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The effects of post-translational side-chain modifications on the stimulatory activity, serum stability and conformation of synthetic peptides carrying T helper cell epitopes

Laszlo Otvos Jr. [a,*], Barbara Cappelletto [a], Istvan Varga [a], John D. Wade [b], Zhi Q. Xiang [a], Kimberly Kaiser [a], LaDonna J. Stephens [a], Hildegund C.J. Ertl [a]

[a] *The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA*
[b] *Howard Florey Institute, Parkville 3052, Victoria, Australia*

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Abstract

Peptides 31D and VF13, corresponding to the rabies virus nucleo- and glycoproteins, respectively, vigorously stimulate T helper cells of the appropriate specificity. Earlier we showed how internal and external glycosylation affects the major histocompatibility complex molecule (MHC)-binding ability and conformation of these T-cell epitopes (Otvos et al. (1994) Biochim. Biophys. Acta 1224, 68–76; Otvos et al. (1995) Biochim. Biophys. Acta 1267, 55–64). In the current report, we examined the T-helper cell stimulatory ability after introduction of a new set of post-translational modifications. To obtain general information concerning the effects of amino acid side-chain modifications on other biochemical properties of protein fragments, we studied the serum stability and the conformation of the 31D and VF13 peptides. We found that the extent of the reduction of the T-cell stimulatory activity depends upon the location in the sequence of the host amino acid residue. Generally, β-linked sugars in mid-chain positions had a greater inhibitory effect than α-linked sugars attached to identical amino acids. In a case where mid-chain glycosylation just marginally reduced the T-cell stimulatory activity, the β-linked glycopeptide was significantly more resistant to serum proteases. This finding suggests that addition of β-linked carbohydrates might be superior to the addition of α-linked sugars for vaccine development, and generally for peptide agonist drug design. In addition, data presented here provide the first documentation that phosphorylation and sulfation of tyrosine residues may retain the MHC-binding ability and T-cell stimulatory activity of class II epitopes. The sulfated and the phosphorylated 31D peptides exhibited considerably increased serum stability compared to the unmodified parent peptide. Finally, all post-translational modifications destabilized the dominant α-helical or turn structures of the peptides presented in aqueous trifluoroethanol mixtures. While the circular dichroism spectra of the α- and β-linked VF13 glycopeptides with monosaccharides were almost indistinguishable, the structure of the glycopeptides depended upon the length of the sugar moiety. Significantly, incorporation of sulfate or phosphate groups resulted in identical peptide conformations.

Keywords: Agonist; Anomer; Antagonist; Antigen; Glycopeptide; Phosphopeptide; Secondary structure; Serum stability; Sulfopeptide

1. Introduction

Many proteins undergo post-translational modifications with a corresponding alteration in their biological and biochemical properties. Synthetic peptides can be good models to study the local conformational and functional changes following attachment of the side-chain moiety including carbohydrate, phosphate and sulfate groups. Up to now, however, the synthetic capability to produce these modified protein fragments was very limited. With the recent availability of appropriately protected amino acid building blocks, the chemical synthesis of phosphopeptides, sulfopeptides, and some glycopeptides is no longer an obstacle to the proposed studies. Comprehensive analysis of the peptide structure and function upon addition of novel side-chain appendages is further prompted by the dual role the modified peptides play in biochemistry and biotechnology. First, they can be used to analyze the fragments of post-translationally modified proteins that occur in nature. Second, the introduction of hydrophilic and protease-resistant sugar [1,2] and phosphate [3,4]

* Corresponding author. Fax: +1 (215) 8985821.

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groups promises to improve the physical and biological properties of peptide drug leads. A comparative analysis of the effects of such modifications is therefore long overdue.

We have studied the ability of some phosphorylated and glycosylated peptides to bind to major histocompatibility complex (MHC) determinants and to stimulate T-cells with appropriate specificity. Internal phosphorylation of a serine residue of an epitope recognized by $CD4^+$ and $CD8^+$ T-cells results in diminished T-cell stimulatory activity, although the phosphopeptide is still capable of binding to the restricting elements [5]. The effect of tyrosine phosphorylation on the T-cell stimulatory activity of class II epitopes has not yet been studied. However, in a recent study, Arsequell and coworkers demonstrate that phosphorylation of a tyrosine residue does not interfere with H-$2D^b$ class I MHC-binding, in contrast with phosphorylation of a threonine that eliminates the binding to all class I MHC alleles tested [6]. The same group shows that another class I MHC-binding peptide can be modified by both α- and β-linked serine glycosylation as well as β-linked asparagine glycosylation without affecting its binding to the MHC, provided the modifications do not involve amino acids located at MHC anchor positions [7]. In our experience, glycosylation of class II epitopes either increases the antigenic potency through increasing the stability of the peptides when the sugar is attached to the N-terminus [8], or reduces the antigenic potency if the sugar is incorporated in mid-chain position [9]. In the latter case the reduction depends upon the anomeric configuration of the added sugar; glycopeptides with β-linked sugars to asparagine residues (the natural anomeric configuration of carbohydrates in extracellular *N*-glycoproteins [10]) lose their ability to bind to class II MHC more than glycopeptides with α-linked sugars to threonine residues [9]. The experimental results were later confirmed by molecular modeling of the MHC:peptide complexes [11]. However, these studies compare α- vs. β-linked glycopeptides when the carbohydrates are incorporated into amino acids in the same position of the peptides, but are attached to different amino acid hosts (β- to Asn and α- to Thr). In the current report we examine the T-helper cell stimulatory activity of the glycopeptides when the same threonine residues are glycosylated with either α- or β-linked sugars. We extend the analysis of the metabolic stability to internally glycosylated and tyrosine phosphorylated peptides as indicated by the functional T-cell assay after incubation of the peptides in human serum. Our models are immunodominant epitopes of the rabies virus nucleo- and glycoproteins. Peptide 31D, AVYTRIMMNGGRLKR binds to murine class II MHC I-E^k and peptide VF13, VVEDEGCTNLSGF binds to I-A^k.

