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IMMUNOGENICITY AND CONFORMATIONAL PROPERTIES OF AN N-LINKED GLYCOSYLATED PEPTIDE EPITOPE OF HUMAN T-LYMPHOTROPIC VIRUS TYPE 1 (HTLV-I)

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The identification and characterization of epitopes of human T-lymphotropic virus type 1 (HTLV-I), which elicit an effective humoral or cell-mediated immune response, remains a central obstacle to the development of a peptide-based vaccine against the virus infection. The objective of the studies presented here was to examine the influence of N-linked glycosylation on peptide structure and immunogenicity. We engineered the 233-253 sequence of gp46 of HTLV-I to contain an N-acetylglucosamine (GlcNAc) residue at Asn^{244}. Secondary structure prediction using computer algorithms indicated that this peptide may contain a β-turn at residues 242-246. Recent work with model glycopeptides suggests that β-turn conformation in peptides may be induced, and probably is stabilized, by the presence of even a single sugar residue. In the present study, the structures of the 233-253 peptide, SC1, and the 233-253(Asn^{244}-GlcNAc) glycopeptide, SC2, were determined. Similar conformation was exhibited by both the glycosylated and nonglycosylated peptide displaying a β-turn at residues 243-246 and extended-chain structure at the peptide/glycopeptide termini. Both peptides were engineered into chimeric constructs with a promiscuous T-cell epitope from measles virus and were used as immunogens in rabbits. Both chimeric peptides were highly immunogenic in rabbits, producing high-titered antibodies as early as primary + three weeks. The antibodies generated against either construct were able to bind to whole virus (ELISA) and to gp46 (radioimmunoprecipitation assay). Additionally, human sera of individuals known to be positive for HTLV-I recognized both the glycosylated and nonglycosylated constructs. It appears that the 233-253 peptide is able to adopt a conformation that mimics the structure in native gp46, and addition of a GlcNAc residue at Asn^{244} does not affect the conformational preference or stability of this construct; nor does glycosylation alter immunogenicity but instead appears to enhance immune recognition.

Keywords: N-link glycosyl epitopes; human T-lymphotropic virus (HTLV 1); conformational immunogenicity; epitopes; peptide vaccines; viral glycoproteins.

INTRODUCTION

Human T-lymphotrophic virus type I (HTLV-I) is the etiologic agent of adult T cell leukemia/lymphoma (ATLL) [1] and is associated with the related neurologic disorders HTLV-I-associated myelopathy and tropical spastic paraparesis (HAM/TSP) [2]. Endemic areas for the virus infection include Southern Japan, the Caribbean basin and Central Africa [3]. In the U.S.A., HTLV-I carriers are found predominantly in high-risk groups such as intravenous drug-users and prostitutes [4].

The *env* of the human retrovirus HTLV-I is composed of two glycoproteins, a surface gp46 and transmembrane gp21 originating from the glycosylated precursor gp68 [5]. The envelope of the virus plays an important role in viral infection and is involved in the induction of neutralizing antibodies. Antibodies against gp46 and gp21 are able to inhibit syncytium formation between HTLV-I-infected and susceptible receptor-bearing cells [6]. Specific antibodies to *env* gp46 have been detected in sera of individuals who have been exposed to the virus [7,8]. For development of vaccines that may prevent HTLV-I infection and/or modify clinical symptoms in already infected individuals, it is important to identify parameters that govern the induction of protective immunity. The identification and characterization of HTLV-1 *env* epitopes responsible for eliciting neutralizing activities has become of major importance. Immunodominant regions of this glycoprotein have been mapped by our group [9,10] and others [7,11-13] using linear synthetic peptides. Neutralizing epitopes have been identified in the gp46 amino acid (aa) region spanning 175-196. Neutralizing antibodies were detected in rabbit antisera generated by this sequence using various strategies: coupling to carrier proteins and hyperimmunizing [9], chimeric constructs with promiscuous T cell epitopes [15], and a MAP construct [16]. The conformational parameters of this region for eliciting high affinity and highly neutralizing antibodies have yet to be determined. In order to ascertain whether this region of the *env* gp46 would be an effective peptide-based vaccine candidate, we have redesigned and synthesized the epitope to include 175-209, and synthesized a chimeric construct incorporating a promiscuous T cell epitope. The efficacy of this epitope is presently undergoing testing.

The precise role and functional importance of carbohydrate moieties in modulating both humoral and cellular immune responses to many viral glycoproteins is poorly understood and remains to be fully elucidated [17-21]. It is noteworthy to

Abbreviations: HTLV-I, human T-lymphotropic virus type 1; CD, circular dichroism; COSY, correlational spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; GlcNAc, N-acetyl glucosamine; TFE, trifluoroethanol; ELISA, enzyme-linked immunosorbent assay; RIPA, radioimmunoprecipitation assay.

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consider that these hydrophilic carbohydrate sidechains may play a critical role in the host's immune recognition and response. A requirement for carbohydrate in group-specific neutralization has been demonstrated for a number of retroviruses [17,18,22,23]. For example, antibodies raised against glycosylated and non-glycosylated forms of gp120 of HIV were compared for specificity of neutralizing activities [23]. Since many conformational determinants were not detected when the N-linked glycosyl moiety was removed, it can be concluded that the immune reactivity of antisera required the stable secondary and tertiary structure of the envelope glycoprotein to be maintained or that the glycosylated moiety is important. In this case, the antigenicity was coincident with glycosylation stabilizing the protein conformation rather than direct participation of the carbohydrate side chains in the antigenic site.

The importance of N-linked glycosylation in the formation of neutralization epitopes for a number of viruses has been described and appears to be involved in directing protein folding and the correct formation of intrachain disulfides links [22]. Many viral envelope proteins are highly glycosylated, especially N-linked glycosylation sites. N-glycans play an important role in modulating glycoprotein antigenicity. The functional significance of glycosylation includes: (1) maintaining and inducing protein conformation, (2) increasing peptide/protein solubility, and (3) protecting the protein from degradation. It has been shown that removal of glycosylation sites directly affects syncytium formation. Thus, N-glycosylation may be involved in modulating neutralizing antibody responses by directing the immune response to selected epitopes. Crystal structure determinations of glycoproteins show that glycosylated asparagines are found in β-turn segments and loop regions. Also, β-turn conformations are produced in peptides which are glycosylated at asparagine and this modification may serve to stabilize turn structure in peptides which exhibit unordered structure in solution.

The aim of this study was to investigate the importance of an N-linked glycosylation of a defined epitope of HTLV-I. The immediate question is whether the conformational aspects of the epitope are maintained or modulated by the sugar moiety, and what are the effects of such modification on generating a humoral response. To do this, we chose a region encompassing residues 230-257. A peptide epitope, 242-257, is recognized by sera from HTLV-I-infected individuals, and harbors a conformational determinant containing a β-turn and a glycosylated asparagine. Lairmore *et al.* [9] were able to induce antibody production to this peptide conjugated to bovine serum albumin (BSA) but these antibodies did not inhibit syncytium formation and failed to protect rabbits against HTLV-I infection, despite the fact that they recognized the native gp46. Even though the 242-257 epitope is predicted to contain a β-turn using the Chou-Fasman algorithms [24], it is not known whether the β-turn conformation was maintained by the peptide in solution or when conjugated to BSA. Consequently, antibodies generated in response to the peptide-carrier protein conjugate were of low affinity, as a result of not mimicking the biologically relevant conformation of this region within gp46, and thereby were unable to neutralize the virus. As part of our studies involving the stabilization of peptide secondary structures within immunogenic constructs, and to further evaluate this region of HTLV-I, we prepared immunogens based on the 233-253 sequence of gp46 which were nonglycosylated (SC1) or contained an N-acetylglucosamine residue at Asn^{244} (SC2). In this paper, we describe the structures of the nonglycosylated and glycosylated forms of the 233-253 sequence by circular dichroism spectroscopy and by two-dimensional NMR techniques. We also report our results on the immunogenicity of these constructs in rabbits as assessed by direct ELISA, whole virus ELISA and radioimmunoprecipitation assays.

