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Characterization of T cell hybridomas raised against a glycopeptide containing the tumor-associated T antigen, (βGal (1–3) αGalNAc-O/Ser)

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T cell hybridomas were raised against the glycopeptide S^{72} (Core-1) containing the tumor-associated disaccharide βGal (1–3) αGalNAc (Core-1) *O*-linked to serine at position 72 in the mouse hemoglobin derived decapeptide Hb (67–76). All hybridomas recognized the glycopeptide S^{72} (Core-1). Two of the selected hybridomas responded, however, much better to the S^{72} (Tn) glycopeptide containing the monosaccharide αGalNAc *O*-linked to serine. In addition, one hybridoma cross-responded to the glycopeptide T^{72} (Core-1) having a threonine at position 72 instead of a serine. No cross-responses were found to other glycopeptides consisting of the same hemoglobin peptide with different glycans attached or to the unglycosylated peptides. The T cell receptor Vα and Vβ usage was clearly diverse. The CDR3α regions demonstrated moreover a predominance of small polar amino acid side chains, and three hybridomas contained a common sequence motif. All the sequenced CDR3β regions contained furthermore a conserved proline-glycine motif. In conclusion, immunization with the disaccharide containing glycopeptides S^{72} (Core-1) created a heterogeneous population of glycopeptide specific T cells with the ability of cross-responding toward related glycopeptides.

Keywords: **glycosylated tumor antigens, T-antigen, T cell hybridomas, glycopeptide antigens, Core-1 glycan**

Abbreviations: **Core-1, glycan consisting of βGal (1–3) αGalNAc; Hb (67–76), peptide VITAFNEGLK derived from mouse hemoglobin; S^{72}, Hb (67–76) substituted with Ser at position 72; S^{72} (αGal), Hb (67–76) substituted with Ser (αGal) at position 72; S^{72} (Core-1), Hb (67–76) substituted with Ser (Core-1) at position 72; S^{72} (Tn), Hb(67–76) substituted with Ser (αGalNAc) at position 72; T^{72}, Hb (67–76) substituted with Thr at position 72; T^{72} (Tn), Hb (67–76) substituted with Thr (αGalNAc) at position 72; T^{72} (αGlcNAc), Hb (67–76) substituted with Thr (αGlcNAc) at position 72; T^{72} (αMan), Hb (67–76) substituted with Thr (αMan) at position 72; T antigen, Core-1 glycan; T^{72} (βGal), Hb (67–76) substituted with Thr (βGal) at position 72; T^{72} (Core-1), Hb (67–76) substituted with Thr (Core-1) at position 72; Tn antigen, αGalNAc glycan; TCR, T cell receptor; CDR, complementarity determining region; V, variable; D, diversity; J, joining.**

Introduction

It is now well-established that both $CD4^+$ and $CD8^+$ T cells can recognize glycopeptides carrying mono- and disaccharides in a MHC restricted manner, and that such T cell responses often display a high degree of specificity for the glycan group [1]. The ability of T cells to recognize peptides, which are partly defined by small carbohydrate groups, can be relevant for the immune response against tumors since aberrant glycosylation is a general characteristic of many cancer cells. The mucin-type *O*-glycans are an interesting group of tumor-associated carbohydrates, which includes Tn (αGalNAc), Sialyl-Tn (αNeuAc (2–6) αGalNAc), T-antigen (βGal (1–3) αGalNAc), and the sialylated T antigen (αNeuAc (2–3) βGal (1–3) αGalNAc). These *O*-glycans have all been found in increased amounts associated to cell surface proteins of a variety of epithelial cancers, but are rarely found on healthy tissue [2].