Glycosylation of asparagine residues in synthetic peptides breaks helices and results in the formation of reverse-turns [12]. Turns themselves are usually stabilized or their geometry is modified after glycosylation [13]. These rules may also apply to *O*-glycosylation [9,13,14], although the resulting glycopeptide structures are less ordered than after glycosylation of asparagine [9]. Increasing the length of carbohydrates from mono- to disaccharide results in just marginally different conformations for *N*-glycosylated peptides as evidenced by circular dichroism (CD) and Fourier-transform infrared (FT-IR) spectroscopy [12], but qualitatively different conformations for *O*-glycopeptides as evidenced by nuclear magnetic resonance (NMR) [15]. We investigated whether the NMR-derived conformational differences between *O*-glycopeptides with different length of sugars can be recorded by CD and whether the anomeric configuration of the attached sugars play any role in the final secondary structures. This latter problem is especially important because while extracellular *O*-glycoproteins carry α- [16], intracellular *O*-glycoproteins carry β-linked carbohydrate side-chains [17]. Phosphorylation of serine residues similarly breaks helices [18], but does not appear to stabilize any special peptide conformation [12], except maybe in cases associated with the pathological aggregation of phosphoproteins, such as those in Alzheimer's disease [19]. In contrast, CD and FT-IR reveals that tyrosine phosphorylation can stabilize both α-helices [20] or β-pleated sheets [21] indicating a possible distinct role of the phosphotyrosine residues in signal transduction.

In spite of the identification of the sulfate binding sites in numerous sulfoproteins [22] and the extended research on the synthesis [23] and biological assay [24] of sulfopeptides (e.g., cholecystokinin, hirudin, gastrin), the general effect of incorporating sulfate moieties on peptide conformation and metabolic stability has, to our knowledge, not been reported. In this paper we study these biochemical properties as well as the T-helper cell stimulatory activity of a sulfated analogue of peptide 31D.

2. Materials and methods

The peptides used in this study are listed in Table 1.

2.1. Synthetic peptides

Peptides were assembled on solid-phase using a Milligen 9050 automated peptide synthesizer employing standard Fmoc-methodology [25]. The synthesis of the Fmoc-Asn-β(Glc_7)-OH and its incorporation into N-terminal positions of peptides has been previously detailed [26]. Fmoc-Tyr(PO_3H_2)-OH was prepared according to Ottinger et al. [27], Fmoc-Tyr(SO_3H)-OH · Ba was purchased from Bachem Bioscience (King of Prussia, PA). Fmoc-Thr-β(Gal)-OH [28] was kindly provided by Professor Raniero Rocchi (University of Padova). Glycoamino acids, phosphotyrosine and sulfotyrosine were incorporated in the same manner as unmodified amino acids, except that Fmoc-Thr-α(Ac_4Gal-1 → 3β-Ac_2GalNAc)-OH, purchased from Bachem California (Torrance, CA) and Fmoc-Thr-

$\alpha(Ac_3GalNAc)$-OH purchased from Oxford Glycosystems (Rosedale, NY) were coupled in stoichiometric amounts to reduce cost. Peptides were cleaved from the solid support by trifluoroacetic acid (TFA) in the presence of thioanisole as scavenger. Deacetylation of the sugar hydroxyl groups was accomplished by a 2-min treatment with 0.1 M NaOH [9,29]. In a most recent paper [29], we thoroughly analyzed different deacetylation methods during the synthesis of an O-glycosylated antibiotic peptide. Significantly, if the peptide was treated with diluted NaOH followed by immediate neutralization, only minor (1.5%) β-elimination occurred. Moreover, capillary electrophoresis of the final product revealed only a single peak, indicating the lack of diastereomer formation (racemization) during the deacetylation step [29]. After cleavage, unmodified peptides were purified, and glycopeptides, the phosphopeptide, the sulfopeptide and the contaminating (unmodified, amino acid deleted) analogs were separated by reversed-phase high performance liquid chromatography (RP-HPLC). The final products were characterized by amino acid analysis and laser desorption mass spectrometry (MS). Table 1 lists the RP-HPLC retention times and the MS data.

