ac-Ile^{260}-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr-Gly-Pro^{275}-NH_2	rCII 260-275
C274	Ac-Ile^{260}-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr-Gly^{274}-NH_2	rCII 260-274
C273, C272, C271 C270, C269, C268 C267, C266, C265	9 peptides	
C264	Ac-Ile^{260}-Ala-Gly-Phe-Lys^{264}-NH_2	rCII 260-264
Reference peptides		
CLIP	H-Ser^{88}-Gln-Met-Arg-Met-Ala-Thr-Pro-Leu-Leu-Met-Arg-Pro^{100}-OH	Ii88-100
m259-273	Ac-Gly^{259}-Ile-Ala-Gly-Phe-Lys-Gly-Asp-Gln-Gly-Pro-Lys-Gly-Glu-Thr^{273}-NH_2	mCII 259-273
r256-270	Ac-Gly^{256}-Glu-Pro-Gly-Ile-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys-^{270}-NH_2	rCII 256-270
r259-273	Ac-Gly^{259}-Ile-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr^{273}-NH_2	rCII 259-273
r259-273Gal	Gal | Ac-Gly^{259}-Ile-Ala-Gly-Phe-Hyl-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr^{273}-NH_2	rCII 259-273, GalHyl 264

rCII, rat collagen type II; mCII, mouse collagen type II; Ii, invariant chain; CLIP, class II-associated invariant chain peptide.

However, removal of the proposed anchor residue, isoleucine 260 (peptides **N261**–**N264**) resulted in a distinct decrease in inhibitory power. An advantage of using whole cells is that this allows binding to native MHC-II molecules under in vivo like conditions, but to the expense of quite a high unspecific binding.

To study the MHC-II binding in a more sensitive system the N-terminally truncated peptides were evaluated in a time-resolved fluoroimmunoassay. In this competition assay, increasing concentrations of the N-terminally truncated peptides were allowed to compete with a fixed concentration of biotinylated CLIP peptide for binding to affinity purified A^q molecules of spleen cell origin. Fluorescence from europium labelled streptavidin was used to detect biotinylated CLIP bound to A^q (Fig. 2C). By increasing the concentration of peptides **N258**, **N259** and **N260** the CLIP peptide was completely competed out from the A^q binding site. Increasing concentrations of peptides **N261**–**N264** did, on the other hand, not affect binding of CLIP. Shortening of the peptide by removal of amino acids Glu, Pro and Gly at positions 257, 258 and 259, respectively, did thus not affect binding to H-2A^q. However, the peptide lost binding, in an all or nothing manner, by removing isoleucine 260, confirming the role of this residue as an anchor position. A minimal peptide must therefore include position 260 to retain MHC-II binding.

To investigate the role of the C-terminal residues a new series of twelve C-terminally truncated peptides based the rat collagen sequence CII260–275 was prepared using solid-phase peptide synthesis. The peptides all began with the anchor residue, isoleucine 260, at the N-terminus and are henceforth referred to as **C264**–**C275**, depending on the final C-terminal amino acid (Table 1). The 12 peptides were all purified by reversed-phase HPLC and they were characterized by mass spectrometry. Their binding to H-2A^q was evaluated in the same assays as described for the N-terminally truncated peptides. Competition of binding with biotinylated CLIP to whole cells was investigated by flow cytometry analysis and revealed a gradual loss in binding as the peptides were shortened (Fig. 2B). This behaviour was confirmed in the time-resolved fluoroimmunoassay using purified A^q molecules (Fig. 2D). The two shortest peptides, **C264** and **C265**, appeared not to bind to A^q as they did not compete with the CLIP peptide. The heptamer CII260–266 (**C266**) thus constitutes the minimal epitope that is able to display at least weak binding to A^q.

A three-dimensional model of the complex between CII256–270 and the A^q molecule has previously been developed based on the structure of the DR1 molecule.[15] This structure was used to develop a computational model for predicting affinities of peptides binding to