We previously investigated T helper cell responses against tumor-associated glycans using the mouse hemoglobin peptide Hb (67–76), VITAFNEGLK as the model peptide for these studies [3–5]. The hemoglobin peptide is known to bind efficiently to the MHC class II molecule E^k using the Ile^{68} and Lys^{76} residues as primary anchors [6]. The central amino acid Asn^{72} is solvent exposed and acts as an important contact residue for

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T cells raised against the decapeptide [6,7]. The hemoglobin peptide was found to be non-immunogenic in the CBA/J (H-2^k) mouse. Substitution of the central Asn^{72} with either serine or threonine *O*-linked to the mono-saccharide αGalNAc created, however, two E^k binding glycopeptides S^{72} (Tn) and T^{72} (Tn), respectively, which were both immunogenic [4]. When T cell hybridomas raised against the glycopeptide T^{72} (Tn) were tested for cross-responses toward closely related glycopeptides, it became clear that the hybridomas recognized the Tn group with a very high degree of specificity [5]. The T cell receptor (TCR) variable (V) gene usage of the hybridomas was found to be heterogeneous. There was, however, a predominant usage of Vα4 in combination with Vβ1 or Vβ15 [8]. The CDR3 regions were furthermore encoded by small polar amino acid residues in addition to an aromatic amino acid present in either the α or β chain. This was interestingly in agreement with the presence of such amino acid residues within carbohydrate recognizing antibodies and proteins [9,10].

We also studied T cell responses against glycopeptides in which the *O*-linked glycan groups were larger and more complex than Tn [3,11]. The glycopeptide S^{72} (Core-1) was selected for these studies in which the disaccharide βGal (1–3) αGalNAc was *O*-linked to serine at position 72 in the hemoglobin peptide. This disaccharide is known as the Core-1 structure or T-antigen, and it is a well-known tumor-associated carbohydrate frequently expressed on adenocarcinomas [2]. The ability of the glycopeptide S^{72} (Core-1) to bind the MHC class II molecule E^k was previously described [3]. It was furthermore demonstrated that the glycopeptide S^{72} (Core-1) could induce strong T cell proliferation when immunized in CBA/J mice [3,11]. The closely related glycopeptide T^{72} (Core-1) in which the Core-1 glycan was attached to the peptide *O*-linked to threonine was, however, not immunogenic despite the fact that it was binding to E^k with the same binding affinity as the S^{72} (Core-1). Immunization of CBA/J mice with the S^{72} (Core-1) was interestingly found to activate T cells cross-responding to the glycopeptide S^{72} (Tn) and to the unglycosylated peptide S^{72}. The observed T cell proliferation indicated that the T cells were recognizing a structural component common for the three antigens. Alternatively, the cross-response could be due to a stepwise and partial deglycosylation of the Core-1 glycan, which, as previously suggested, was taking place during the priming phase of the immune response *in vivo* [3,4]. Due to this, mice immunized with the glycopeptide S^{72} (Core-1) could be primed by S^{72} (Core-1), S^{72} (Tn) and S^{72}.

In the present study, we generated a small number of T cell hybridomas in order to characterize the T cell recognition of the glycopeptides S^{72} (Core-1) in more details at the clonal level. We showed that the T cell hybridomas were all S^{72} (Core-1) specific although some of them also had prominent cross-responses to the related glycopeptides, T^{72} (Core-1) and S^{72} (Tn). Immunization with S^{72} (Core-1) created moreover a heterogeneous T cell population illustrated by a diverse TCR Vα and Vβ repertoire of the few studied T cell hybridomas. Sequence similarities were, however, found within the CDR3α and β regions, and we consider whether the glycan specificity could be associated to this. The possible role of partial deglycosylation during *in vivo* priming is discussed.

Materials and methods

Peptides and glycopeptides

The synthesis of the Hb (67–76) derived peptides S^{72} and T^{72} and the glycopeptides S^{72} (Core-1), T^{72} (Core-1), S^{72} (Tn), T^{72} (Tn), S^{72} (αGal), T^{72} (αGal), T^{72} (αGlcNAc), T^{72} (αMan), and T^{72} (βGal) was performed by multiple column techniques using fluoren-9-yl-methoxycarbonyl amino acid pentafluorophenyl esters, as described in detail elsewhere [12]. The glycopeptides and peptides used in this study are illustrated in Table 1.