2.2. *Proliferation assay (for VF13 peptides)*

Irradiated C3H/He mouse spenocytes ($5 \cdot 10^5$) in 100 μl of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10^{-5} M 2-mercaptoethanol and 2% FBS in 96-well round-bottom microtiter plates were co-cultured with antigen at the indicated concentration in 25 μl medium for 30 min at 37°C. D2rab.gp T-cells ($2 \cdot 10^4$) were then added in 50 μl of medium. Proliferation of T-cells was measured 48 h later by a 6 h [^{3}H]thymidine pulse [30].

2.3. *Cytokine release assay (for 31D peptides)*

The 9C5.D8 T-cell hybridoma was tested for recognition of peptides presented by irradiated H-2 compatible splenocytes by a lymphokine release assay as described [30].

2.4. *Serum stability*

To assess the metabolic stability of the peptides, these were incubated in 50% human serum in a concentration of 0.1 mg/ml for 3 h or overnight. One batch was kept at 37°C to promote degradation and the other control batch was kept for the same period of time at 4°C. The serum-preincubated peptides were subsequently diluted in culture medium and subjected to the T-cell stimulation assays as described in Section 2.2 and Section 2.3.

2.5. *CD*

CD spectra were taken on a Jasco J-720 instrument at room temperature in a 0.2 mm path-length cell. Double distilled water and spectroscopy grade trifluoroethanol (TFE) were used as solvents. Peptide concentration was approximately 0.5 mg/ml, as determined by quantitative HPLC. The CD spectra shown are baseline-corrected and smoothed by the algorithm provided by Jasco. The glycosylated VF13T peptides exhibited a time-dependent conformational change, as the dimerization of the peptide in solution progressed. The CD spectra shown in Fig. 5 represent the conformation of the monomers. Similar to our earlier reports, the spectral contributions of the *O*-glycosides [9,13] and the tyrosine-phosphates [20] were not subtracted. Accordingly, the CD contribution of the tyrosine sulfate was not considered. In fact, no major perturbation of the CD below 225 nm is expected to occur due to the presence of a single tyrosine side-chain [31] and its contribution of intensity to the spectrum of the 15-mer 31D is negligible. Mean residue ellipticity $[\Theta]_{MR}$ is expressed in deg · cm^2/dmol using mean residue weights calculated from the molecular weight divided by the number of amino acids in the peptides.

Table 1
Synthetic peptides and their characterization

Peptide	Sequence	Side-chain modification (X)	RP-HPLC retention time (min)	LD-MS * $(M+H)^+$
31D	AVYTRIMMNGGRLKR	-	22.6	1765
31D-YP	AVY(X)TRIMMNGGRLKR	Phosphate	21.3	1848
31D-YS	AVY(X)TRIMMNGGRLKR	Sulfate (barium salt)	22.0	1847
31D-Tα-GalGalNAc	AVYT(X)RIMMNGGRLKR	Gal(β1 → 3)GalNAc(α → O)	20.2	2132
31D-Tβ-Gal	AVYT(X)RIMMNGGRLKR	Gal(β → O)	21.6	1930
VF13N	VVEDEGCTNLSGF	-	25.5	1370
VF13N-Tα-GalGalNAc	VVEDEGCT(X)NLSGF	Gal(β1 → 3)GalNAc(α → O)	25.1	1755 $(M+Na)^+$
VF13N-Tβ-Gal	VVEDEGCT(X)NLSGF	Gal(β → O)	21.8	1532
VF13T	VVEDEGCTTLSGF	-	26.1	1359
VF13T-T8α-GalGalNAc	VVEDEGCTT(X)LSGF	Gal(β1 → 3)GalNAc(α → O)	25.4 * *	1743 $(M+Na)^+$
VF13T-T8α-GalNAc	VVEDEGCTT(X)LSGF	GalNAc(α → O)	21.8	1583 $(M+Na)^+$
VF13T-T8β-Gal	VVEDEGCTT(X)LSGF	Gal(β → O)	21.4	1556 $(M+K)^+$

* The accuracy of the LD-MS instrument used at the examined mol wt. range is ±4 Da.
* * Retention time data taken from [9] by using a somewhat slower eluting HPLC system.

