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Immunology

Antigenic peptides containing large PEG loops designed to extend out of the HLA-A2 binding site form stable complexes with class I major histocompatibility complex molecules

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ABSTRACT **Recognition of peptides bound to class I major histocompatibility complex (MHC) molecules by specific receptors on T cells regulates the development and activity of the cellular immune system. We have designed and synthesized *de novo* cyclic peptides that incorporate PEG in the ring structure for binding to class I MHC molecules. The large PEG loops are positioned to extend out of the peptide binding site, thus creating steric effects aimed at preventing the recognition of class I MHC complexes by T-cell receptors. Peptides were synthesized and cyclized on polymer support using high molecular weight symmetrical PEG dicarboxylic acids to link the side chains of lysine residues substituted at positions 4 and 8 in the sequence of the HLA-A2-restricted human T-lymphotrophic virus type I Tax peptide. Cyclic peptides promoted the *in vitro* folding and assembly of HLA-A2 complexes. Thermal denaturation studies using circular dichroism spectroscopy showed that these complexes are as stable as complexes formed with antigenic peptides.**

The activation of T-cell receptors (TCRs) on T cells is thought to involve the simultaneous interaction of the TCR with a major histocompatibility complex (MHC) molecule and the bound antigenic peptide. Antigenic peptides are typically 8–10 residues long and bind in an extended, kinked conformation to class I MHC molecules (for reviews, see refs. 1 and 2). Recognition of the compound surface formed by atoms of the MHC molecule and of the bound peptide provides a mechanism for the observed MHC restriction of antigens by T cells (3). Studies using analogs of antigenic peptides and mutants of MHC molecules indicate that residue substitutions in the peptide sequence or in and adjacent to the peptide binding site on the MHC molecule affect recognition by T cells (for review, see ref. 4).

We have designed and synthesized cyclic peptides that incorporate high molecular weight PEG within the ring to study whether a large, bulky structure on antigenic peptides still permits binding to class I MHC molecules and the formation of stable complexes. Homobifunctional PEG derivatives have been used to link covalently the side chains of lysine residues substituted at position 4 (P4) and P8 in the sequence of the human T-lymphotrophic virus type I Tax peptide (LLFGYPVYV) (5) to form large nonpeptidic loops designed to extend out of the peptide binding site and interfere with interactions between TCRs and class I MHC–peptide complexes. PEGs were used as linkers because of their water solubility, conformational flexibility, and biocompatibility.

MATERIALS AND METHODS

Synthesis of PEG Dicarboxylic Acids I. Commercially available PEG 300, PEG 400, and PEG 600 (Union Carbide) were functionalized as reported for monomethoxyPEG (6). Intermediates and products were characterized by fast atom bombardment (FAB) mass spectrometry: PEG 300 dicarboxylic acid **I** $[M + H]^+$ 311 ($n = 4$), 355 ($n = 5$), 399 ($n = 6$), 443 ($n = 7$), 487 ($n = 8$), 531 ($n = 9$), 575 ($n = 10$) where n corresponds to the number of oxyethylene units making up each PEG chain in the distribution. FAB mass spectra for PEG 400 and PEG 600 dicarboxylic acids **I** showed the expected $[M + H]^+$ ions.

Synthesis and Purification of Peptides. Cyclic peptides were synthesized manually on 4-hydroxymethylphenoxy (HMP) resin using N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) amino acids except for the last residue (see Fig. 1). Tyrosine was protected with *tert*-butyl (Bu^t) groups and lysine residues were protected with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethyl (Dde) groups (see Fig. 1). Double couplings were performed for each amino acid using preformed hydroxybenzotriazole active esters. Initial substitution level was determined to be 0.25 mmol/g resin by picric acid test (7). After assembly of the protected peptide, selective and quantitative removal of Dde groups was achieved with 1% hydrazine hydrate in *N*-methylpyrrolidinone (NMP) (6 × 4 min). The peptidyl resin was then divided into three equal parts for cyclization with PEG dicarboxylic acids **I**. Ring closure was achieved by mixing peptidyl resin suspended in dichloromethane (DCM)/dimethylformamide (DMF) (1:9) with either of the PEG dicarboxylic acids **I** (2 eq) and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (4 eq) for 5 min. *N,N*-diisopropylethylamine (DIEA) (8 eq) was then added and the mixture was shaken for 24 hr, after which a negative ninhydrin test (8) was obtained. Cyclization was repeated a second time as above. Linear peptides were synthesized using an automated synthesizer (Applied Biosystems model 431A) on HMP resin with N^{α}-Fmoc amino acids. Standard side chain protecting groups were used except for lysine residues, which were protected with Ac groups.