EXPERIMENTAL

Synthesis of Fmoc-Asn(GlcNAc)OAc-COOH derivative

2-Deoxy-2-acetamido-β-D-glucopyranosyl amine was formed by reaction of N-acetylglucosamine with ammonium bicarbonate [25]. Following extensive lyophilization, the 2-deoxy-2-acetamido-β-D-glucopyranosyl amine derivative was treated first with Fmoc-OSu, then with acetic anhydride, in pyridine to produce the 3,4,6-tri-O-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyl Fmoc derivative [26]. After purification of this derivative by crystallization, the Fmoc group was removed using piperidine in tetrahydrofuran. 3,4,6-Tri-O-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyl amine was reacted with Fmoc-Asp-O*t*Bu in the presence of 1 equiv each of N-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC) in dimethylformamide at 25°C overnight [27]. The dicyclohexylurea by-product was removed by filtration and the Fmoc-Asn$(GlcNAc)_{OAc}$-OtBu derivative was precipitated by addition of excess dry diethyl ether. The *t*-butyl group was removed by treatment of this derivative with trifluoroacetic acid (TFA); the TFA was removed by rotary evaporation and the residue triturated with dry diethyl ether and used directly in glycopeptide synthesis.

Glycopeptide and peptide synthesis

All peptides were synthesized on a Milligen/Biosearch 9600 synthesizer using Fmoc/*t*-butyl strategy. Side-chain protection was as follows: *t*-butyl ether (Ser, Thr), pentamethylchroman sulfate (Arg), trityl ether (His), *t*-butyl ester (Asp, Glu) and *t*-butyloxycarbonyl (Lys). The carboxyl terminal Thr was attached to 4-methyl benzhydrylamine resin (0.57 mmol/g substitution, bearing the 4-hydroxymethylphenoxyacetic acid linker), by double-coupling of the pre-formed pentafluorophenyl (Pfp) ester in the presence of 1 equiv HOBt and 0.1 equiv dimethylaminopyridine for 1 hr each coupling. To sequentially synthesize these peptides, derivatives were activated *in situ* using BOP/HOBt, except for the Fmoc-Asn$(GlcNAc)_{OAc}$-COOH and Fmoc-Asn-OPfp derivatives. The former was coupled using 1.5 equiv in the presence of equimolar amounts of HOBt and DCC for 4 hours at 25 °C, and the latter in the presence of 1 equiv of HOBt for 2 hours.

When the synthesis was complete, the peptide/glycopeptide was cleaved from the resin, and side-chain protecting groups were removed, by use of the following reagents: (1) 90% TFA/5% anisole/3% thioanisole/2% ethanedithiol for SC1 and SC2, and (2) 87.5% TFA/5% water/5% phenol/5% thioanisole/2.5% ethanedithiol for SC1MVF and SC2MVF. Crude peptides were purified by gel filtration and semi-preparative RP-HPLC.

The O-acetyl groups of the carbohydrate moieties of SC2 and SC2MVF were removed by treatment of the peptide, suspended in dry methanol, with sodium methoxide (pH of the solution was approximately 12) (moist pH paper) for 45 min at room temperature. The solution was neutralized, and the methanol removed, by bubbling carbon dioxide. The peptides were dissolved in H_2O and purified by HPLC.

Peptide/glycopeptide characterization

All peptide constructs were analyzed by mass spectrometry, amino acid analysis, reverse-phase HPLC, and circular dichroism (CD) spectroscopy; SC1 and SC2 were analyzed by capillary electrophoresis (CE) and were structurally characterized by two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy.

Reverse-phase HPLC. Analytical HPLC was performed on a Vydac C4 column (4.6 mm x 25 cm, 5 μm) at 32.5°C and a flow rate of 1 mL/min. Peptides were chromatographed using 20- to 30- min linear gradients of 0-60% (SC1 and SC2) or 10-90% (SC1MVF and SC2MVF) acetonitrile in water, both solvents containing 0.1% TFA as counter ion. Absorbance was monitored at 214 and 280 nm. All peptides were purified by semi-preparative RP-HPLC to homogeneity.

Mass spectrometry. Fast atom bombardment mass spectrometry (FAB-MS) was run for SC1 and SC2 at the Ohio State University Chemical Instrument Center using a Finnegan Mat - 900 instrument. Electrospray MS were determined for SC1MVF and SC2MVF by Dr. Yiping Sun of the Procter and Gamble Company, Cincinnati, OH using a Sciex API-III instrument. Masses (*calculated*, **found**) are: SC1 (*2235.6*, **2235.6**), SC2 (*2438.8*, **2438.8**), SC1MVF (*3850.9*, **3849.4**), and SC2MVF (*4054.1*, **4053.3**).

Amino acid analysis. Amino acid composition and peptide/glycopeptide concentrations were determined at the OSU Biochemical Instrument Center. Peptides were hydrolyzed in 6 N HCl; subsequently, amino acids were derivatized and analyzed as their phenylthiohydantoins using a Waters PicoTag System. Compositional values are as follows [observed(theory)]: SC1: Asx, 1.01 (1); Ser, 7.08 (8); His, 1.16 (1); Thr, 1.82 (2); Pro, 1.88 (2); Tyr, 1.16 (1); Val, 3.09 (3); Leu, 2.59 (3). SC2: Asx, 1.35 (1); Ser, 8.29 (9); His, 1.09 (1); Thr, 1.78 (2); Pro, 2.03 (2); Tyr, 1.33 (1); Val, 3.49 (3); Leu, 2.52 (2). SC1MVF: Asx, 0.99 (1); Glx, 1.13 (1); Ser, 7.66 (9); Gly, 3.57 (2); His, 2.00 (2); Arg, 0.94 (1); Thr, 1.55 (2); Pro, 2.33 (2); Tyr, 1.00 (1); Val, 5.70 (6); Ile, 1.39 (2); Leu, 5.53 (5); Lys, 1.09 (1). SC2MVF: Asx, 1.10 (1); Glx, 1.35 (1); Ser, 8.54 (9); Gly, 2.86 (2); His, 2.06 (2); Arg, 1.09 (1); Thr, 1.73 (2); Pro, 2.13 (2); Tyr, 1.00 (1); Val, 6.49 (6); Ile, 1.47 (2); Leu, 6.02 (5); Lys, 1.06 (1).