3. Results

3.1. T-cell stimulation

The T-cell stimulatory activity of glycosylated VF13 peptides was measured at 5 μg/ml. This high peptide concentration is suboptimal for side-chain unmodified peptides VF13N and VF13T owing to the generally observable 'pro-zone' activity of T-cell epitopic peptides and peptides recognized by monoclonal antibodies. However, a high peptide concentration is required to obtain any detectable activity of the glycosylated peptides on the D2 rab.gp T cell line (raised against rabies virus glycoprotein unglycosylated on Asn37) [9]. As shown in Fig. 1, attachment of α-linked sugars to both peptides VF13N and VF13T considerably decreased the T-cell stimulatory activity. This effect was more prominent for the peptide pair VF13N – VF13N-Tα-GalGalNAc where the glycopeptide was only slightly more stimulatory than the background control. Nevertheless, α-glycosylated peptide VF13T-T8α-GalGalNAc retained some stimulatory activity for the T-cells. In contrast, peptides carrying a β-linked monosaccharide on identical amino acids (VF13N-Tβ-Gal and VF13T-T8β-Gal) lost their T-cell stimulatory activity almost entirely. This is probably because β-glycosylated VF13 peptides have a very limited ability to bind to the MHC determinant [9,11].

We studied the serum stability of peptides at two temperatures. The resistance of the peptides to serum proteases was probed by preincubating the peptides with human serum at 37°C before the T-cell proliferation assay (see next chapter). As short incubation at 4°C is not expected to considerably affect peptide stability, the T-cell stimulation of peptides treated with human serum at low temperatures serves as a starting point to access the inherent T-cell stimulatory activity of the peptides during identical assay conditions. For these experiments analogues of peptide 31D were used. As with the VF13 peptides, incorporation of carbohydrates to the threonine located in mid-chain position of peptide 31D resulted in decreased T-cell stimulatory activity at low teperature (Fig. 2). Also in agreement with data obtained with the VF13 peptides, the 31D-derived glycopeptide carrying an α-linked sugar moiety (31D-Tα-GalGalNAc) retained a greater T-cell stimulatory activity than the glycopeptide carrying a β-linked side-chain substitution (31D-Tβ-Gal) (Fig. 2). Major differences between the 31D and the VF13-derived peptides were; (a) the 31D-glycopeptides were generally much more stimulatory than the VF13-glycopeptides, and (b) even the β-glycosylated 31D-peptide retained considerable activity.

Incorporation of phosphate or sulfate side-chains to the tyrosine residue, located in mid-chain position of peptide 31D, did not interfere with the T-cell stimulation at 4°C (Fig. 3). In fact, at low peptide concentrations the modified peptides exhibited higher T-cell stimulatory activity than the unmodified peptide. This was probably due to some remaining serum protease activity on the unmodified peptide during the assay procedure. The remaining protease activity at 4°C was generally apparent when the peptides were incubated overnight, and during those reaction conditions most of the peptides entirely lost their T-cell stimulatory activity. The phosphopeptide and the sulfopeptide exhibited indistinguishable T-cell stimulatory activity after 3 h at 4°C (Fig. 3). While the T-cell stimulation of peptide 31D carrying palmitic acids at the N-terminus without any serum treatment slightly exceeded those of peptides 31D-YP and 31D-YS, the phosphopeptide and the sulfopeptide reproducibly stimulated the T-cells to an almost identical extent (data not shown).

3.2. Serum stability at 37°C

Both T-cell agonist and antagonist peptide drug leads should possess increased serum half-life at physiological

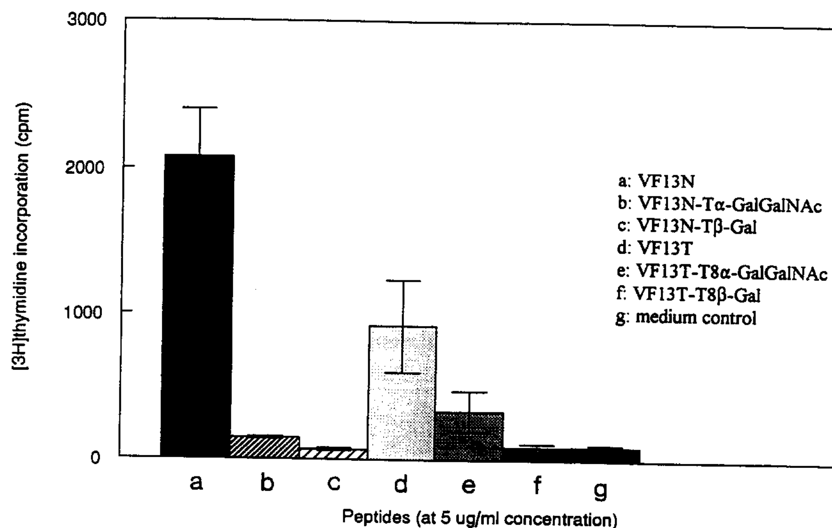

Fig. 1. Response of T-cells to VF13 peptides and glycopeptides. [^{3}H]Thymidine incorporation was measured in cpm as described in Section 2. The standard deviation of triplicated samples is represented by the error bars.