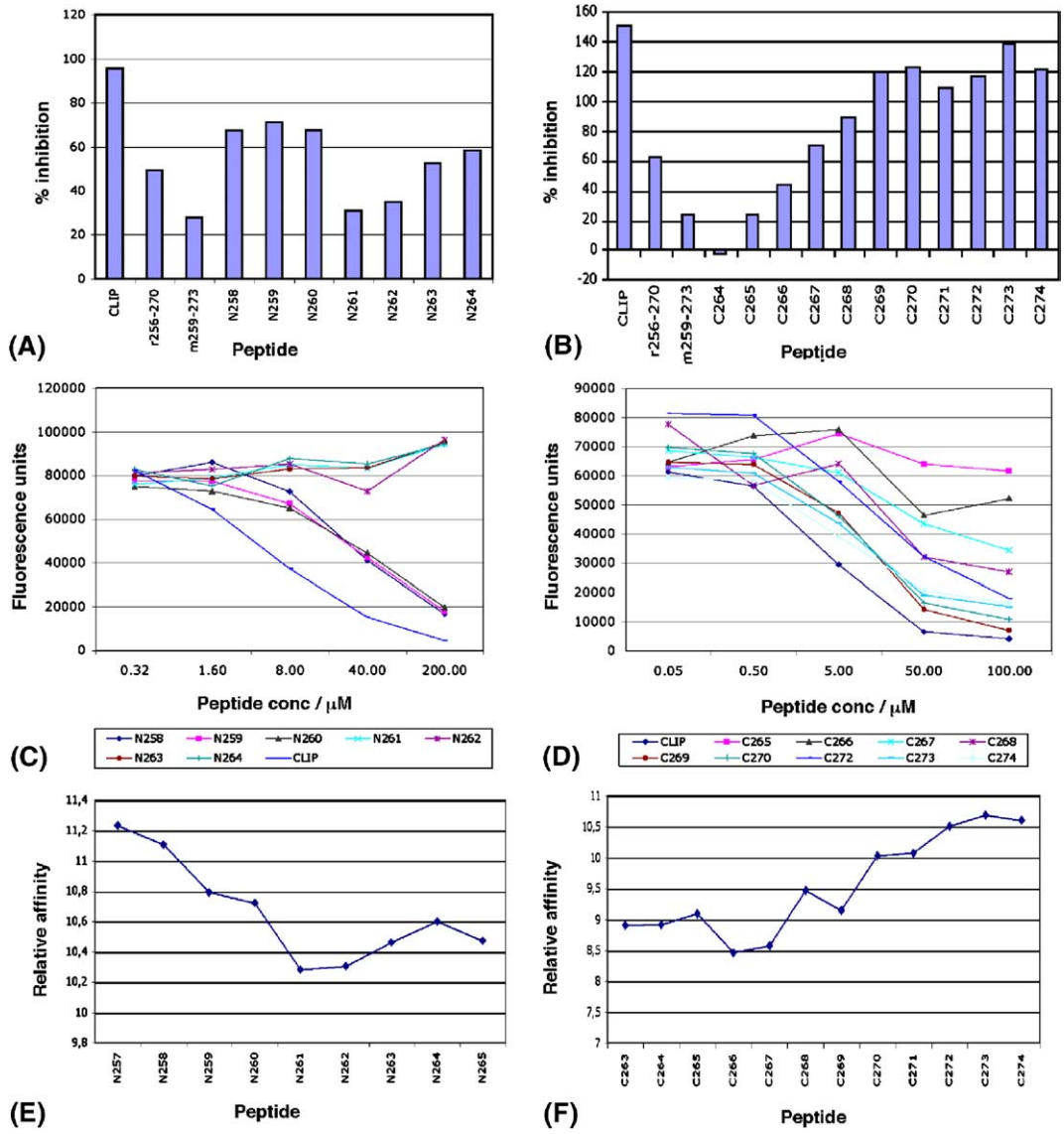


Figure 2. Binding of N- and C-terminally truncated peptides to A^q. (A and B) Inhibition of binding of a biotinylated CLIP peptide to the A^q molecule on whole cells (APCs) upon incubation with peptides from the N- and C-terminally truncated series, respectively. The peptides were allowed to compete for binding to the APCs for 2.5 h, after which the biotinylated CLIP peptide was detected by a streptavidin conjugate (SAPE) and quantified by flow cytometry analysis. Data are displayed as % inhibition as compared to biotinylated CLIP in the absence of competing peptide. (C and D) Binding of biotinylated CLIP to affinity purified A^q molecules in a competition assay with increasing concentrations of peptides from the N- and C-terminally truncated series, respectively. A^q bound biotinylated CLIP was detected by time-resolved fluoroimmunoassay using europium labelled streptavidin. (E and F) Binding affinity of N- and C-terminally truncated peptides to the A^q molecule as calculated based on a structural model of the A^q molecule. The peptides were docked into the active site of the MHC-II model and the affinity was calculated using a PLS model based on VALIDATE fields.