CD spectroscopy. The CD spectra were obtained using a Jasco J500 instrument interfaced with an IBM computer. The spectropolarimeter was calibrated with a 0.06% aqueous solution of ammonium-*d*-10-camphor sulfonate. CD spectra were obtained at 25°C, under continuous nitrogen purge of the sample chamber, using 0.1 cm path length quartz cuvettes. An average of three scans was taken for each spectrum. Peptides were dissolved in degassed, nitrogen-purged, water and an aliquot was removed for amino acid analysis. Samples were diluted over the concentration range of 62 μM to 2 mM with water or aqueous trifluoroethanol. Molar ellipticity ($[\theta]_{M,\lambda}$) values were calculated using the following equation:

$$[\theta]_{M,\lambda} = (\theta \times 100 \times M_r) \div (n \times c \times l)$$

where θ is the recorded ellipticity (deg), M_r the molecular weight of the peptide, n the number of residues in the peptide, c the peptide concentration (mg/mL), and l the cell pathlength (cm).

Capillary electrophoresis. Electropherograms were acquired using a Beckman PAC/E 2100 instrument equipped with a 50 cm fused-silica capillary (50 μm ID) at 25°C under an applied field of 15 kV. Samples were separated using 50 mM sodium borate, pH 8.2; detection was at 214 nm. HPLC-purified peptides showed single peaks by CE.

Proton NMR spectroscopy. Peptides were dissolved in 90% ^1H_2O/10% ^2H_2O to a final concentration of 5 mM. The pH of each solution was 3.2. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate-2,2,3,3-*d*4. ^1H NMR spectra were acquired using a Bruker DMX600 spectrometer. Phase-sensitive COSY [28], TOCSY [29] and NOESY [30] spectra were acquired with a States-TPPI method [31]. Spectral widths in all two-dimensional NMR experiments were 8012.82 Hz in both dimensions. A 4K x 512 time domain matrix was recorded, multiplied by proper window functions and zero-filled to a 4K x 1024 matrix prior to Fourier transformation. The mixing times in the TOCSY and NOESY experiments were 40 ms and 300 ms, respectively. Temperature coefficients of the amide proton resonances were derived from the gradient of the plot of chemical shift versus temperature. COSY spectra obtained at 10°C, 20°C and 30° C were used to calculate temperature coefficients.

Immunogenicity of SC1MVF and SC2MVF constructs.

Immunization protocols. New Zealand white rabbits were housed in the Ohio State University vivarium in accordance with a University approved animal care and use protocol and in compliance with National Institutes of Health guidelines for the utilization and care of research animals. Each rabbit was immunized subcutaneously at 4-5 sites with a total of 500 μg of peptide immunogen emulsified in squalene/mannide monooleate (4:1) supplemented with 100 μg of muramyl dipeptide (MDP) [32]. Secondary and tertiary immunizations were given at three week intervals using the same protocol. Sera were collected weekly, aliquoted and stored frozen at -20°C prior to testing.

Direct ELISA to determine antibody production. U-bottom polyvinyl chloride assay plates were coated with 100 μg antigen at a concentration of 2 μg/mL in phosphate-buffered saline (PBS). The plates were incubated overnight at 4°C, then excess liquid was removed and sites of non-specific binding were blocked by addition of 200 μL of PBS containing 1% bovine serum albumin (incubation for 60 min at room temperature). The plates were washed twice with PBT (PBS containing 1% horse serum, 0.05% Tween-20 and 0.02% thimerosal). Rabbit antiserum diluted 1/500 to 1/1000 in PBT was added to antigen-coated plates, serially diluted (1:2) in PBT, and incubated for two hours at room temperature. The plates were washed with PBT and 50 μL of goat anti-rabbit IgG, conjugated to horseradish peroxidase, were diluted 1/500 with PBT and 100 μL of this solution were added to each well. Following incubation at room temperature for 60 min, excess antibody-conjugate was removed by washing the plates with PBT and water. Bound antibody was detected using 50 μL of 0.15% H_2O_2 in 24 mM citric acid, 5 mM sodium phosphate (pH 5.2) with 0.5 mg/mL 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) as chromophore. Color development was allowed to proceed for 10 min before the reaction was stopped by addition of 25 μL of 1% sodium dodecyl sulfate. Absorbance at 410 nm was determined using a Dynatech MR700 ELISA reader. Titers are expressed as the serum dilution at which the absorbance is > 0.2 absorbance units above the background.

Whole virus enzyme immunoassay (whole virus EIA). To test for rabbit antibodies against complete disrupted HTLV-I a commercially available ELISA for human antibodies was modified

(Cambridge Biotech Corp., Worcester, MA). Serial two-fold dilutions of sera were evaluated to determine titers. HTLV-I (Hut-102, B2) coated microtiter plates and ELISA reagents were used according to manufacturer's instructions with the exception of a substitution of anti-human antibody conjugates with alkaline phosphatase conjugated anti-rabbit whole IgG conjugates (Sigma Chemical Co., St. Louis, MO).

Radioimmunoprecipitation assay (RIPA). RIPA was performed as described previously [8]. Briefly, HTLV-I infected MT-2 cells were metabolically labeled (200 μCi) with [^{35}S]cysteine and [^{35}S]methionine (Translabel, ICN Biomedical Inc.). Labeled cells were washed and disrupted in RIPA lysing buffer and lysates were prepared by centrifugation. Labeled lysate preparations were incubated with 20 μL of test serum for 16 hr at 4°C. Immune complexes were precipitated with protein A-Sepharose CL4B resin, and bound immune complexes were washed with RIPA lysing buffer and then eluted by incubation of the sample at 100°C. Samples were analyzed by electrophoresis in 10% SDS-PAGE gels with visualization by autoradiography.

Peptide/Glycopeptide Reactivity against HTLV-I$^+$ Sera.

HTLV-I positive human serum specimens were also tested for reactivity to our peptide (SC1, SC1MVF, MVF) and glycopeptide (SC2, SC2MVF) constructs by direct ELISA. Samples included a World Health Organization panel of ten serum samples obtained from HTLV-I-infected persons previously tested positive by immunoblot assay for HTLV-I antibodies (kindly provided by Dr. Harvey Holmes, National Institute for Biological Standards and Controls, Hertfordshire, England).

RESULTS

Peptide and Glycopeptide Engineering.

By use of numerous computer algorithms for the prediction of protein secondary structures (α-helix, β-turn and β-sheet [24]) and antigenic potential (solvent exposure index [33], protrusion index [34], antigenicity [35,36], segmental mobility [37], hydrophilicity [38]), we have identified several regions of gp46 of HTLV-I that are good candidates for vaccine development. One of these regions, sequence 220-257, contains two predicted β-turns linked by a β-strand, two glycosylated asparagine residue in each turn as well as two cysteine residues at the amino terminus. Also, Lairmore *et al.* have established the immunogenicity of the 242-257 region [9] and other researchers [11,14] have shown that sera from HTLV-I-infected individuals cross-react with the 235-257 peptide. We chose to restrict our epitope selection to the 233-253 carboxy-terminal portion, so as not to complicate interpretation of the effects of glycosylation on peptide structure and immunogenicity. Thus, the 233-253 sequence was designed to incorporate a single glycosylated residue, with the β-turn segment near the middle of the sequence, and no cysteine residues. The peptide and glycopeptide constructs are illustrated in Figure 1.

To provide helper T cell activity for antibody production, a "promiscuous" T cell epitope from measles virus fusion protein (MVF) [15,39] was designed to be incorporated into the non-glycosylated sequence 233-253 (SC1) sequence as well as the glycosylated 233-253 (SC2) sequence. The MVF T cell epitope was designed to be located at the amino terminus of the chimeric constructs SC1MVF and SC2MVF.