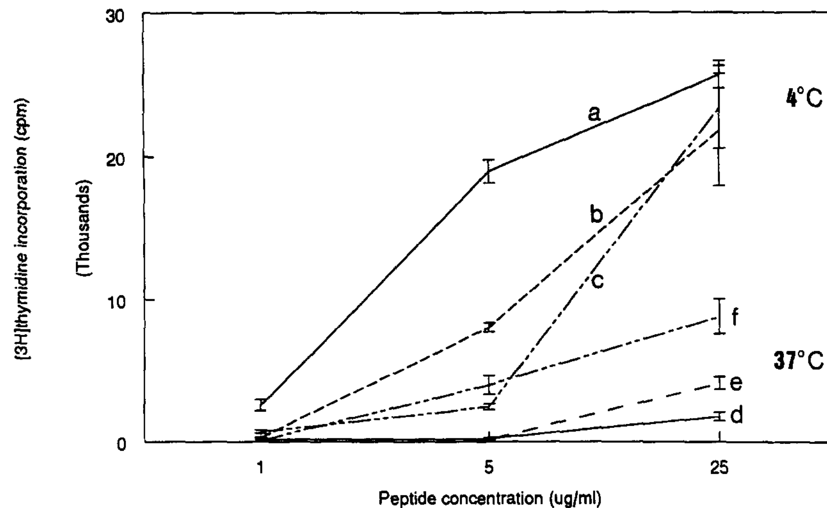

Fig. 2. Response of T-cells to peptide 31D and its glycosylated analogs after incubation in 50% human serum for 3 h. Solid traces **a** and **d** represent unmodified peptide 31D; broken traces **b** and **e** represent the α-glycosylated peptide 31D-Tα-GalGalNAc; dots and dashes **c** and **f** represent the β-glycosylated peptide 31D-Tβ-Gal. Traces **a–c** indicate preincubation at 4°C; traces **d–f** indicate preincubation at 37°C.

temperatures compared to the unmodified parent analogs. To test whether internal glycosylation provided T-cell modulatory peptide leads, we applied the serum stability assay to the 31D-derived glycopeptides. We selected the 31D peptides over the VF13 peptides, because the former glycopeptides retained much higher T-cell stimulatory activity without serum treatment. Preincubation of the peptides in 50% human serum considerably influenced their T-cell stimulation. Unmodified peptide 31D was affected the most; it went from most stimulatory at 4°C to least stimulatory at 37°C (Fig. 2). α-glycosylated peptide 31D-Tα-GalGalNAc was marginally more resistant to preincubation than peptide 31D. β-glycosylated peptide 31D-Tβ-Gal, however, exhibited remarkably high serum stability after 3 h (a customary end point of our studies), and was clearly superior to the other two peptides (Fig. 2). Moreover, after incubation at 4°C overnight, only the β-glycosylated peptide stimulated the cells more than the background (data not shown).

The phosphorylated and the sulfated tyrosine residue (third amino acid in the 31D sequence) is located one residue closer to the N-terminus (a frequent peptidase cleavage site [2]) than the threonine, and consequently the phosphopeptide and the sulfopeptide were expected to exhibit more pronounced protease resistance than the glycopeptides. Indeed, both the phosphorylated and the sulfated 31D analogs remained considerably more stimulatory to the T-cells after 3 h incubation in 50% human serum at

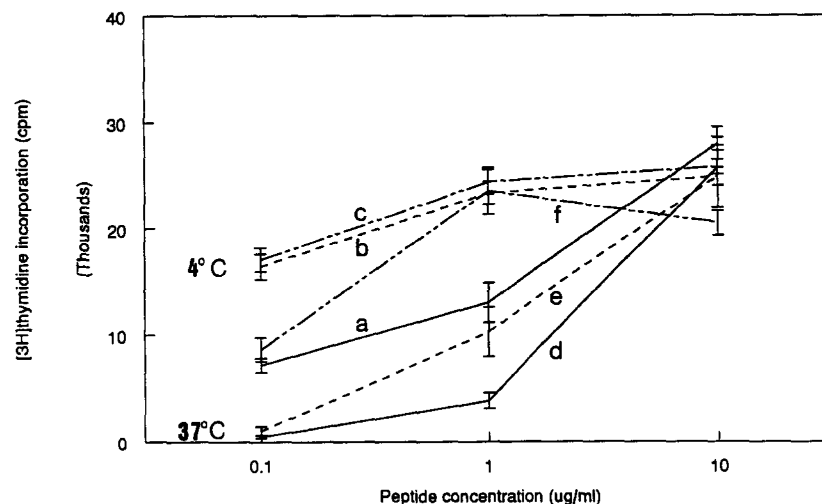

Fig. 3. Response of T-cells to 31D peptides after incubation in 50% human serum for 3 h. Solid traces **a** and **d** represent the unmodified peptide 31D; broken traces **b** and **e** represent the tyrosine-phosphorylated peptide 31D-YP; dots and dashes **c** and **f** represent the tyrosine-sulfated peptide 31D-YS. Traces **a--c** indicate preincubaion at 4°C; traces **d–f** indicate preincubation at 37°C.