Preparation of T cell hybridomas

7–8 weeks old female CBA/J mice (H-2^k) were immunized in the hind foot pads and at the base of the tail with 0.1 ml of water-in-oil emulsion containing equal amounts of glycopeptide S^{72} (Core-1) in PBS and Freund's complete adjuvant with *Mycobacterium butyricum* (Difco Laboratories, Detroit, USA). A total of 100 μg of glycopeptide was injected into each mouse. Lymph node cells from popliteal and inguinal lymph nodes of the immunized mice were prepared 10 days after immunization, and the lymph node cells were cultured in tissue culture media RPMI1640 containing 10% FCS (Gibco BRL, Life Technologies, Roskilde, Denmark) for 72 hours in Cluster Trays (Nunc A/S, Roskilde, Denmark) using 2 mg/ml glycopeptide for restimulation. The restimulated T cells were fused with the $\alpha\beta$TCR deficient thymoma BWα-β- [13–15] using 50% (w/v) polyethylen glycol (PEG). All hybridomas were cloned by limiting dilution in 96-well microtiter plates containing 1, 0.3, and 0.1 cell/well, and growing T cell hybridomas were tested for glycopeptide specificity as described below. The A4 and T4 nomenclature, which is used to name the individual

Table 1. Peptides and glycopeptides used in the present study

Peptides	
S^{72}	VITAFSEGLK
T^{72}	VITAFTEGLK
Glycopeptides	
S^{72}(Core-1)	VITAFS(βGal(1–3)αGalNAc-*O*)EGLK
T^{72}(Core-1)	VITAFT(βGal(1–3)αGalNAc-*O*)EGLK
S^{72}(Tn)	VITAFS(αGalNAc-*O*)EGLK
T^{72}(Tn)	VITAFT(αGalNAc-*O*)EGLK
S^{72}(αGal)	VITAFS(αGal-*O*)EGLK
T^{72}(αGal)	VITAFT(αGal-*O*)EGLK
T^{72}(αMan)	VITAFT(αMan-*O*)EGLK
T^{72}(βGal)	VITAFT(βGal-*O*)EGLK
T^{72}(αGlcNAc)	VITAFT(αGlcNAc-*O*)EGLK

hybridomas, refers to the glycosylated amino acid building block used for glycopeptide synthesis. A4 refers thus to the serine-*O*-Core-1 building block, whereas the T4 refers to the threonine-*O*-αGalNAc building block.

Assay for IL-2 production

T cell hybridomas were incubated in 96-well flat-bottomed microtiter plates (1×10^5 cells/well) together with the mitomycin C treated B cell lymphoma, CH27 (H-2^k) (1×10^5 cells/well). Peptides or glycopeptides were added to the *in vitro* culture at 10, 3, 1, 0.3, 0.1, and 0.03 μg/ml. Background proliferation was measured by addition of PBS. The final volume per well was 220 μl. Culture supernatant (80 μl) was harvested after 24 hours, and the contents of IL-2 in the culture medium was measured by proliferation of the IL-2 dependent cell line HT-2 (4000 cells per well). [^{3}H]-Thymidine (1 μCi/well) (Amersham, Amersham Denmark ApS, Denmark) was added to the culture, and the cells were harvested on a Filtermate 196 (Packard Instruments, Downers Grove, Ill., USA) after 20 hours in culture. The [^{3}H]-thymidine incorporation was determined by liquid scintillation counting (Topcount, Packard Instruments, Downers Grove, Ill., USA). Data shown represents one of at least three similar experiments performed.

FACS analysis

The T cell hybridomas were tested for surface expression of CD4. T cell staining was performed by the use of FITC-conjugated (rat anti-mouse) CD4 antibody (H129.19) (PharMingen, San Diego, CA, USA). To each FACS tube was added 1×10^6 cells. The cells were incubated with primary antibody diluted in FACS buffer (PBS containing 0.1% NaN_3 and 5% FCS) for 30 minutes at 4°C in the dark. After incubation, the cells were washed three times in FACS buffer and fixed in FACS buffer containing 1% paraformaldehyde before the FACS analysis. The samples were analyzed with a Becton Dickinson FACS-Scan (Becton Dickinson).