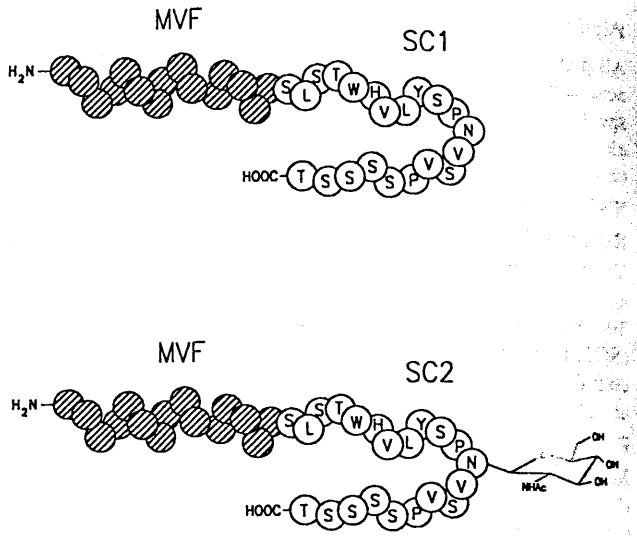

Figure 1. Schematic representation of the SC1, SC2, SC1MVF and SC2MVF constructs used in this study. The MVF promiscuous T-cell epitope is shown with cross-hatching.

Peptide/Glycopeptide Synthesis.

The synthesis of the SC1 and SC1MVF peptides was achieved using Fmoc/*t*-butyl strategy with BOP/HOBt activation and coupling protocols. For synthesis of the glycopeptides SC2 and SC2MVF, the N-acetylglucosamine moiety was incorporated by using Fmoc-Asn(GlcNAc)OAc-COOH which was activated *in situ* with DCC and HOBt. Peptides were purified by HPLC to homogeneity as indicated by HPLC and CE analyses (not shown). Analysis by FAB-mass spectroscopy and amino acid analysis show that the purified peptides had the correct amino acid composition and molecular weight.

Peptide/Glycopeptide Characterization by CD Spectroscopy.

Circular dichroic (CD) spectroscopy for SC1 and SC2 indicates the presence of β-turn structure as shown in Figure 2. The spectrum of each peptide in aqeuous solution shows a weak minimum ($[\theta]_{M,\lambda}$ approximately -800 deg·cm^2/dmol) at 200 nm.

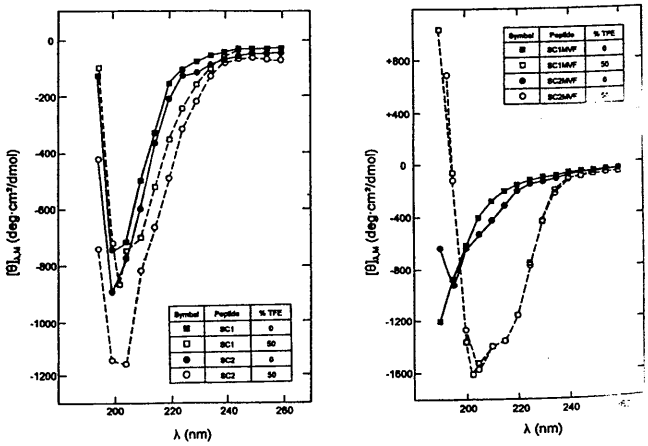

Figure 2. CD spectral analysis of the SC1, SC2, SC1MVF and SC2MVF constructs. General conditions: SC1 and SC2 concentration was 250 μM; SC1MVF and SC2MVF concentration was 65 μM in aqeuous solution or in water/TFE (1:1). Triplicate scans were obtained at 25°C in a nitrogen-purged sample chamber.

Very little change in the CD spectrum for either peptide is seen upon addition of trifluoroethanol (TFE) to these samples.

The CD spectra of SC1MVF and SC2MVF in aqeuous solution (Figure 2) are similar to SC1 and SC2, perhaps exhibiting a more random coil conformation for the chimeric peptide constructs. However, upon addition of TFE to these peptides, the content of α-[illegible]ses as evidenced by pronounced minima at approximately 208 nm and 220 nm and positive $[\theta]_{M\lambda}$ values below 195 nm. The presence of α-helical structure is most likely due to the presence of the MVF sequence in the chimeras, shielding the β-turn minima. Neither peptide exhibited concentration-dependent CD spectra over the range 62 μM to 1mM, indicating that they are monomeric in solution.

NMR Spectroscopy of SC1 and SC2.

Complete ^1H NMR assignments of SC1 and SC2 were obtained using standard two-dimensional methods [28-30]. A small portion of minor forms (<5%) of resonances, which probably arise from *cis-trans* isomerization of Pro11 and/or Pro16, was observed in the NMR spectrum. Tables 1 and 2 summarize the assignments of the major forms of SC1 and SC2. The major forms in SC1 and SC2 contain the *trans* isomer of proline, as determined by the sequential $d_{\alpha\delta}$(Xaa-Pro) connectivities where Xaa defines a preceding residue (not shown). Significant changes in the chemical shifts of protons in residues 9-13 (the predicted β-turn region) are seen between the glycosylated peptide SC2 and the non-glycosylated SC1 construct as shown in Tables 1 and 2. The difference in chemical shifts is not surprising considering the existence of the GlcNAc residue on Asn12 in SC2. The amide proton of Val13 undergoes the most perturbation (Tables 1 and 2). TOCSY spectra of SC1 and SC2 (Figure 3) show the complete spin system connectivities, except those between NH-H^δ for the Leu residues.

Table 1. Proton Assignments[a] of SC1 at 20°C, pH 3.2.

Residue	H^N	H^α	H^β	H^γ	Others
Ser1	---	4.14	3.98, 3.99		
Leu2	8.72	4.45	1.63	1.64	H^δ 0.89, 0.93
Ser3	8.57	4.48	3.79, 3.85		
Thr4	8.15	4.31	4.22	1.14	
Trp5	8.14	4.61	3.22		NH1 10.12, CH2 7.20, CH4 7.55, CH5 7.12, CH6 7.22, CH7 7.45
His6	8.09	4.49	2.93, 3.11		H^δ 7.09, H^ϵ 8.52
Val7	8.03	3.87	1.92	0.81, 0.87	
Leu8	8.31	4.28	1.46, 1.53	1.50	H^δ 0.81, 0.89
Tyr9	8.29	4.57	2.92, 2.94		H^δ 7.07, H^ϵ 6.77
Ser10	8.19	4.68	3.73, 3.77		
Pro11	---	4.37	1.93, 2.27	1.98	H^δ 3.54, 3.67
Asn12	8.46	4.68	2.72, 2.83		H^δ 6.97, 7.65
Val13	8.08	4.19	2.11	0.91	
Ser14	8.46	4.48	3.79, 3.84		
Val15	8.27	4.44	2.08	0.93, 0.97	
Pro16	---	4.43	1.93, 2.31	1.98, 2.04	H^δ 3.69, 3.86
Ser17	8.55	4.46	3.86, 3.93		
Ser18	8.49	4.53	3.88, 3.94		
Ser19	8.43	4.57	3.91		
Ser20	8.20	4.42	3.88, 3.91		
Thr21	8.07	4.36	4.34	1.18	

[a]Proton chemical shifts are ±0.01. Dashes indicate that there are no observable NH protons in prolines and the amino-terminal residue (Ser1). The resonances whose chemical shifts differ by ≥0.02 ppm from those of SC2 (Table 2) are underlined.

Table 2. Proton Assignments[a] of SC2 at 20°C, pH 3.2.