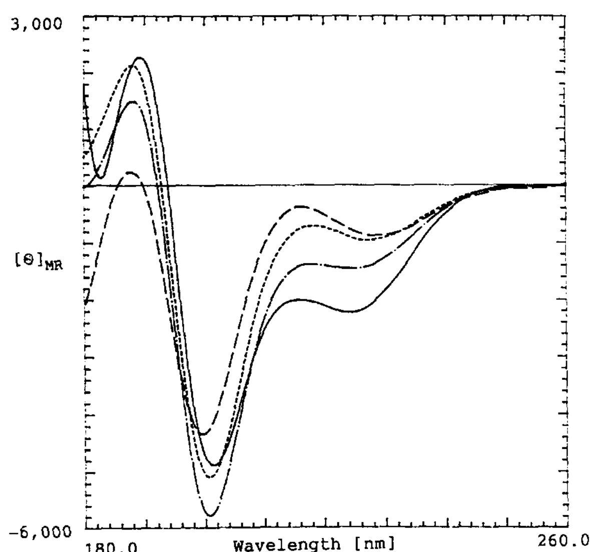

Fig. 4. CD spectra of peptide VF13T and its Thr8-glycosylated analogs. Solid line: unmodified peptide VF13T; dots: VF13T-T8α-GalNAc (α-linked monosaccharide); dashes: VF13T-T8α-GalGalNAc (α-linked disaccharide); dots and dashes: VF13T-T8β-Gal (β-linked monosaccharide).

37°C than their unmodified parent analogue (Fig. 3). The sulfopeptide remained comparatively less affected by the proteases and this is unlikely to be due to any size difference between the phosphate and the sulfate anions (the S-O distance in the sulfate anion is 149 pm, and the P-O distance in the phosphate anion is 152 pm). It needs to be emphasized that these assays, by nature, are highly complex and variable, as can be seen in the comparison of the shapes of the curves and the values for peptide 31D in Figs. 2 and 3. The relative serum stability and T-cell stimulatory activity of the peptides, however, indicate the trends among the properties of the peptide analogs examined.

3.3. Peptide conformation

Internal *O*-glycosylation generally destabilized the helical (31D) or turn (VF13T) structures of the unmodified peptides in aqueous TFE mixtures [8,9]. In the current study, we examined the conformation-modifying effects of α- vs. β-glycosylation of Thr8 in peptide VF13T, and the effect on the CD spectra of the length of the sugars that are attached to Thr8 in peptide VF13T, or Thr4 in peptide 31D. Unmodified peptide VF13T exhibited a type C CD spectrum in 50% TFE (Fig. 4), characteristic of type I (III) β-turns [32] or type I (III) and type II β-turn mixtures [33]. The blueshift of the 202 nm band from that observed in ideal type C spectra (204–206 nm) indicated some unordered contribution. Unordered peptides (or polypeptides with left-handed polyproline II structure) exhibit a strong negative band at or below 200 nm accompanied by a weak positive band above 210 nm [34]. The intensity of the negative 224 nm $n\pi^*$ band of peptide VF13T was decreased and that of the further blueshifted negative $\pi\pi^*$ band (201 nm) was increased after addition of monosaccharides in either α- or β-anomeric configuration (Fig. 4), indicating a greater contribution of the unordered structure to the conformational equilibrium. The ratio of the 225–227 nm:201–202 nm bands, the measure of ordered structure for helical or turn peptides, was 0.46 for the unmodified peptide, 0.21 for the α-linked glycopeptide, and 0.19 for the β-linked glycopeptide. The CD of the two monosaccharide-coupled glycopeptides were very similar including the identical wavelength of all of the bands. The only notable difference was that the whole spectrum of the α-linked glycopeptide was slightly shifted to the positive direction. This shift was approx. 700 deg · cm^2 /dmol at the $\pi\pi^*$ bands which likely reflected the contribution of the single acetamido group present on the carbohydrate portion of the α-linked glycopeptide. The β-linked glycopeptide lacks this functionality on the sugar moiety. The positive shift of glycopeptides carrying acetamido groups is documented by the CD of 1,2-diacetamido-1,2-dideoxy-β-D-glucopyranose, which compound exhibits a broad positive band centered near 194 nm with an intensity, calculated to the mean residue ellipticity of peptides the size of VF13, of approx. 2300 [12]. Taken together, the data indicated that addition of monosaccharides in either α- or in β-anomeric configuration similarly destabilized the turn structure of peptide VF13T. Nevertheless, turns were still significantly present in the conformational equilibrium for the glycopeptides carrying monosaccharides, but were almost absent for the glycopeptide carrying the disaccharide side-chain. The negative $\pi\pi^*$ band of glycopeptide VF13T-T8α-GalGalNAc was further blueshifted to 199 nm, and the positive 188 nm band nearly disappeared. This spectral feature, together with the almost zero-line crossing of the 216 nm band, characterized the CD as type U spectrum, and indicated that the peptide existed in predominantly unordered conformation (Fig. 4).