Peptides were deprotected and cleaved from polymer support by treatment with 95% trifluoroacetic acid (TFA) for 2.5 hr (1.5 hr for linear peptides). Crude peptides were precipitated by the addition of cold ether and were purified by reverse-phase (RP)-HPLC on a Vydac C_{18} preparative column (Hesperia, CA). All purified peptides showed expected composition by amino acid analysis. FAB mass spectra were: Tax 300 peptide $[M + H]^+$ 1336 ($n = 3$), 1380 ($n = 4$), 1424 ($n = 5$), 1468 ($n = 6$), 1512 ($n = 7$), 1556 ($n = 8$), 1600 ($n = 9$), 1644 ($n = 10$); Tax peptide $[M + H]^+ = 1070$ (calculated = 1070); and [Lys^4(Ac),Lys^8(Ac)]Tax peptide $[M + H]^+ = 1190$ (calculated = 1190). The FAB mass spectra for Tax 400 and Tax 600 peptides showed the expected $[M + H]^+$ ions.

Enzymatic Hydrolysis of Cyclic Peptides. Tax 300 peptide (1 μM) was incubated with proline specific endopeptidase (Seikagaku Kogyo, Co., Tokyo) at enzyme/substrate ratio of

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Abbreviations: TCR, T-cell receptor; MHC, major histocompatibility complex; FAB, fast atom bombardment; RP-HPLC, reverse-phase HPLC; T_m, melting temperature.

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1:900 (wt/wt) in 25 mM phosphate buffer (pH 7.0) at 30°C. Aliquots from the digest were taken at different times and analyzed by RP-HPLC on a Vydac C_4 analytical column. Fractions corresponding to the digested peptide were analyzed by FAB mass spectrometry: $[M + H]^+$ 1398 ($n = 4$), 1442 ($n = 5$), 1486 ($n = 6$), 1530 ($n = 7$), 1574 ($n = 8$), 1619 ($n = 9$).

***In Vitro* Assembly and Purification of Class I MHC–Peptide Complexes.** Assembly of class I MHC complexes was initiated by dilution of denatured changes *Escherichia coli*-expressed heavy chain (HLA-A*0201) (1 μM) and β_2-microglobulin (β_2m) (2 μM) in the presence of excess synthetic peptides (10–30 μM) (9). The mixture was incubated at 10°C for approximately 48 hr, concentrated, and purified by gel filtration HPLC. Concentrations of stock complex solutions in 10 mM 4-morpholinepropanesulfonic acid (Mops) (pH 7.5) were determined by amino acid analysis, using norleucine as internal standard, and spectrophotometrically using ε = 94,240 $M^{-1}\cdot cm^{-1}$ at 280 nm.

Thermal Denaturation of Class I MHC–Peptide Complexes. CD studies were done using an Aviv Associates (Lakewood, NJ) model 62DS spectropolarimeter equipped with a thermoelectric temperature controller. Thermal denaturation curves for HLA-A2 complexes (0.18 mg/ml) were obtained in triplicate by monitoring the change in CD signal at 218 nm in the range of 20–90°C (10).

RESULTS

Peptide Design and Synthesis. The x-ray structure of HLA-A2 complexed with Tax peptide shows that peptide side chains at P4 and P8 point out of the binding site (11), suggesting that lysine residues could be substituted at those positions without affecting binding to the MHC molecule. High molecular weight PEG dicarboxylic acids were used to covalently link the side chains of these lysine residues. The design of cyclic peptides includes anchor residues Leu at P2 and Val at P9 to retain the specificity of interactions between peptides and HLA-A2.