Identification of TCR Vα and Vβ gene usage

Total RNA was purified from 0.5–1 $\times 10^7$ T cell hybridomas using the Qiaquick RNeasy purification kit (Qiagen, Struers Kebo Lab, Denmark). Five μg of purified total RNA was converted into cDNA by using the Ready-to-go cDNA synthesis kit (Pharmacia Biotech, Sweden). The TCR Vα usage was determined by PCR using 20 variable Vα specific primers and one constant α chain primer. The TCR Vβ usage was determined by PCR using 20 variable Vβ specific primers and one constant β chain primer [16,17]. Hot-Start PCR was used for all the TCR typing [18]. The PCR primers (20 pmol each) were aliquoted and mixed in 1× PCR buffer (1× PCR Buffer (10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM each dNTP). The tubes were added one Wax-Aid bead (Advanced Biotechnologies Ltd, Epsom, UK) each, and the wax beads were melted by heating the tubes for 5 minutes at 80°C followed by cooling to room temperature. PCR buffer mix (1× PCR buffer (10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl) including Taq DNA polymerase (1.25 U per 50 μl reaction (Amersham Pharmacia Biotech)) was added to the top. 1/25 of the prepared cDNA was finally added to each PCR tube, and the PCR was cycled using the following parameters: initial denaturation at 92°C for 2 minutes followed by 32 cycles of 92°C for 1 minute, 60°C for 30 seconds and 72°C for 1 minute. The PCR was terminated by a final run-off at 72°C for 7 minutes. PCR reactions were performed with a PTC-100 PCR machine.

Direct sequencing of the TCR CDR3 regions

The PCR products were used directly for sequencing. Three identical reactions were set up and analyzed in order to check for base mis-incorporations by the *Taq* DNA Polymerase during the PCR reaction. Before sequencing the PCR products, the DNA was treated with exonuclease 1 and shrimp alkaline phosphatase (Amersham Life Sciences, Cleveland, Ohio, USA) to remove residual single stranded primers and PCR products as well as any remaining dNTPs. DNA sequencing was performed by Thermo sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio, USA) using [^{33}P] labeled terminators. The sequencing was performed on a Hoefer SQ3 Sequencer (Hoefer Pharmacia Biotech, Uppsala, Sweden), and all gels were analyzed on Kodak MS X-Ray films. The variable (V), and junctional (J) gene sequences were verified by Arden et al. [19] and Koop et al. [20].

Results

Creation of T cell hybridomas recognizing the glycopeptide S^{72} (Core-1)

$CD4^+$ T cell hybridomas were produced and cloned in order to determine the pattern of glycopeptide recognition. Five hybridomas were selected for the study, and the glycopeptide specificity of each hybridoma was identified by its ability to recognize the glycopeptide S^{72} (Core-1), as well as related glycopeptides and peptides. The related glycopeptides were all derived from the Hb (67–76) peptide by *O*-glycosylation of serine or threonine at position 72, but each with a different glycan group attached (Table 1). All five hybridomas recognized the S^{72} (Core-1) against which they were raised (Figure 1). The T cell hybridoma A4.2.C6 showed high specificity to the glycopeptide S^{72} (Core-1) and showed no signs of recognizing any other of the used glycopeptides and peptides. This also applied for the T cell hybridoma A4.1.E3.C6. But the specificity of the recognition was clearly lower compared with A4.2.C6, as the hybridoma A4.1.E3.C6 only responded to the glycopeptide S^{72} (Core-1) when used in the highest glycopeptide concentration.

The T cell hybridoma A4.1.B2 cross-responded to the closely related glycopeptide T^{72} (Core-1) even though the response was five-fold lower compared with the response to S^{72} (Core-1). The cross-response was surprising as the glycopeptide T^{72} (Core-1) by itself was non-immunogenic when immunized into the CBA/J mouse [3].