Incorporation of a mono- or a disaccharide to the threonine, as well as phosphorylation or sulfation of the tyrosine residue resulted in markedly decreased α-helical content of peptide 31D. Peptide 31D undergoes an unordered → α-helix conformational transition when assayed in dilutions from water to TFE [35]. Compared to ideal α-helical peptides [31], in 50% TFE the CD curve intensity of 31D revealed a medium level of helicity [35] which was further supported by the 2 nm blueshift of the bands representing the $\pi\pi^*$ transition (Fig. 5). Another 2 nm blueshift, and decrease in the band intensities were detected when the monosaccharide was added in peptide 31D-Tβ-Gal (Fig. 5), indicating that glycosylation destabilized the helical structure. Elongation of the carbohydrate in glycopeptide 31D-Tα-GalGalNAc further enhanced this effect without generating new conformers, as evidenced by the further decrease of all band intensities and unchanged wavelength of the peak maxima compared to the CD of the glycopeptide carrying the monosaccharide moiety (Fig. 5).

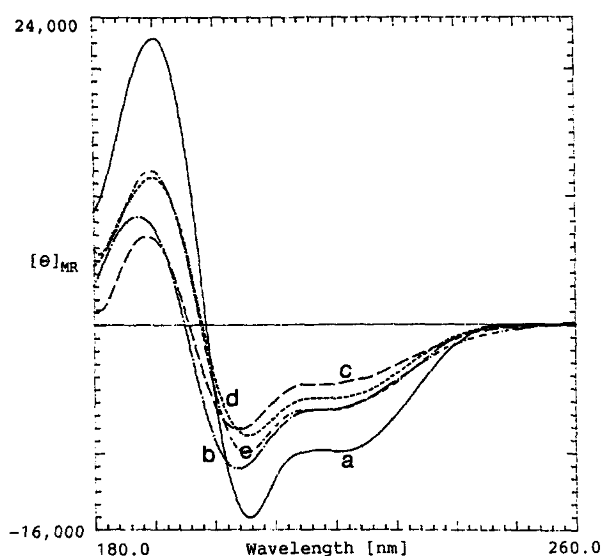

Fig. 5. CD spectra of peptide 31D and its modified analogs. Solid line (a): unmodified peptide 31D; dots and dashes dashes (b): threonine-glycosylated peptide 31D-Tβ-Gal carrying a monosaccharide side chain; dashes (c): threonine-glycosylated peptide 31D-Tα-GalGalNAc carrying a disaccharide side-chain; dots (d): phosphopeptide 31D-YP; dots and short dashes (e): sulfopeptide 31D-YS.

The α-helix content, measured at 208 nm [36], dropped approx. 20% after phosphorylation or sulfation of the tyrosine residue (Fig. 5). Accordingly, the 206 nm band and the zero-line crossing were bluesifted by 0.5–1 nm after the side-chain modifications. Owing to a possible minor contribution of the tyrosine side-chain at higher wavelengths, the determination of the helix-content based on the higher energy $\pi\pi^*$ transition is more realistic than computer analyses of the CD curves that consider the entire CD spectra. Remarkably, the CD spectra of the phosphopeptide and the sulfopeptide were identical within the experimental error of the CD analysis and concentration determination of the samples.

4. Discussion

These studies had two major goals: (a) to model and characterize various biochemical properties of glycoprotein, phosphoprotein and sulfoprotein fragments, and (b) to identify carbohydrate structures that are useful for peptide modification in general drug design, particularly that of T-cell agonists and antagonists. Earlier we found that internal glycosylation with α-linked carbohydrates reduces, while β-linked carbohydrates fully diminishes the T helper cell stimulatory ability [8,9]. Here we further corroborated these observations by noting that the class II MHC-binding and T-cell stimulatory ability of β-linked glycopeptides did not always decline to zero. When measurable T-cell activity remained after attachment of β-linked sugars, the design of T-cell antagonist peptides could benefit from the vast variety of available carbohydrate structures. Our serum stability assay indicated that β-glycosylated 31D peptide was more resistant to serum proteases than the α-glycosylated analogue. Moreover, the α-linked carbohydrate in peptide 31D-Tα-GalGalNAc was a disaccharide, while the β-linked carbohydrate in 31D-Tβ-Gal was only a monosaccharide, indicating that the anomeric configuration had a greater effect on the serum stability than the length of the sugar (and presumably the distance from the cleavage site). In drug design terms, this means that if mid-chain glycosylation is planned, incorporation of sugars in β-anomeric configuration is more beneficial for T-cell antagonist production than in α-anomeric configuration provided the β-glycosylated peptide retains its ability to bind to the MHC. Because glycopeptides commonly are less stimulatory to the T-helper cells, internal glycosylation is not advised for such agonist design. Stability studies in human serum are crucial in the investigation of the therapeutic utility of a peptide [2]. When the biological activity of a possible peptide drug lead can tolerate the addition of a carbohydrate side-chain, we recommend β-linked sugars over α-linked sugars. In general biochemistry terms our data indicate that peptide degradation is not only stereochemically, but also regiospecifically controlled. Alternatively, if the cleavage of the sugar precedes the cleavage of the peptide bond, the human serum may retain more glycoprotein α-glycosydase activity than protein β-glycosydase activity. Finally, if the peptide serum stability truly models the general intracellular protein and glycoprotein stability, our findings may explain why intracellular O-glycoproteins predominantly carry β-linked carbohydrates (extracellular *O*-glycoproteins carry α-linked carbohydrate moieties).