A^q. Using VALIDATE fields,[26] where the variables describing the number of rotable bonds and log *P* were excluded to allow a better comparison between molecules having large difference in size, binding affinities to the A^q molecule were calculated for the two truncation series (Fig. 2E and F). The trends in the calculated affinities correlate well with the experimental data for binding (cf. Fig. 2E and F vs A–D). The drop in affinity when isoleucine 260 is removed in the N-terminal truncation series is predicted by the model, as well as the more successive loss in affinity for the C-terminally truncated peptides. Thus the VALIDATE based model appears to be useful for predicting trends in peptide binding. This predictive model could possibly be validated with an external test set to evaluate how general it is, and how it could be used for further prediction of binding of different peptides, as well as non-peptidic compounds, to the MHC-II A^q molecule.

In order to study the response of T cells to the peptide library, the N-terminally truncated peptides were incubated with antigen presenting spleen cells expressing A^q class II MHC molecules and either of two peptide specific T-cell hybridomas (HCQ.4 and HDB2) obtained previously in CIA.[16] The response of the hybridomas on incubation with increasing concentrations of peptides was measured as interleukin-2 (IL-2) production, detected by proliferation of IL-2 dependent CTLL T cells

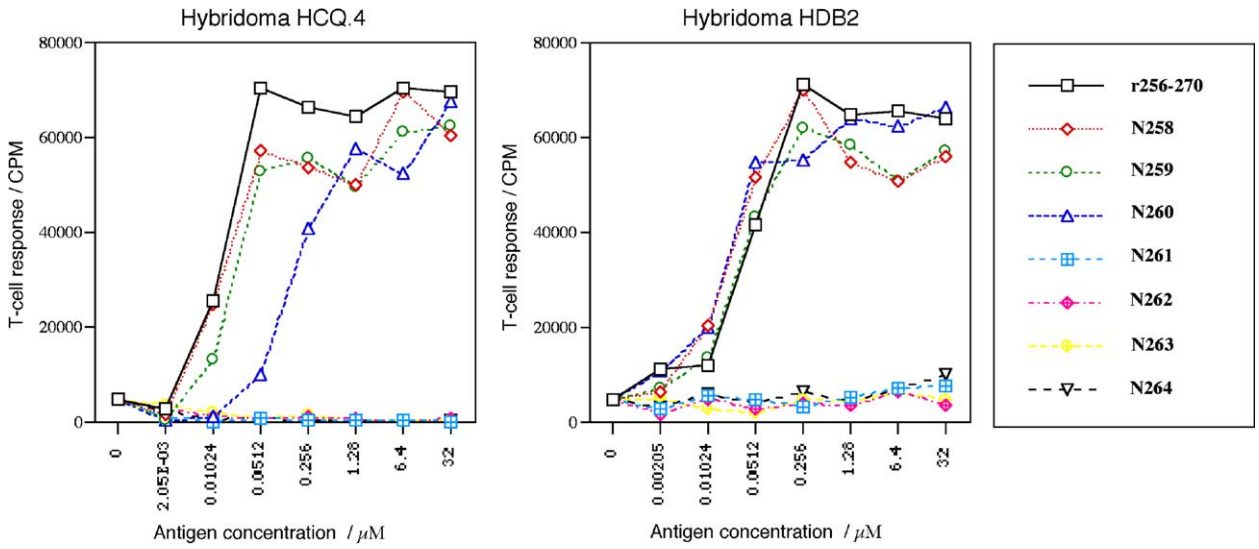


Figure 3. Response of two T-cell hybridomas to increasing concentrations of peptides from the N-terminally truncated series presented by spleen cells from A^q mice. The two hybridomas were chosen for their ability to recognize collagen type II in its non-glycosylated form. Recognition of the peptide—A^q complex by the T-cell hybridomas resulted in secretion of interleukin 2 (IL-2). The production of IL-2 in the supernatant of the T-cell cultures was measured in a radioassay based on proliferation of the IL-2 sensitive T-cell clone CTLL.

(Fig. 3).[27] This revealed that both hybridomas respond well to **N258**, **N259** and **N260**, but as expected not at all to **N261**–**N264**, which lacks the MHC anchor isoleucine 260. Taken together with the binding data for the N-terminally truncated peptides it can be concluded that as long as this series of truncated peptides retain binding to the class II MHC molecules, the hybridomas are able to recognize them. The C-terminally truncated peptides were investigated for A^q restricted recognition by the HDB2 and HCQ.4 hybridomas in the same way (Fig. 4). The two hybridomas give a response towards peptides **C275**–**C267** that is slightly stronger than, or equal to, that generated by the longer peptide CII256–270. However once another amino acid is truncated from the peptide, as in the case of **C266**, the response drops drastically by at least two orders of magnitude. As expected the two shortest peptides **C264** and **C265**, which did not bind to A^q, were unable to elicit a response from the two hybridomas. It thus seems that once the binding of the peptides to MHC-II gets to weak the response of the two T-cell hybridomas is lost. Thus, CII260–267 is concluded to be the minimal *peptide* epitope capable of binding to A^q with sufficient affinity and presenting appropriate T-cell contact residues so that a full response is produced from the T-cell hybridomas.