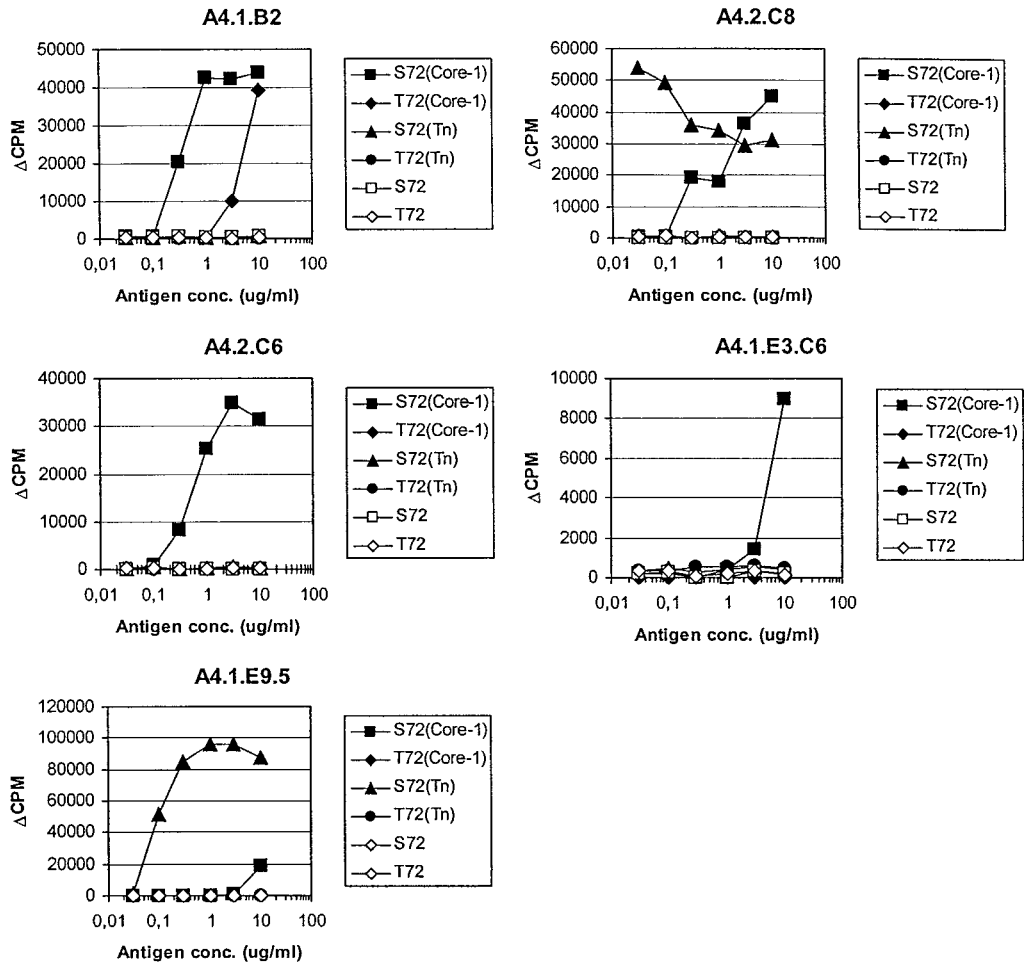

Figure 1. Dose-response curves for the five T cell hybridomas raised against the glycopeptide S^{72} (Core-1). The T cell hybridomas were tested against the glycopeptide S^{72} (Core-1) and the related glycopeptides and peptides. Abscisse: Concentration of added glycopeptide or peptide used to stimulate the hybridoma. Ordinate: [^{3}H]-thymidine incorporation (Δcpm: cpm (glycopeptide or peptide) – cpm (PBS)) of IL-2 sensitive HT-2 cells cultured with hybridoma supernate. Data shown represents one of at least three similar experiments performed.