The other possible way to generate T-cell antagonist glycopeptides is by adding long sugars to the termini [11]. Tetra and heptasaccharide moieties in influenza hemagglutinin peptides $N(Glc_4)$-CTLIDALLGDPH and $N(Glc_7)$-CTLIDALLGDPH partially blocked the recognition of the MHC:glycopeptide complex by the TcR [37]. Our preliminary results indicated a 5–15% reduction in the T-cell stimulatory activity of N-terminally glycosylated N-VF13 peptides, when the Asn(GlcNAc) residue was replaced to $Asn(Glc_7)$ (at 1–10 μg/ml). This alternative design of T-cell antagonists requires the identification of the minimal epitope, as blocking TcR recognition is dependent upon the distance between the N-terminal sugar and the TcR binding site [37], and the current synthetic methodology limits the length of the carbohydrate that can be efficiently added. The approach is nevertheless attractive, as N-terminal glycosylation is generally superior in terms of peptide stability [2].

The T-cell stimulatory ability of the VF13 derived glycopeptides were more sensitive to the anomeric configuration of the attached sugars than those of the 31D glycopeptides. Moreover, in contrast with a serine-phosphorylated, class II rabies peptide delineated from the nominal phosphoprotein [5], peptide 31D also retained its

T-cell stimulatory ability after either phosphorylation or sulfation. This is especially surprising since the best alignment of peptide 31D positions the tyrosine residue into the first binding pocket of murine class II MHC I-E^k and the human equivalent HLA-DR1 [11]. Since the phosphopeptide and the sulfopeptide stimulated T-cells far better than the unmodified peptide after incubation in human serum, the conserved T-cell simulation cannot be explained by removal of the phosphate or sulfate group during the assay procedure. A more credible explanation is that peptide 31D and its fragments or analogs can assume multiple positions in the MHC groove. This idea is supported by the seemingly unusual observation that short tri- and tetrapeptide fragments of peptide 31D can stimulate T-cells, albeit only upon significantly elevated peptide concentrations [30]. Nevertheless, the maintenance of class II MHC-binding and T-helper cell stimulatory ability after phosphorylation or sulfation indicated that the T-cell epitope repertoire of phospho- and sulfoproteins may include modified peptides. This finding may be of significance in signal transduction research where the study of phosphotyrosine-specific humoral immunity can be complemented with investigation of phosphotyrosine-specific cellular immune responses.

Phosphorylation and sulfation of the tyrosine residue in peptide 31D equally reduced the α-helicity of the unmodified peptide. The similarity of the secondary structures can be explained by the approximately similar size of the phosphate and the sulfate anions. As no new conformers were generated and only the helix content decreased, the bulky side-chains appear to exclude part of the peptides from the helical network without any special interaction between the negatively charged side-chains and the peptide backbone. This is in contrast with earlier observations that suggest stabilizatory effects of tyrosine phosphorylation on α-helices [20] and β-pleated sheets [21]. Recognition of the importance of the conformation of phosphotyrosine-containing peptides in signal transduction [38] and in the design of inhibitors of these processes [39] therefore demands more caution in the treatment of this problem and requires the conformational analysis of a statistically satisfying number of tyrosine phosphorylated peptides.

It was suggested that the monosaccharides in short *O*-glycopeptides do not interact with the peptide backbone and that their role is only to exclude certain conformations present in the non-glycosylated parent analogs [14]. The same research group used NMR to show that elongation of the carbohydrate to a disaccharide results in markedly different turn conformations compared to the glycopeptide carrying a monosaccharide moiety [15]. Both observations are supported by our current CD studies on turn peptides. The α- or β-glycosylated versions of peptide VF13T studied here exhibited very similar CD spectra. These intermediate spectra represented a mixture of turn and unordered peptide conformations and were less ordered than those of the unmodified parent peptide. The contribution of the unordered conformer was significantly increased after the carbohydrate side-chain was extended. This latter observation did not hold true for glycosylated analogs of the helical 31D peptide. Incorporation of the monosaccharide destabilized the helical conformation of 31D, an effect which was more pronounced following elongation of the sugar but without generating new structures. This is in contrast with the behavior of the turn-like peptides but is in accordance with the conformational analysis of *N*-glycosylated analogs of another helical rabies peptide [12]. In summary, it appears that the conformation-modifying effect of incorporating different sugar structures depends upon both the length of the sugar and the initial secondary structure of the peptide.

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