Functionalization of PEGs was achieved as reported (6). In brief, the lithium salts of the hydroxyl end groups were alkylated with methyl bromoacetate followed by alkaline hydrolysis to yield desired symmetrical PEG dicarboxylic acids **I**. PEG 300, PEG 400, and PEG 600, which contain an average number of repeating oxyethylene units (n value) of 6.4, 8.7, and 13.2, respectively, were used as starting materials.

Cyclic peptides were synthesized as outlined in Fig. 1. After selective cleavage of Dde groups, cyclization of the liberated lysine side chains proceeded via PEG dicarboxylic acids. In this cyclization strategy, ring formation occurs on the polymer support using a symmetrical homobifunctional linker. The symmetrical nature of the linker allows the cyclization reaction to occur in a single step and to yield structurally homogeneous cyclic peptides. Final deprotection and cleavage from the polymer support yielded crude cyclic Tax 300, Tax 400, and Tax 600 peptides with ring sizes varying between ≈46 atoms (Tax 300) and 66 atoms (Tax 600).

Peptide Characterization. Cyclic peptides eluted as sharp (Tax 300 and Tax 400) or rather broad (Tax 600) peaks from RP-HPLC C_{18} preparative column. In all studies, the desired cyclic monomeric peptides were obtained as the major product with no evidence for formation of cyclodimers as analyzed by RP-HPLC. Sequence composition were confirmed by amino acid analysis and the presence of PEG was demonstrated by FAB mass spectrometry. Fig. 2*A* shows a typical FAB mass spectrum for purified Tax 300 peptide, revealing a series of distinct peaks corresponding to $[M + H]^+$ ions. Each peak is separated by intervals of m/z 44, corresponding to the mass of the repeating oxyethylene group, and is identified by the appropriate n value. The distribution of peaks in Fig. 2*A* is centered between $n = 6$ and $n = 7$ as obtained in the FAB mass