Two of the hybridomas A4.1.E9.5 and A4.2.C8 recognized the glycopeptide S^{72} (Tn), and both T cells responded much stronger to the glycopeptide S^{72} (Tn) compared with S^{72} (Core-1) (Figure 1). The response to the glycopeptide S^{72} (Tn) measured by A4.2.C8 was inversely related to the glycopeptide concentration, and the T cell proliferation actually increased as the glycopeptide concentration was lowered. This indicated that A4.2.C8 was highly sensitive to the glycopeptide S^{72} (Tn), and that the maximal response and a full dose-response curve could only be reached by lowering the glycopeptide concentration even further. The strong cross-response toward the S^{72} (Tn) suggested that the cells were recognizing a common structure present within the two glycopeptides. The recognition of S^{72} (Tn) could, however, also imply that the two hybridomas were derived from T cell clones, which, during the T cell priming *in vivo*, responded to S^{72} (Tn) formed by enzymatic removal of the distal galactose from the Core-1 glycan. Finally, none of the T cells recognized the unglycosylated peptides S^{72} and T^{72} or the related glycopeptides containing small glycans such as αGal, αGlcNAc, αMan or βGal (not shown).

Vα and Vβ gene usage of hybridomas raised against the glycopeptide S^{72} (Core-1)

The pattern of glycopeptide recognition made it clear that the five isolated T cell hybridomas were not identical. But they all recognized the glycopeptide S^{72} (Core-1) and it was of interest to see if the TCR of the hybridomas displayed certain structural similarities, which could refer to the glycopeptide specificity. Due to the high diversity of the TCR repertoire, only a small fraction of all available T cells are recognizing a given peptide epitope [21,22]. However, in many disease models the TCR repertoire toward a disease causing epitope is biased as evident by a restricted TCR Vα and Vβ gene usage [23,24]. Thus, total RNA was isolated from the hybridomas in order to determine if the TCR Vα and Vβ gene usage was biased toward certain gene

Table 2. TCR Vα and Vβ gene usage of 5 T cell hybridomas raised against S^{72}(Core-1)

Hybridoma	*Vα*	*Vβ*
A4.1.B2	1,2,16	15
A4.1.E3.C6	3,16	4
A4.1.E9.5	5,16	ND
A4.2.C6	3,16	1
A4.2.C8	4,8,16	2

ND, not determined.

elements. The TCR α and β chain transcripts were analyzed by RT-PCR designed to amplify the murine Vα and Vβ gene families [8]. Table 2 illustrates the TCR Vα and Vβ gene usage identified for each hybridoma. The Vα and Vβ usage was clearly heterogeneous as the variable α gene segments Vα1, Vα2, Vα3, Vα4, Vα5, and Vα8 as well as the variable β gene segments Vβ1, Vβ2, Vβ4, and Vβ15 were expressed by the five T cell hybridomas. It was unfortunately impossible to identify the Vβ usage of the hybridoma A4.1.E9.5. Thus, the identification of ten different Vα and Vβ gene segments within only five selected hybridomas clearly indicated that the T cell repertoire toward the glycopeptide was diverse. All the hybridomas expressed a transcript encoding the Vα16 gene segment (Table 2). This transcript has been reported as being derived from the BWα-β-T cell fusion partner and to be non-functional due to a frame-shift insertion [13]. Sequencing of the Vα16 amplified PCR products confirmed that it was identical to the non-functional Vα16 transcript described by Letourneur et al. [13].

The hyper-variability within the TCR CDR3α and CDR3β regions plays an important role during T cell recognition of an MHC-bound peptide [21,22,25–28] and the amino acid composition of the TCR CDR3 regions of glycopeptide-specific T cell may reveal important information about the antigen specificity. Consequently, all the PCR products were purified and sequenced in order to identify the TCR CDR3α and β regions. The translated amino acid sequences are illustrated in Table 3. Even though the RNA transcript of a given Vα or Vβ gene segment was found by RT-PCR, it was, however, not necessarily part of a functional transcript. In accordance to this, two hybridomas were found to express two different TCR α-chain transcripts (Table 2). The hybridoma A4.1.B2 expressed two TCR α-transcripts containing either Vα1 or Vα2. Sequencing of the two PCR products revealed nevertheless that the Vα2 containing transcript was out of correct reading frame concluding that the Vα1 containing transcript was used by the hybridoma. In contrast, the hybridoma A4.2.C8 had both transcripts in the correct reading frame. Expression of two functional TCR α-chains by the same T cell is a common phenomenon [29,30], but the simultaneous presence of both TCR α-chain transcripts makes it difficult to determine which one is used for the glycopeptide recognition. The Vβ2 containing transcript expressed by A4.2.C8 was finally also found to be out of correct reading frame. This leaves unfortunately the TCR Vβ transcripts of two T cell hybridomas A4.2.C8 and A4.1.E9.5 unidentified.