Since most murine T-cell hybridomas obtained in CIA recognize the glycosylated form of type II collagen, we synthesized the minimal peptide (CII260–267) carrying a β-D-galactose residue on hydroxylysine 264, henceforth referred to as **Gal-C267** (Fig. 5). This glycopeptide was evaluated for T-cell recognition by four representative hybridomas, selected from each of four groups of hybridomas that were previously found to display different fine specificity for the galactose moiety linked to hydroxylysine 264.[24] The octamer glycopeptide **Gal-C267** is recognized by the four hybridomas to the same extent as denaturated collagen (dCII) and the longer glycopeptide counterpart **r259–273 Gal** (Fig. 6). As expected, **Gal-C267** was not recognized by hybridoma HCQ.4, which is specific for non-glycosylated CII. Denaturated type II collagen serves as a positive control for both glycopeptide and peptide recognizing hybridomas since it contains a mixture of lysine and galactosylated hydroxylysine at position 264. The fact that all four galactose specific hybridomas recognize the octamer glycopeptide **Gal-C267** reveals that all structural features for eliciting a proper T-cell response are found within this minimal epitope. These findings suggest that short CII-derived glycopeptides, or drug-like analogues, could be used to interfere with development of autoimmune rheumatoid arthritis, and potentially for treatment of the disease. For instance, the minimal glyco-epitope **Gal-C267** may be used to develop altered peptide ligands (APL's) in which only one, or a few, of the amino acids have been modified. It is known that introduction of minor modifications in an immunodominant peptide may result in different effects on the T cell such as partial agonism or antagonism, which subsequently may give different T-cell activation states.[28] A partial activation or deactivation may cause a T cell to go into anergy, that is, a passive state in which the T cell no longer responds towards its antigen. Other APL's may induce regulatory T cells resulting in an active and sustained suppression of the immune response. In addition, recent vaccination attempts using CII peptides have shown promising results in CIA,[29] and the potential of **Gal-C267** in such studies could also be investigated further.

3. Conclusion

Two series of truncated peptides derived from type II collagen have been prepared and tested for binding to A^q, a MHC-II molecule associated with high incidence and severity of rheumatoid arthritis (RA). The subsequent response of T-cell hybridomas obtained in collagen induced arthritis, a common mouse model for RA, was also determined for the two series of peptides. In