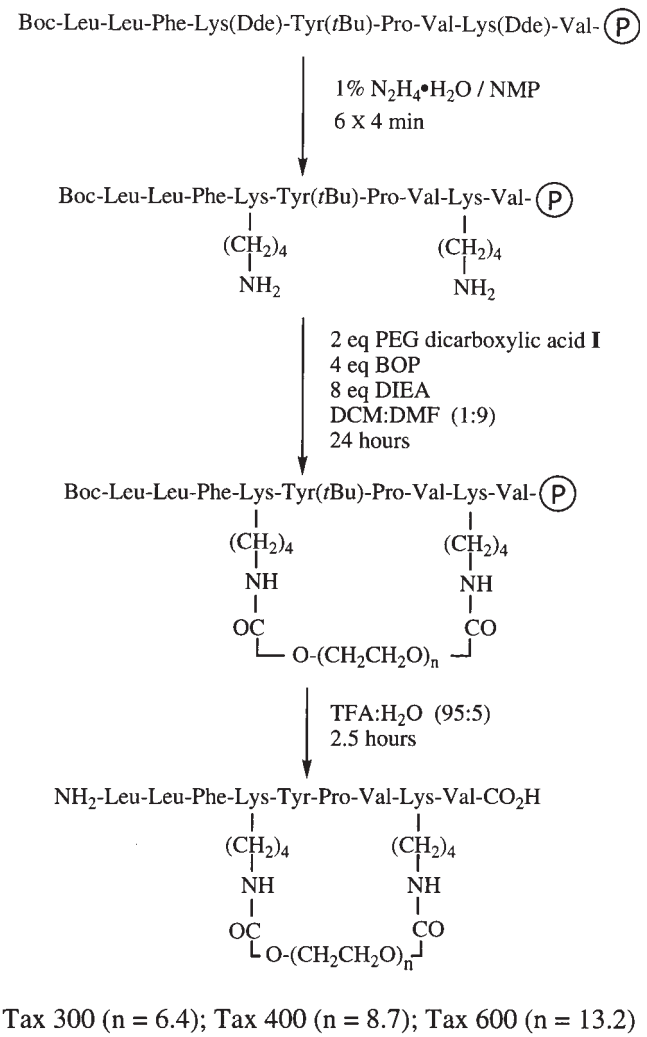

FIG. 1. Solid-phase synthesis of cyclic peptides containing PEG in the ring structure. Values of n in parentheses correspond to the average number of repeating oxyethylene units in the PEG loop.

spectrum for PEG 300 dicarboxylic acid (spectrum not shown), suggesting that the cyclization reaction is compatible with all PEG chains in the distribution. Similar results were obtained for Tax 400 and Tax 600 peptides.

The cyclic nature of peptides was further confirmed by digestion of the Tax 300 peptide by proline specific endopeptidase. This enzyme cleaves specifically peptide bonds on the carboxyl side of proline residues (12). Fig. 2*B* shows the RP-HPLC chromatograms of two aliquots from proline specific endopeptidase digestion of Tax 300 peptide taken at different times. Analysis of the digest after 3.5 hr reveals the presence of a single additional peak that becomes increasingly dominant after 16.5 hr. Analysis of the purified digested peptide by FAB mass spectrometry (Fig. 2*C*) shows a series of peaks shifted by m/z +18 (corresponding to the mass of a water molecule) in comparison with those in the FAB mass spectrum for the undigested peptide (Fig. 2*A*). These results indicate that enzymatic hydrolysis on the carboxyl side of proline at P5 causes the predicted linearization of the ring structure with the addition of one equivalent of water, thus confirming the cyclic structure of the PEG-containing peptides shown in Fig. 1.

***In Vitro* Assembly of Class I MHC–Peptide Complexes.** HLA-A2 complexes were reconstituted from *E. coli*-expressed human heavy chain and β_2m in the presence of excess cyclic peptides and purified by gel filtration chromatography (Fig.