The length of the functional CDR3α regions was between 7 and 10 amino acids, and the small, polar amino acids Asn, Ser, Thr, as well as Gly dominated all the sequences. The enrichment of these amino acids was also observed in hybridomas raised against the glycopeptide T^{72} (Tn) [8]. Nearly all CDR3α regions interestingly contained a polar sequence motif Ser-X-polar-polar (X = Arg or Thr), where the two polar residues encoded two serines or two asparagines. The length of the TCR CDR3β regions varied between 8 and 10 amino acids, but did not reveal any clear amino acid dominance. However, the three available CDR3β regions all contained an identical proline-glycine amino acid motif. Finally, an aromatic amino acid was present in either the CDR3α or β region of those hybridomas in which both CDR3 sequences were available. This observation was also made in the T^{72} (Tn) specific hybridomas [8].

Discussion

The data presented in this study and our previous results [3,11] are a clear demonstration of the ability of T cells to recognize MHC class II bound glycopeptides in which the carbohydrate part is defined by the tumor-associated Core-1 structure. Only a few studies have shown the ability of T cells to recognize MHC class II bound glycopeptides in which the glycan is a disaccharide [31–34]. Deck et al. previously showed that the

Table 3. $\alpha\beta$TCR CDR3 sequences derived from glycopeptide specific T cell hybridomas raised against S^{72}(Core-1)

Hybridoma	*Vα*	*CDR3α*[1,2]		*Jα*	*Vβ*	*CDR3β*		*Jβ*
A4.1.B2	Vα1	CAA	NTGNYKY	VFG J33	Vβ15	CGAR	PGHRNSDY	TFG J1S2
	Vα2	Out of frame						
A4.1.E3.C6	Vα3	CAV	SRNNYAQGL	TFG J20	Vβ4	CASS	QEPGGMDAEQ	FFG J2S1
A4.1.E9.5	Vα5	CAV	SEPGTGSNRL	TFG J22		Missing		
A4.2.C6	Vα3	CAV	STNNAGAKL	TFG J32	Vβ1	CASS	PGQTYAEQ	FFG J2S1
A4.2.C8	Vα4	CAL	STSSGSWQL	IFG J17	Vβ2	Out of frame		
	Vα8	CAL	SDLGAGNKL	TFG J14				

[1]Only TCR CDR3 sequences in correct reading frame are shown.
[2]The sequence data are available from EMBL under accession number: AJ404689–AJ404697.

disaccharide galabiose (βGal (1–4) βGal), if attached to the central amino acid of the A^k binding peptide HEL (52–61), primed T cells recognizing the galabiose and the peptide in a MHC restricted manner [33]. T cell hybridomas generated against the glycopeptide showed moreover high specificity toward the distal βGal residue together with the exposed peptide residues [34]. In the present study, the limited panel of available glycopeptides made it impossible to reveal the fine specificity of the individual hybridomas recognizing the glycopeptide S^{72} (Core-1). We have, however, obtained important characteristics of the glycopeptide recognition at the clonal level. All the selected T cell hybridomas recognized the glycopeptide S^{72} (Core-1) even though the extent of the response differed among the tested clones. The central positioned serine residue and the intact *O*-linked disaccharide were both essential for the recognition by the hybridomas A4.2.C6 and A4.1.E3.C6, as none of the related peptides and glycopeptides could stimulate these hybridomas. In contrast, the central serine residue seemed to be of less importance for the glycopeptide recognition by the hybridoma A4.1.B2, as the cells could also recognize the closely related T^{72} (Core-1) in which the Core-1 structure was *O*-linked to threonine instead.