Residue	H^N	H^α	H^β	H^γ	Others
Ser1	---	4.14	3.98, 3.99		
Leu2	8.72	4.45	1.63	1.63	H^δ 0.89, 0.93
Ser3	8.57	4.48	3.79, 3.85		
Thr4	8.14	4.30	4.22	1.13	
Trp5	8.14	4.60	3.22		NH1 10.11, CH2 7.20, CH4 7.55, CH5 7.11, CH6 7.21, CH7 7.45
His6	8.09	4.48	2.92, 3.10		H^δ 7.08, H^ϵ 8.52
Val7	8.03	3.87	1.92	0.81, 0.87	
Leu8	8.31	4.28	1.46, 1.53	1.50	H^δ 0.80, 0.89
Tyr9	8.27	4.57	2.92, 2.95		H^δ 7.07, H^ϵ 6.77
Ser10	8.21	4.66	3.73, 3.78		
Pro11	---	4.36	1.93, 2.28	1.98	H^δ 3.54, 3.66
Asn12[b]	8.48	4.71	2.72, 2.86		H^δ 8.71
Val13	7.99	4.19	2.11	0.91	
Ser14	8.45	4.47	3.80, 3.85		
Val15	8.27	4.43	2.08	0.92, 0.97	
Pro16	---	4.43	1.93, 2.31	1.98, 2.04	H^δ 3.69, 3.86
Ser17	8.54	4.45	3.88, 3.93		
Ser18	8.48	4.52	3.88, 3.94		
Ser19	8.42	4.57	3.91		
Ser20	8.21	4.42	3.88, 3.91		
Thr21	8.07	4.36	4.34	1.18	

[a]Proton chemical shifts are ±0.01 ppm. Dashes indicate that there are no observable NH protons in prolines and the amino-terminal residue (Ser1).The resonances whose chemical shifts differ by ≥0.02 ppm from those of SC1 (Table 1) are underlined.

[b]The assignments for the N-acetyl glucosamine residue linked to Asn12 are as follows: H_1. 5.02, H_2. 3.82, H_3. 3.58, H_4. 3.48, H_5. 3.73, H_6. 3.81,3.84, NH 8.25, CH_3 2.00.

SC1 and SC2 show strong sequential $d_{\alpha N}(i,i+1)$ NOEs (Figure 4) throughout each polypeptide, except for the Asn12-Val13 segment, which exhibits rather weak interaction for SC1 and moderate NOEs for SC2. Instead, moderate $d_{NN}(i,i+1)$ NOEs were observed between Val13 and Ser14 for SC2 (data not shown). For the SC1 peptide, a moderate NOE is observed at 8.08/8.46 ppm which may be assigned to either Asn12-Val13 or Val13-Ser14 since the amide resonances of Asn12 and Ser14 coincide. Considering that SC1 and SC2 show remarkably similar NOE patterns in other regions of the peptide chain, it is believed that the $d_{NN}(i,i+1)$ NOE should be arising from Val13-Ser14. Figure 5 shows a summary of sequential and medium-range NOEs observed for SC1 and SC2. Both peptides most likely reside in extended-chain conformations with a β-turn configuration near Asn12 as deduced from the following NOE characteristics: (1) weak intraresidue $d_{\alpha N}$ NOEs, (2) strong sequential $d_{\alpha N}(i,i+1)$ NOEs except for the Asn12-Val13 segment (3) few sequential $d_{NN}(i,i+1)$ NOEs except for the Val13-Ser14 segment, and (4) few medium-range NOEs. The weak medium-range NOEs observed in SC1 and SC2 indicate that a small ensemble of peptides may be adopting a helical or turn structure in the Thr4-Val7 region.

Temperature coefficients can also be used to monitor structured conformations [40]. Figure 6 shows a plot of temperature coefficients *versus* residue number for the SC1 and SC2 constructs. The unusually low temperature coefficient observed for the His6 amide proton (-1.6 ppb/K) indicates that this amide bond is protected from solvent. Also, the temperature dependence of this amide proton may be due to hydrogen bonding occuring from weak, medium-range NOE interactions observed near His6. The amide protons in the β-turn region (Asn12 to Ser14) did not show appreciably lower temperature coefficients, which indicates

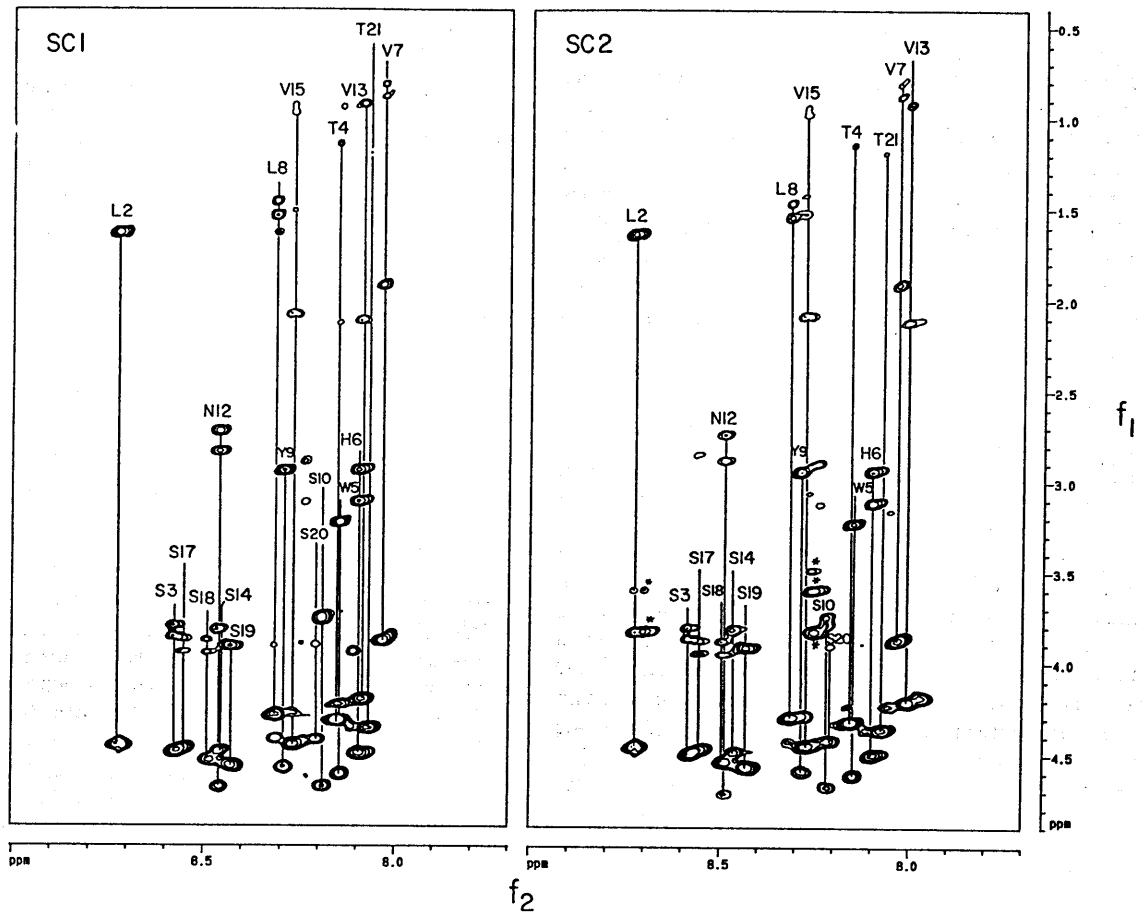

Figure 3. NH-aliphatic connectivities from the TOCSY spectra of SC1 and SC2 constructs. Samples were dissolved at 5 mM concentration in 90% ^1H_2O/10% ^2H_2O. Spectra were obtained at 20°C using a mixing time of 40 ms. The cross peaks arising from direct and relayed coherence transfers in the same spin systems are connected by vertical lines. The cross peaks arising from the GlcNAc group and the side-chain $N^{\delta}H$ proton of Asn12 (Asn^{244} in the native gp46 sequence) are indicated with an asterisk.