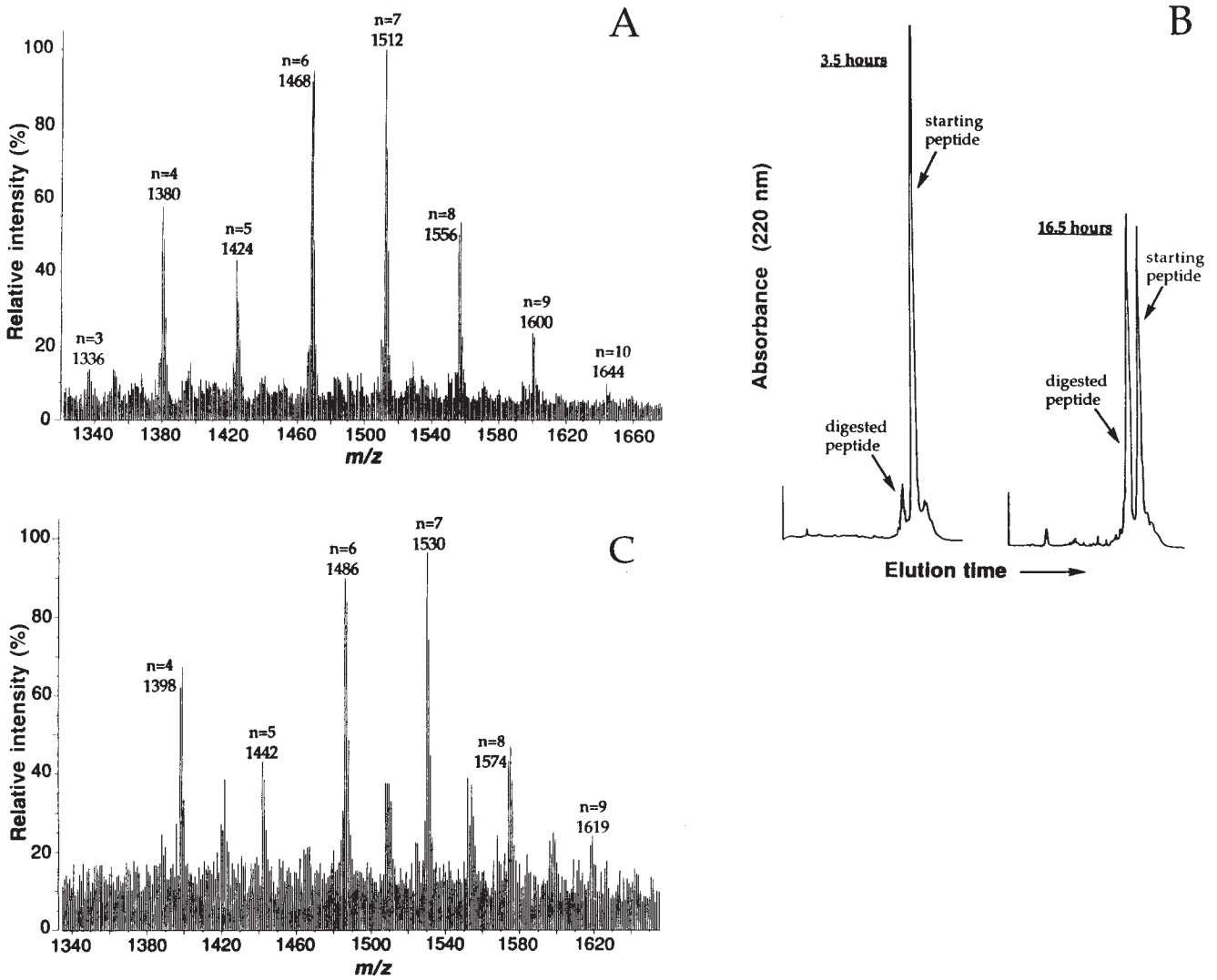

FIG. 2. Characterization of purified Tax 300 peptide. *(A)* FAB mass spectrum for Tax 300 peptide showing $[M + H]^+$ ions. Each peak is identified by a value of *n* corresponding to the number of repeating monomer units in the PEG loop. *(B)* RP-HPLC chromatograms of two samples from proline specific endopeptidase digest of Tax 300 peptide taken at different times. *(C)* FAB mass spectrum for digested peptide showing $[M + H]^+$ ions. The unmarked major peaks correspond to peptide $[M + Na]^+$ ions.

3*A*) (9). All cyclic peptides promoted folding of class I MHC complexes as indicated by the presence of a single peak at the expected elution time (11.5 min). Adjacent peaks are aggregated heavy chain (6.5 min) and free β_2m (13 min). Analysis of purified HLA-A2 complexed with Tax 300 peptide by FAB mass spectrometry (Fig. 3*B*) shows the expected peptide $[M + H]^+$ ions (compare Fig. 2*A* with Fig. 3*B*), confirming the binding of cyclic peptides to HLA-A2. Results also show that the distribution of peaks is identical in both Fig. 2*A* and Fig. 3*B*, suggesting that folding and assembly of HLA-A2 occurs independently of the particular size of the peptide ring structure. The relatively high background in Fig. 3*B* is typical for low concentration FAB mass spectrometry analysis of class I MHC complexes. Similar results were obtained for HLA-A2 complexed with Tax 400 and Tax 600 peptides.