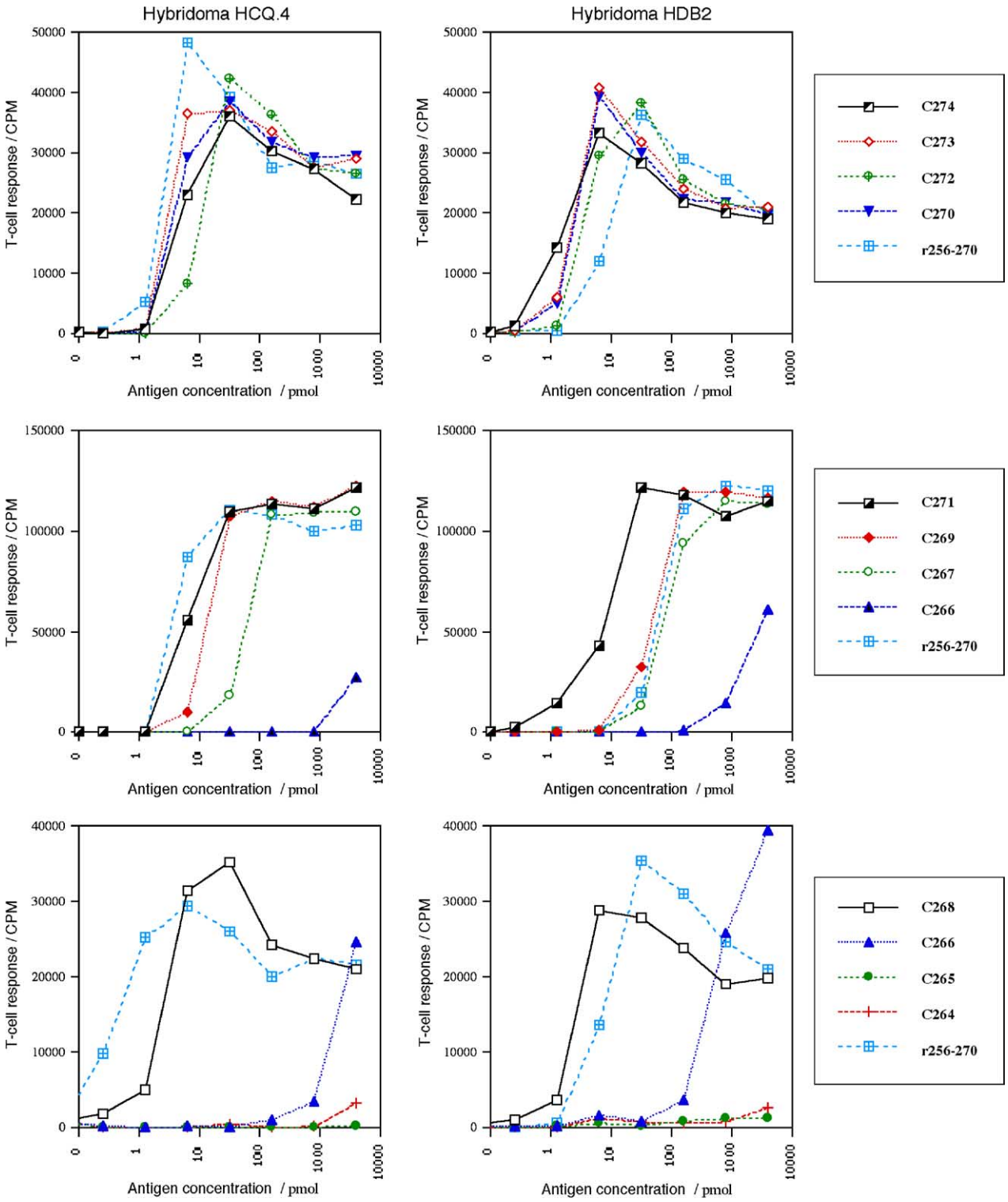
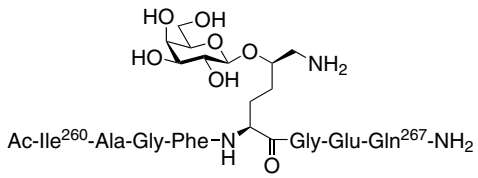


Figure 4. Response of two T-cell hybridomas to increasing concentrations of peptides from the C-terminally truncated series when presented by spleen cells from H-2A^q mice. The resulting IL-2 production was determined as described in the legend of Figure 3.

binding to A^q, the importance of isoleucine 260 as an anchor position at the N-terminus was confirmed, while a gradual loss of binding was seen on truncation of the C-terminus. The minimal truncated peptide that shows significant binding to the A^q molecule was found to be CII260–266. However, an additional amino acid (Gln 267) was required at the C-terminus in order to elicit a proper T-cell response, making CII260–267 the minimal T-cell epitope. A computer-based model able of predicting the binding trends was also developed. Synthesis and evaluation of the minimal T-cell epitope CII260–267 in glycosylated form, that is, carrying a β-D-galactosylated hydroxylysine moiety at position 264, revealed that the glycopeptide contained all structural elements required to stimulate carbohydrate-specific T-cell hybridomas obtained in arthritic mice.



Gal-C267

Figure 5. The glycopeptide **Gal-C267**, corresponding to residues 260–267 of type II collagen with a galactose moiety linked to hydroxylysine 264.

4. Experimental section

4.1. General methods and materials

Hydroxylysine (Hyl) was protected in several steps to give (5*R*)-N^{α} (flouoren-9-ylmethoxycarbonyl)-N^{ε}-benzyloxycarbonyl-5-hydroxy-L-lysine allyl ester.[30] This derivative was used in a silver silicate promoted glycosylation with acetobromogalactose to yield a protected Gal-Hyl building block.[31] After removal of the allyl ester (5*R*)-N^{α}-(fluoren-9-ylmethoxycarbonyl)-N^{ε}-benzyloxycarbonyl-5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-5-hydroxy-L-lysine (Gal-Hyl) was obtained ready for use in solid-phase peptide synthesis. Dimethylformamide (DMF) was distilled and used immediately, or stored for a short time over 3 Å molecular sieves. Analytical reversed-phase HPLC was performed on a Kromasil C-8 column (250 × 4.6 mm, 5 μm, 100 Å), eluted with a linear gradient of MeCN (0 → 100%, alternatively 0 → 80%, over 60 min) in H_2O; both eluents containing 0.1% TFA. A flow rate of 1.5 mL/min was used and detection was at 214 nm. Preparative reversed-phase HPLC was performed on a Kromasil C-8 column (250 × 20 mm, 5 μm, 100 Å), with the same eluents, a flow rate of 11 mL/min and detection at 214 nm. The T-cell hybridomas used in this study originate from DBA/1 (HDB.2, HM1R.2) and C3H.Q (HCQ.2, HCQ.4, HCQ.10) mice and were established earlier.[16,19,21,22]