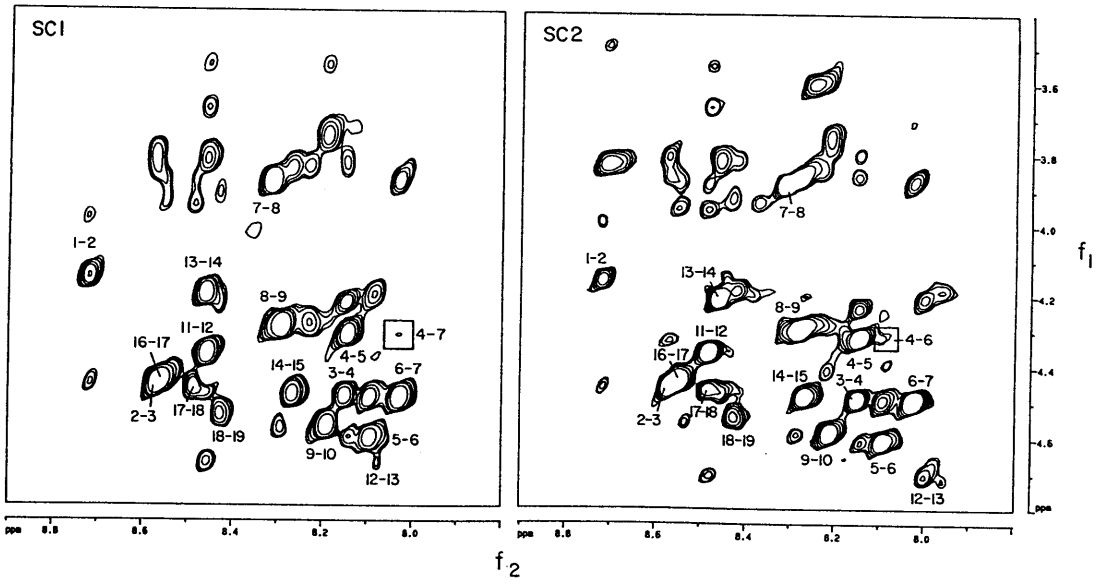

Figure 4. Fingerprint region of the NOESY spectra of SC1 and SC2. Samples were dissolved at 5 mM concentration in 90% ^1H_2O/ 10% ^2H_2O. Spectra were obtained at 20°C using a mixing time of 300 ms. Sequential $d_{\alpha N}$ (i,i+1) NOE cross peaks are indicated. The unlabeled cross peaks arise from intraresidue $d_{\alpha N}(i,i)$ and $d_{\beta N}(i,i)$, sequential $d_{\beta N}(i,i+1)$, and other connectivities. The medium range NOEs are enclosed within a box.

Figure 5. Summary of sequential and medium range NOE connectivities for SC1 and SC2. Ser1 corresponds to Ser^{233} of the gp46 sequence. The thickness of the horizontal bars qualitatively corresponds to NOE intensities. The hatched areas represent the connectivities to the $H^{\delta\delta}$ protons of proline as a substitute for the H^N proton. The asterisk indicates that the $d_{NN}(i,i+1)$ connectivity cannot be assigned unambiguously since the amide resonances of Asn12 and Ser14 coincide.

that this segment does not form a tight reverse-turn stabilized by hydrogen bonding interactions. Except for a few residues around Asn12, both peptides show nearly identical temperature coefficients for the amide protons. The small difference observed for Tyr9, Ser10, Asn12 and Val15 may arise from glycosylation in SC2. These results indicate that the addition of the GlcNAc residue to Asn12 may affect the hydrogen-bonding capacity of the amide protons within the β-turn peptide segment.

Immune Response to SC1MVF and SC2MVF.

New Zealand white rabbits (two/immunogen) were immunized with the peptide construct emulsified in squalene/mannide monooleate vehicle containing muramyl dipeptide as adjuvant. Rabbits were boosted with peptide at three-week intervals and

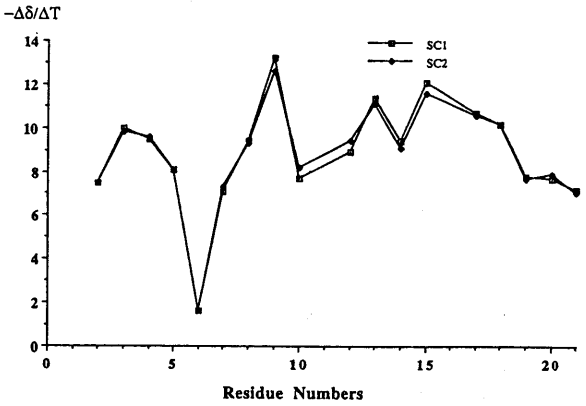

Figure 6. A plot of temperature coefficients (-Δδ/ΔT) versus residue numbers for SC1 and SC2. Peptides were dissolved at 5 mM concentration in 90% ^1H_2O/10% ^2H_2O, and COSY spectra were obtained at 10°C, 20°C and 30°C.

sera collected weekly were titered against the immunogen and the various constructs. High-titered (8,000-32,000 for SC1MVF and 16,000-32,000 for SC2MVF) antipeptide antibodies were produced against these immunogen as early as primary + three week sera. Following the tertiary immunization, the titers increased to 256,000 for SC1MVF and 128,000 for SC2MVF. Figures 7A and 7B show the antibody titers of each construct during the primary through tertiary response. The anti-SC1MVF antibodies recognized the SC1 construct, and the anti-SC2MVF antibodies bound SC2 in direct ELISA. Since neither antisera reacted with the MVF T cell epitope sequence (data not shown), we conclude that the antibodies were specific for the B-cell epitope. Cross-reactivities observed between anti-SC1MVF antibodies and SC2 constructs, as well as between anti-SC2MVF antibodies SC1 (Figure 7), indicate overlapping reactivities between the constructs. This qualitative observation by direct ELISA is not entirely unanticipated, since the only difference between SC1 and SC2 is the presence of the GlcNAc moiety on asparagine in the SC2 sequence. Further studies are in progress