Stability of Class I MHC–Peptide Complexes. Thermal denaturation experiments were done to determine the effect of the peptide ring structure on the stability of HLA-A2 complexes. Denaturation curves were obtained by monitoring the change in CD signal at 218 nm and were fit by a nonlinear least squares analysis to determine the midpoint of the unfolding transition, T_m (melting temperature) (10). Results show that T_ms for HLA-A2 complexed with cyclic peptides are very similar and range between 70.7°C and 72.1°C, suggesting that stabilization of complexes is independent of the particular size of the peptide ring structure. The stability of these complexes is comparable with that of HLA-A2 complexed with either the Tax peptide (T_m = 73.6°C) or the [Lys^4(Ac),Lys^8(Ac)]Tax peptide (T_m = 68.9°C). Since measurement of T_ms by CD has been shown to correlate with peptide binding affinity for class I MHC complexes (10, 13), these results suggest that cyclic peptides and linear Tax peptides adopt similar bound conformations with most likely the same contacts made to MHC residues. The presence of PEG loops thus appear to have no destabilizing effect on the structure of class I MHC complexes. Crystals of HLA-A2 complexed with cyclic peptides have been obtained.

DISCUSSION

X-ray structures of several complexes between class I MHC molecules and antigenic peptides have shown that the bound conformations of peptides share common structural features (for reviews, see refs. 1 and 2): the amino and carboxyl termini form a network of hydrogen bonds with conserved MHC residues located at both ends of the binding site, anchor residues are buried in pockets formed by polymorphic MHC residues, and much of the central portion of the peptide is

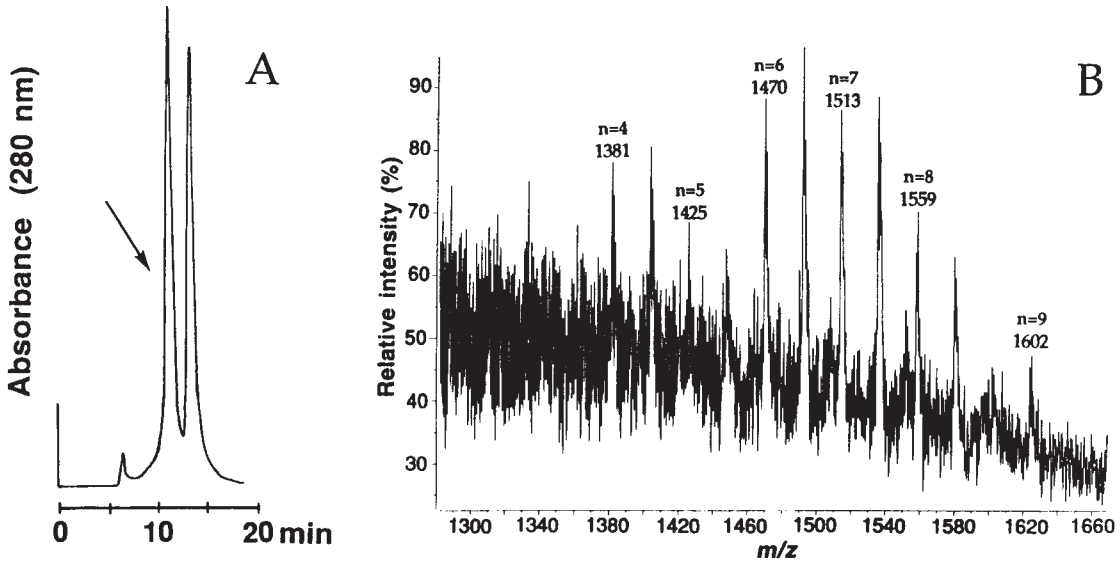

FIG. 3. Assembly and characterization of a HLA-A2 complex. (*A*) Gel filtration chromatogram of HLA-A2 complexed with Tax 300 peptide. Complex eluted as a single peak identified by an arrow. *(B)* FAB mass spectrum for purified HLA-A2 complexed with Tax 300 peptide showing peptide $[M + H]^+$ ions (compare with spectrum shown in Fig. 2*A*). A series of unmarked peaks corresponding to peptide $[M + Na]^+$ ions is also apparent. Similar results were obtained for Tax 400 and Tax 600 peptides.

directly accessible on the surface of the MHC molecule for recognition by a TCR. These structural features suggest that the presence of bulky substituents in the central region of an antigenic peptide (or other MHC ligands) might be compatible with binding of the ligand to the MHC molecule but could prevent engagement of the MHC–ligand complex by any TCR. For the purpose of blocking engagement of TCRs in ternary complexes, cyclic peptides that incorporate a large nonpeptidic ring structure designed to project out of the binding site were synthesized (Fig. 1). PEGs were used as linkers because of their water solubility, conformational flexibility, biocompatibility, and weak immunogenicity. PEGs have been successfully used to modify proteins and peptides conferring resistance to proteolysis, increased *in vivo* half-life, and decreased antigenicity and immunogenicity (14–16), to link small molecules (17, 18), and as anchors on polymer supports for the synthesis of peptides (19, 20). Cyclic peptides are also favored because of their prolonged *in vivo* stability.