4.2. General procedure solid-phase peptide synthesis (SPPS)

All peptides, and glycopeptide **Gal-C267**, were synthesized as C-terminal amides on a Tentagel–S–NH_2 resin

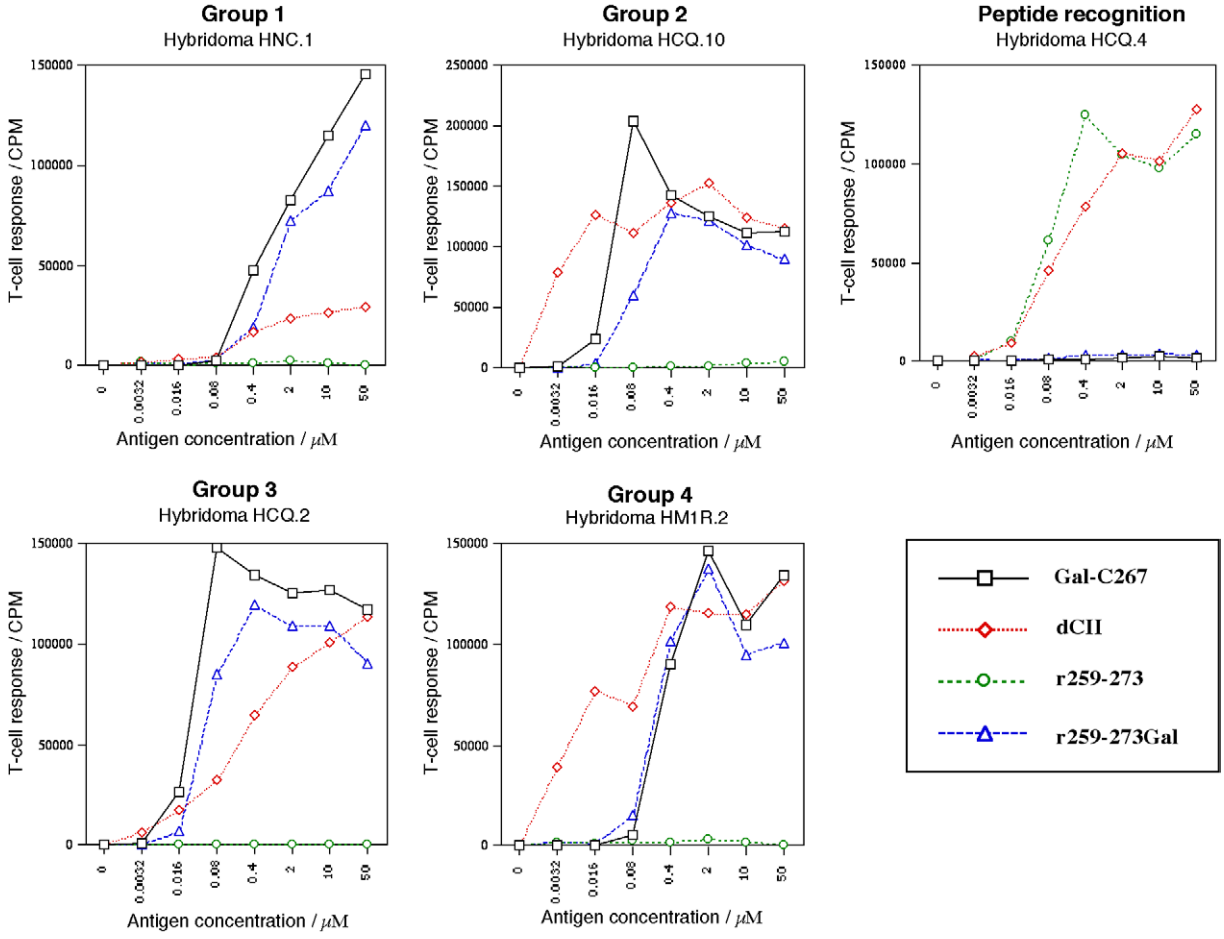


Figure 6. The minimal glycopeptide **Gal-C267**, evaluated for stimulation of T-cell hybridomas obtained in collagen induced arthritis (CIA). Increasing concentrations of (glyco)-peptides (**Gal-C267** and reference peptides) were incubated with spleen cells from A^q expressing-mice and the indicated hybridomas. The hybridomas were chosen from each of four groups having different selectivity towards the galactose moiety. One hybridoma recognizing non-glycosylated peptides was also included. The T-cell response result in IL-2 production, which was determined as described in the legend of Figure 3.