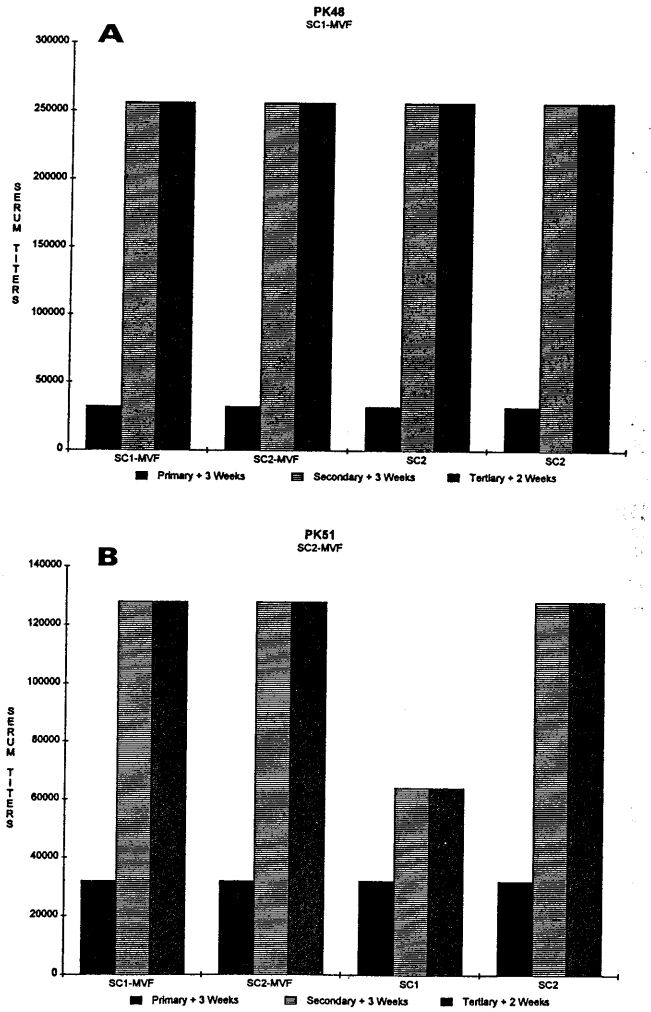

Figure 7. Antibody titers in rabbits immunized with SC1MVF (A) and SC2MVF (B) chimeric constructs. Rabbits were immunized with 500μg peptide emulsified in squalene/mannide monooleate (4:1) containing muramyl dipeptide adjuvant. Booster immunizations were given at three-week intervals. Sera were titered against immunogen and the various constructs by direct ELISA. Titers are defined as the serum dilution which gives an absorbance value ≥ 0.2 units above background.

to determine the neutralizing activities of the glycosylated *versus* the non-glycosylated peptides.

To determine whether the antibodies elicited by these constructs recognized HTLV-I viral preparations, whole virus enzyme immunoassay (whole virus EIA) was utilized to determine the binding of anti-SC1MVF and anti-SC2MVF antibodies to native gp46 immobilized to ELISA plates following disruption of HTLV-1 virus. Antisera from all four rabbits exhibited binding to whole virus in this assay. Rabbits inoculated with SC2MVF at 1:40 dilution of each serum specimen (Figure 8), as well as serial dilutions of these same serum specimens (1:40 to 1:640) had similar reactivity to HTLV-I by ELISA. In addition, both rabbits inoculated with SC2MVF reacted to HTLV-I envelope protein (gp68) in RIPA, while only one of two rabbits immunized with SC1MVF reacted to the envelope protein (data not shown).

To determine whether the glycosylated peptide was capable of recognition by human sera, a panel of 10 human serum specimens from HTLV-I-infected individuals was tested against SC1, SC2, SC1MVF, SC2MVF and MVF T-cell epitope constructs. ELISA plates were coated with these peptides, then reacted with sera from HTLV-I$^+$ individuals. The mean absorbance values (±one standard deviation) were: MVF, 0.491±0.1; SC1, 1.01±0.9; SC2, 0.984±0.8; SC1MVF, 1.59±0.9; and SC2MVF, 1.78±0.9. Statistical analyses (ANOVA and Student-Newman-Keuls post-test) of these data indicate significant differences in reactivities between the SC1MVF and SC2MVF compared with the MVF peptide only.

The SC2MVF glycopeptide had a slightly greater significance value ($p < 0.01$) than SC1MVF peptide ($p < 0.05$). The reactivities of the human sera against SC1 and SC2 were lower than compared with either SC1MVF or SC2MVF. An explanation for this observation is that the shorter peptides have a less stable conformation in solution. Alternatively, the T-cell epitope in the chimeric constructs helps stabilize a more native conformation of the peptide.

DISCUSSION

Synthetic peptides are useful in delineating antigenic and immunogenic regions of proteins, but there are several obstacles that need to be surmounted before an effective peptide vaccine can be constructed that elicits specific immune responses [41].

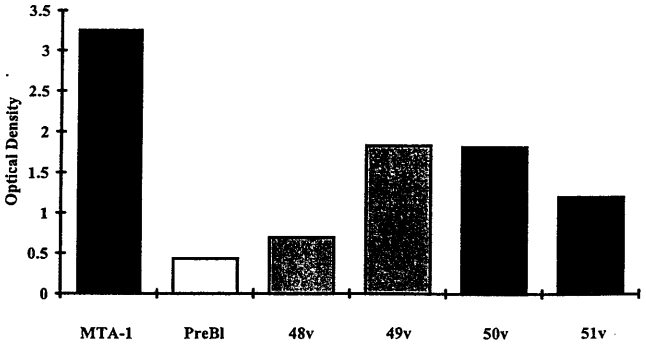

Figure 8. HTLV-I ELISA for antibodies against whole disrupted HTLV-I. East rabbit serum was tested at 1:40 dilution from either prebleed (PreBl, representative sample from rabbit 48) or secondary + two weeks sera. MTA-1 is a positive control serum from a rabbit hyperimmunized with a recombinant form of gp46 (aa162-209). Rabbits 48 and 49 were immunized with SC1MVF, and rabbits 50 and 51 were immunized with the SC2MVF construct.

Foremost in this regard is the rational engineering of highly structured peptides, which mimic antibody recognition sites [42]. The development of efficacious peptide based vaccines requires the engineering of antigenic regions of proteins into peptide constructs that mimic the corresponding structure in the native protein. We have been successful in engineering an α-helical segment from the protein LDH-C_4 (lactate dehydrogenase C_4 isozyme) into supersecondary motifs such as the αα-fold, four-helix bundle, αβ, βαβ, and βαβα [43-45]. In addition, we have designed and synthesized loop regions of LDH-C_4 and of the β subunit of human chorionic gonadropin (β-hCG) using the zinc-finger peptide motif as a template for mimicking antigenic loop segments of native proteins [46]. Another important aspect relating to the design of peptide-based vaccines is not only in the enhancement of immunogenicity, but in being able to elicit antibodies in a divergent outbred population. To achieve this, we have utilized "promiscuous" T cell epitopes in colinearly synthesized chimeric constructs and have successfully demonstrated the usefulness of this approach to bypass haplotype-restricted immune responses [15,39,47].

Antibodies directed against both linear and conformational epitopes in viral envelope glycoproteins have been shown to possess neutralizing activities. There have also been reports that carbohydrate moieties influence the antigenicity of viral glycoproteins, and that many conformational epitopes lose their activity when these moieties are removed. Viral envelope proteins are often glycosylated, usually via attachment of carbohydrate moieties to asparagine residues [17-21]. In the majority of cases, asparagine is glycosylated when it is present in a sequence motif -Asn-Xaa-Ser/Thr-, where Xaa may be any amino acid except proline. N-linked glycosylation sites, predominantly found on β-turns, are usually located on the surface in hydrophilic, protruding, mobile and accessible regions of the polypeptide chain. Although the role of N-linked glycosylation in directing and maintaining folding of some proteins is well founded, its effect on antigenicity is not well understood. In the work presented here, we extend our basic studies relating to the design of conformational epitopes to examine the effects of N-linked glycosylation on both peptide structure and on immunogenicity.