The covalent attachment of PEG linkers to the antigenic Tax peptide at positions where peptide side chains point out of the binding site (P4 and P8) results in large loops that are positioned to extend toward the TCRs of an approaching T cell. PEG loops will adopt a conformation or (more likely) many conformations that either extend out of the binding site into solution (Fig. 4*A*) or collapse against the top surface of the MHC molecule covering part of the two α-helices that border the peptide binding groove (Fig. 4*B*) or both. Bacterial protein superantigens that block the top surface of the MHC molecule by covering part of the α-helices are known to interfere with TCR binding (21, 22). In either case, modeling suggests that such large loops will block MHC-restricted T cells that bind to residues on the top surface of the α-helices of an MHC molecule.

Cyclic peptides were shown to bind to class I MHC molecules by their ability to promote the *in vitro* assembly of HLA-A2 (Fig. 3). The thermodynamic stability of the resulting complexes was shown to be comparable with that of HLA-A2 complexed with the linear Tax peptides. These results suggest that the bound conformation of cyclic peptides is likely to be identical to that of typical antigenic peptides bound in the binding site of class I MHC molecules. Although no other cyclic peptide has been shown to associate with class I MHC molecules, the *in vivo* presentation of a disulfide-linked form of insulin by class II MHC molecules has been reported (23).

To our knowledge, only one study has reported the use of linkers to achieve ring closure on a polymer support (24); in that report, the amino terminus of the peptide was linked to the side chain of a lysine residue via succinic acid. The cyclization reaction was achieved by two cycles of deprotection/coupling steps using succinic anhydride as the activated form of the linker. Our strategy for cyclizing peptides on polymer supports is different and involves the simultaneous and selective deprotection of the two moieties engaged in ring closure, followed by coupling of PEG dicarboxylic acid in a single step (Fig. 1). *In vitro* degradation studies show that the amide linkage formed is stable to peptidase cleavage in mouse serum (M.B. and D.C.W., unpublished results). The cyclization reaction could also be extended to include other symmetrical PEG derivatives with complementary natural and nonnatural amino acids; for example, the use of PEG diamines to link carboxyl groups. In addition, homobifunctional PEG derivatives could be used to link other parts of the peptide together and ring structures of varying sizes can be designed owing to the availability of PEGs in a wide range of molecular weights. Heterobifunctional PEG derivatives (17, 25) could also be used. The design of other potential blocking ligands could include the covalent attachment of high molecular weight PEG derivatives (e.g., monomethoxy PEG) to the side chain of one or more residues from the central portion of an antigenic peptide, thus creating long linear PEG chains that extend out of the binding site. Recently, peptide analogs in which several residues from the central portion of antigenic peptides were replaced by nonpeptidic moieties such as 4-aminobutyric acid, 6-aminohexanoic acid, and phenanthridine have been reported (26, 27). These nonnatural ligands were shown to bind to their designated class I MHC molecules without significant changes in binding properties.