(Rapp Polymere, Germany) in a manually operated reactor, or a parallel synthesis set-up, using standard SPPS-methodology.[23,30] The linker Fmoc-2,4-dimethoxy-4′-(carboxymethyloxy)-benzhydrylamine (Rink, 4 equiv) was first coupled to the resin, followed by the N^{α}-Fmoc amino acids. N^{α}-Fmoc amino acids carrying standard side-chain protective groups (Bachem, Switzerland and Neosystem S.A., France, 4 equiv) as well as the Rink linker were coupled to the resin in DMF using diisopropyl carbodiimide (DIC, 3.9 equiv) in the presence of 1-hydroxy-benzotriazole (HOBt, 6 equiv). The completion of the reaction was monitored by the naked eye using bromophenol blue as indicator. The glycosylated amino acid Gal-Hyl (1.2–1.4 equiv) was coupled in DMF using DIC (1.2–1.4 equiv) and 1-hydroxy-7-azabenzotriazole (1.8–2.1 equiv) for 24 h. Removal of Fmoc protective groups between each coupling cycle was performed using 20% piperidine in DMF. After completing the synthetic sequence, the N-terminus of the peptide was acetylated for 1 h using Ac_2O/DMF (1:2). Cleavage and deprotection of the peptides was performed with TFA/H_2O/thioanisole/ethanedithiol (35:2:2:1), during 4 h at rt, or 3 h at 40 °C (glycopeptide **Gal-C267**). Following repeated concentration from HOAc the (glyco) peptide(s) were precipitated from Et_2O and the crude products freeze–dried. Deacetylation of glycopeptide **Gal-C267** was performed using NaOMe (20 mM) in MeOH for approximately 2 h, after which the solution was neutralized by addition of 10% HOAc in MeOH and concentrated. Purification on reversed-phase HPLC and freeze–drying gave the homogeneous compounds **N257**–**N264**, **C257**–**C264** and **Gal-C267**. The identity of the (glyco)peptides was confirmed by MS and amino acid analysis (peptides **N257** and **C257**). MS (FAB): **N257** calcd 1896, found 1897/1898; **N258**–**N263** not tested (the synthetic procedure implies correct MS for these peptides); **N264** calcd 1225, found 1225; **C274** calcd 1516, found 1516; **C273** calcd 1459, found 1459; **C272** calcd 1358, found 1358; **C271** calcd 1229, found 1229; **C270** calcd 1172, found 1172; **C269** calcd 1044, found 1044; **C268** calcd 946, found 947; **C267** calcd 889, found 890; **C266** calcd 761, found 762; **C265** calcd 632, found 633; **C264** calcd 575, found 576.

4.3. Flow cytometry analysis

The binding of peptides to cell bound A^q MHC class II molecules was studied in an inhibition assay, performed with cell-line M12Q 14-7 transfected with A^q. Increasing concentrations of the competitor peptides **N257**–**N264**, **C275**–**C264** were incubated with a fixed concentration of biotinylated CLIP peptide (5 μM) and M12Q 14-7 cells (3.5×10^5) in a total volume of 100 μL in a 96-well plate (Nunc) for 2.5 h at 37 °C. Excess peptide was removed by washing three times with PBS containing 1% foetal calf serum (FCS) and 0.1% sodium azide. The cells were stained with 0.2 μL streptavidin–phycoerythrin (SAPE) for 30 min at 4 °C in the dark, which bound to the biotinylated CLIP peptide. Following another wash (three times as above), the cells were dissolved in 500 μL of PBS buffer and transferred to FACS tubes. Propidium iodide was added to detect living cells. For flow cytometry analysis, a typical forward and side scatter gate for lymphocytes was set to exclude dead cells, and the phycoerythrin (PE) dye was detected in FL2. In total, 2000 events were typically collected within the gate and analyzed using the FACSort (Becton Dickingson, San Jose, CA) and Becton Dickingson software. The percentage of inhibition for each peptide was calculated from the gated mean fluorescence by comparing with the positive control (no inhibiting peptide) after subtracting the negative control (no biotinylated CLIP peptide).