The two hybridomas A4.1.E9.5 and A4.2.C8 responded much better to the glycopeptide S^{72} (Tn) compared with S^{72} (Core-1). This strongly indicated that the two hybridomas were recognizing a common structure present within the two different glycopeptides (i.e. parts of the αGalNAc *O*-linked to serine). In fact, the hybridoma A4.1.E9.5 only responded to the S^{72} (Core-1) when used in a very high glycopeptide concentration. It could be speculated whether the T cell clones represented by A4.1.E9.5 and A4.2.C8 were primed by the glycopeptide S^{72} (Tn) formed by removal of the terminal β (1–3)-linked galactose of Core-1 *in vivo*. Such a partial deglycosylation of glycoproteins does occur during antigen processing within the antigen presenting cell as naturally occurring glycopeptides have been eluted from human MHC class II molecules [35,36]. A partial deglycosylation could thus explain why S^{72} (Core-1) primed T cells were found to crossrespond to the glycopeptide S^{72} (Tn) and the unglycosylated peptide S^{72} [3]. We previously made a similar observation when T cell hybridomas were raised against the glycopeptide T^{72} (Tn) [4,5]. The vast majority of these T cell hybridomas responded to the glycopeptide T^{72} (Tn), and none of these recognized the unglycosylated peptide T^{72}. A few of the isolated hybridomas were, however, recognizing the peptide, but they could not recognize the glycopeptides. Thus, the presence of T cell clones, which only recognized the unglycosylated peptide, strongly indicated the occurrence of a partial deglycosylation during the priming phase of the immune response. In the present study, none of the isolated hybridomas were, however, found to recognize the unglycosylated peptides S^{72} or T^{72}. One possible reason could be the low frequency of such clones as our previous study showed that the peptide specific T cell hybridomas constituted less than 1–2% of the total number of tested hybridomas [5].

In this study, we have shown that immunization of CBA/J mice with a MHC class II binding glycopeptide carrying the tumor-associated βGal (1–3) αGalNAc generated a population of S^{72} (Core-1) recognizing T cells having various glycopeptide specificities. Examination of the TCR Vα and Vβ gene usage of the hybridomas further indicated that the T cell repertoire against the glycopeptide S^{72} (Core-1) was highly diverse as five different Vα and at least three different Vβ gene segments were isolated from the small number of clones. Sequencing of the CDR3α and β regions revealed, however, some closely related sequence "motifs", and we cannot exclude that such "motifs" are involved in the T cell recognition of the glycopeptide i.e. by interacting with the exposed carbohydrate group. Importantly, a possible correlation between the TCR α chain and the glycopeptide specificity was seen between the hybridomas A4.1.E3.C6 and A4.2.C6, which both only recognized the glycopeptide S^{72} (Core-1). Both hybridomas used the TCR Vα3 gene segment indicating the expression of a similar TCR α chain except for the CDR3 α region. Furthermore both TCR α chains contained the polar sequence "motif" within the CDR3α and the proline-glycine "motif" in the CDR3β. They even expressed the same Jβ segment, J2S1. Unfortunately, no general sequence identity was seen between the other two hybridomas, A4.2.C8 and A4.1.E9.5 showing a similar response pattern. However, important sequence information could be present within the two TCR β transcripts, which were not identified.

We previously analyzed the TCR CDR3α and β regions of a large panel of T cell hybridomas raised toward the glycopeptide T^{72} (Tn) [8]. Even though most of the T^{72} (Tn) specific hybridomas showed very similar glycopeptide responses and expressed a predominant usage of Vα4, Vβ1 and Vβ15, no direct relation was found between the response pattern and the CDR3α or β sequences [8]. However, the S^{72} (Core-1) hybridomas in this study and the T^{72} (Tn) hybridomas [8] demonstrated the same occurrence of small and polar amino acids. The polar nature of these amino acids makes them well suited for creating a strong hydrogen bond network to the hydroxyl groups of the carbohydrate group. An aromatic amino acid was moreover also present in the CDR3α or β region of those three S^{72} (Core-1) hybridomas in which both CDR3 region sequences were available. Aromatic residues have important functions in carbohydrate binding proteins often by the creation of hydrophobic patches [9,10], and their presence was also found in the T^{72} (Tn) specific hybridomas [8].

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