As shown in this report, the ability of peptides containing large PEG loops to bind in the peptide binding site of a class I MHC molecule and to form stable, long half-life complexes comparable to antigenic peptides suggests that these PEG-containing peptides could be administered like a vaccine but rather than stimulating the production of MHC-restricted T cells would instead be nonimmunogenic. Such a finding would

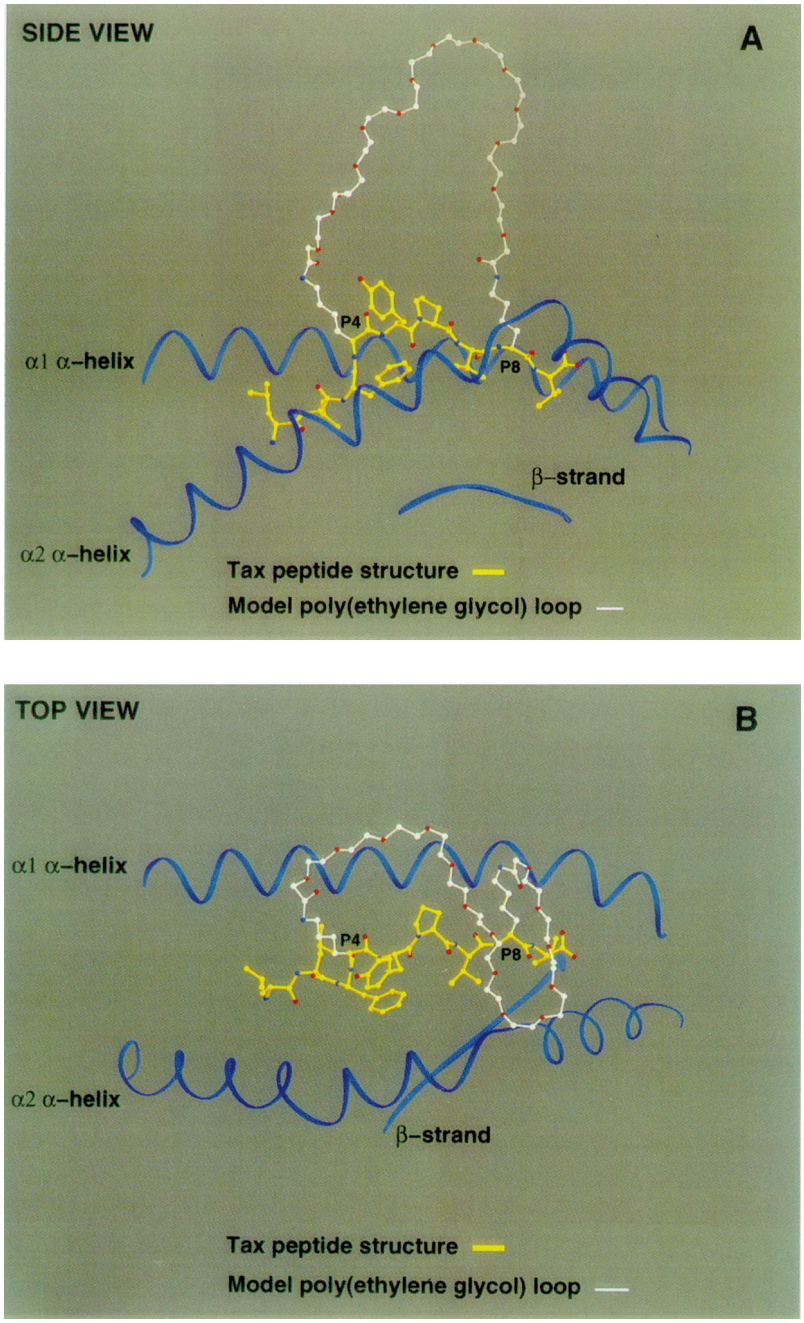

FIG. 4. Model structures of Tax 600 peptide showing PEG loop (n = 13). Structures are modeled on the conformation of HLA-A2 complexed with Tax peptide (11) in which P4 and P8 were substituted by lysine residues. Both α-helices and one β-strand forming the binding site are shown. *(A)* Side view showing extension of the PEG loop out of the binding site. *(B)* Top view showing the PEG loop contacting the top surface of the two α-helices.

indicate that ligands, peptidic or not, that are designed to inhibit the function of specific MHC molecules could be rendered nonimmunogenic by adding bulky groups to extend out of the binding site and interfere with MHC-restricted TCR binding.

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