4.4. Affinity purification of A^q molecules

Spleens from A^q mice were used as a source of MHC class II molecules. The spleens were homogenized and solubilized in PBS containing 1% Nonidet P-40 and a cocktail of protease inhibitors (Complete™, Boehringer, Mannheim) at a concentration of 10^8 cells/mL. Nuclei and debris were removed by centrifugation at 10,000*g* for 45 min, followed by filtration through 1F (Munktell) 0.8, 0.45, and 0.22 μm filters (Millipore). The lysates were then passed four times through a pre-equilibrated affinity column, with the mAbs Y3-P or 7.16.17 coupled to Affi-Gel (Bio-Rad). Following a wash using five column volumes of equilibration buffer (PBS, 0.5% NP-40, Complete™), the detergent concentration was reduced by washing to 0.1% and the MHC class II molecules were eluted with a 0.1 M glycine buffer (0.15 mM NaCl, 0.1% NP-40, pH 10.9). The protein containing fractions were pooled and concentrated by centrifugation (Sorvall SS34, 4500 rpm) through a microsep™-10 kD membrane (Filtron, Northborough, MA). The supernatant was kept at 4 °C throughout the purification. The protein content was evaluated by the BCA protein assay (Pierce, Rockford, IL) and the A^q MHC class II molecules were characterized by SDS-PAGE (Bio-Rad).

4.5. Peptide binding to A^q MHC class II molecules

To study the binding of peptides to purified MHC class II molecules an inhibition assay was performed essentially as described.[32,33,15] Increasing concentrations of peptides **N257**–**N264**, **C257**–**C264** were incubated for 60 h at room temperature (or 6–12 h, 37 °C, with shaking)[34] with a fixed concentration of biotinylated CLIP peptide ('CLIPbio', 2.0 μM) and affinity purified A^q molecules (0.1 μM) in 100 mM phosphate buffer (pH 7.0), containing 0.2% NP-40 and a cocktail of protease inhibitors (Complete™, Boehringer, Mannheim). A^q—peptide complexes were captured by incubating for 2 h at room temperature in a 96-well microtiter plate pre-coated with the mAb Y3-P and blocked with PBS, containing 3% BSA, 0.02% sodium azide. Excess peptide was removed by washing with 50 mM Tris buffer containing 150 mM NaCl and 0.1% Tween 20. CLIPbio—MHC class II complexes were quantified using the dissociation enhanced lanthanide fluoroimmunoassay (DELFIA®) kit system based on the time-resolved fluoroimmunoassay technique with europium labelled streptavidin (Wallac, Turku) according to the manufacturers instructions. Following incubation with europium labelled streptavi-

din for 1 h at 4 °C, the plates were washed and treated with enhancement solution, which releases europium from streptavidin and forms a highly fluorescent micellar solution that can be measured by reading the fluorescence on a fluorometer (Wallac).

4.6. Determination of T-cell hybridoma response

The response of each T-cell hybridoma, that is, IL-2 secreted on incubation of the hybridoma with antigen presenting spleen cells and increasing concentrations of (glyco)peptides **N257**–**N264**, **C257**–**C264** and **Gal-C267** was determined in a standard assay using the T-cell clone CTLL.[27] In brief, 5×10^4 T-cell hybridomas were co-cultured with 5×10^5 syngeneic, 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 mCi of ^{3}H-TdR and incubated for an additional 15–18 h. The cells were harvested on glass fibre 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.

4.7. Prediction of binding of peptides to A^q MHC-II molecules

The affinity predictions were carried out essentially as described before.[35] The peptides were docked manually into the active site of A^q using Sybyl 6.3.[36] Docking was performed by superimposition on the backbone of the peptide moiety of CII in the modelled complex with A^q.[15] Energy minimization to relieve strain caused by addition of hydrogen atoms to the complex, and to allow the peptides to assume a favourable conformation, was run on an SGI R12000 Octane computer using the batchmin program from Macromodel 6.5,[37] together with the Amber all-atom force field. The values for shell and core were set to 10 and 8 Å, respectively. The gradient convergence was 0.1 and an iteration limit of 10,000 was used. No solvation model was employed. After minimization the peptide and A^q were extracted to separate files, which were then used as input for calculations of the VALIDATE fields. All fields except $H\log P$ and the induction enthalpy were calculated with VALIDATE.[26] $H\log P$ values were derived using Hint 2.3[38] implemented in Sybyl. Batchmin was used to minimize the extracted peptide using the Amber all-atom force field with the GB/SA solvation model; the starting and the final energies were then used to calculate the induction enthalpy field. The affinity of the peptide for A^q ($-\log K_{dis}$) was predicted using a PLS model based on the VALIDATE fields, generated in SIMCA-P 8.0[39] by Svensson et al.[35] From this final model, the variable describing the number of rotable bonds (RBs) and $\log P$ were excluded to give a fair chance of comparison between peptides that differ significantly in size.

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