The HTLV-I gp46 glycoprotein contains four glycosylation sites, and the region around Asn^{244} is predicted by various computer algorithms to be in β-turn conformation and to have a high antigenic value. Thus, the HTLV-I 233-253 sequence (SC1) constitutes a conformational, N-linked glycosyl epitope. Recent studies indicate that asparagine glycosylation may confer stability to certain peptide conformations, notably β-turn structures. The design of the glycosylated peptide SC2, containing GlcNAc at Asn^{244}, was intended to stabilize the β-turn and mimic the structure of the peptide as found in native gp46. In terms of asparagine glycosylation, addition of a monosaccharide unit produces the most profound effect on the peptide structure and the addition of larger saccharide moieties do not enhance this effect in any appreciable manner. Thus, the monosaccharide N-acetyl-glucosamine was incorporated into the Asn^{244} site in the design of the glycopeptide construct SC2.

The structure determination by CD indicates that there is slightly greater degree of β-turn conformation in SC2 than SC1 (Figure 2). Both the SC1 and SC2 constructs exhibited β-turn structure in aqueous solution, as shown by CD and NMR determinations. This result is comparable to others. For example, circular

dichroic analysis of peptide T, a peptide inhibitor fragment of HIV gp120, in water shows mainly unordered conformation. However, addition of a single GlcNAc residue to the Asn residue in this sequence (ASTTTNYT) induces β-turn conformation [48]. The principal neutralizing determinant peptide of HIV gp120, GPGRAFY, is predicted to contain a Type II β-turn in the GPGR sequence. In solution, this peptide is a mixture of interconverting forms [49] with a significant population of β-turn structure exhibited. Addition of glycosylated asparagine at the amino terminus stabilized the β-turn configuration of this peptide [50].

Using 2D-NMR, the structure of the 233-253 peptide (SC1) was shown to be a β-turn from residues 242-245, the rest of the peptide showing extended conformation. The two strands connected by the β-turn do not interact to any significant degree as evidenced by the lack of cross-peaks in the NOESY spectrum. In SC2, the spectra exhibit subtle differences, but the overall structure is very similar to SC1 (*i.e.*, two extended peptide segments connected by a β-turn). There are no detectable medium-range NOE cross-peaks in the sequence around Asn12 for SC2 as shown in the NOESY spectrum (Figure 4). This cross-peak may be too weak to detect, or there is no such interaction due to a loose turn structure. The NMR evidence indicates that the presence of the GlcNAc group on the Asn12 side-chain barely perturbs the overall conformation of the peptides. The chemical shifts of all the protons except those from the residues around the asparagine residue are nearly identical (Tables 1 and 2). Also, the same pattern of inter-and intra-residue NOEs, except that of $d_{\alpha N}$ (Asn12-Val13), is observed in both peptides. However, it is interesting that the Val13 NH proton undergoes the largest chemical shift perturbation (-0.09 ppm) and the $d_{\alpha N}$ (Asn12-Val13) NOE intensity becomes intense by addition of GlcNAc at the Asn12 position. This observation indicates that SC1 and SC2 may adopt a slightly different turn structure. However, the 2D NMR spectra do not provide convincing evidence of enhanced stability of the β-turn structure in the SC2 glycopeptide as opposed to the SC1 peptide. The relatively flexible nature of these peptides hinders further detailed analysis of the turn structure. Some investigators have used model di- and tri-glycopeptides to examine the role that sugar residues play in stabilization of peptide structures [51]. It has been shown that there is hydrogen bonding interactions between the N-acetyl group of GlcNAc and the amide bond C-terminal to the Asn residue. These interactions restrict the rotational freedom of the sugar, the peptide backbone, and the asparagine side-chain and as a result the β-turn conformation is stabilized at these sites. However, such hydrogen bonding is not likely in SC2, because no NOEs connecting the sugar and the peptide were observed in the NOESY spectrum of this glycopeptide.

The effects of glycosylation on protein antigenicity and immunogenicity are poorly understood and remain to be fully elucidated. There are some studies which indicate that carbohydrate moieties are important parts of some glycoprotein antigenic sites. For instance, monoclonal antibodies were generated against the differentially glycosylated C-terminal peptide of human chorionic gonadotropin β-subunit, which was conjugated to a carrier protein [52]. These antibodies were specific to the glycopeptide form from which they were derived, exhibiting binding to both carbohydrate and peptide moieties [53]. In the case of viral glycoproteins, one of the antigenic sites of influenza virus hemagglutinin contains carbohydrate side-chains, and another is proximal to two saccharide moieties on the surface of the glycoprotein [54]. For HTLV-1, neutralization of the virus requires antibodies raised against conformational determinants that appear to require the presence of sugar residues at the appropriate glycosylation sites, Asn^{222} and Asn^{244} [55]. The SC1 and SC2 constructs were engineered into immunogenic peptides SC1MVF and SC2MVF by colinear synthesis with a promiscuous T cell epitope from measles virus MVF. The structures of SC1MVF and SC2MVF are very similar in aqueous solution, as shown by CD measurements, exhibiting some β conformation and random structure. Upon addition of TFE to these peptides in solution, the degree of helicity increases, which is probably due to adoption of α-helix conformation as a result of the MVF sequence. Both SC1MVF and SC2MVF were highly immunogenic, eliciting high titer antibodies as early as primary + three weeks. Upon boosting, the titers of the antibodies increased dramatically, indicating that these antipeptide antibodies recognized the immunogenic sequence as well as the individual SC1 and SC2 peptides. Our data indicate that rabbits immunized with the glycosylated form of the 233-253 sequence (SC2MVF) recognize the native form of the viral peptide in whole virus EIA. While the number of rabbits in this study precludes interpretation of the relative strength of the immune response to the viral peptide, it is of note that both rabbits immunized with the SC2MVF glycopeptide recognized the HTLV-I envelope precusor gp68 in the RIPA. Studies in progress are aimed at analyzing the neutralizing activities of these antibodies, by syncytia inhibition and vesicular stomatitis virus pseudotype assays, as well as to test the immunoprotective capacity of these N-linked chimeric epitopes by challenging immunized rabbits with irradiated HTLV-I-infected cell inocula.

The reactivities of human serum specimens from persons known to be positive for HTLV-I infection implies that both SC1 and SC2 were recognized. The lower values for the SC1 and SC2 constructs compared to the chimeric constructs SC1MVF and SC2MVF are likely due to a differential adsorption of the shorter peptides to the ELISA plates. Interestingly, the human sera had a slightly greater reactivity to SC2MVF than to SC1MVF, suggesting stabilization of the construct by glycosylation or presentation of a conformation more like the structure of native gp46. These results suggest that glycosylation of this HTLV-I-derived peptide did not alter the immunogenicity of the chimeric construct and, alternatively, may enhance the immune recognition of the peptide.

CONCLUSION

This work represents a first attempt at designing an N-linked glycopeptide based on a human retrovirus for the purpose of examining the humoral immune response, and more importantly to gain insight into the dependence of conformational epitopes on N-glycosylation and the elucidation of neutralizing antibodies. The role of neutralization in vaccine development is still largely unexplained, and the requirement of carbohydrates or the importance of epitope glycosylation in infectivity remains to be elucidated. Clearly, a vaccine that targets discontinuous, glycosylation-dependent neutralizing determinants has the potential to be more effective than a linear unconstrained peptide.

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