Total chemical synthesis, characterization, and immunological properties of an MHC class I model using the TASP concept for protein de novo design

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Abstract

The design, total chemical synthesis, and immunological properties of a four- α -helix bundle template-assembled synthetic protein (TASP) mimicking some of the structural features of the major histocompatibility complex (MHC) class I is described. In a first approach, the native sequence 58–74 of the α_1 heavy chain domain of HLA-A2 was modeled in order to increase helix stability and amphiphilicity of the 17-mer peptide, preserving the residues for potential T-cell receptor (TcR) binding properties. According to the TASP concept, these helical segments were covalently attached to a cyclic template molecule designed for the induction of a four-helix-bundle topology of the assembled peptide blocks.

After extensive HPLC purification, stepwise solid-phase synthesis resulted in a TASP molecule of high chemical purity as demonstrated by analytical HPLC, mass spectrometry, and amino acid analysis. CD spectroscopic investigations are consistent with the onset of a partial α -helical conformation in aqueous buffer as well as in TFE. Antibodies raised directly against this four- α -helix bundle TASP molecule (without prior conjugation to a carrier molecule) were detected by ELISA. Flow cytometry studies showed that these antibodies recognize the native MHC class I molecule on the surface of HLA-A2-positive cells. The results indicate that the TASP approach represents a versatile tool for mimicking conformational epitopes.

Keywords: anti-TASP antibodies; flow cytometry analysis; MHC model; protein de novo design; secondary structure formation; solid-phase synthesis; TASP

The design and chemical synthesis of small proteins mimicking the structural and functional properties of natural proteins have become a most challenging goal in recent protein de novo design (Moser et al., 1983; Mutter, 1985;

Regan & De Grado, 1988; Mutter & Vuilleumier, 1989; Richardson & Richardson, 1989; Hecht et al., 1990). In order to overcome some intrinsic problems in creating protein-like molecules arising from our limited understanding of the parameters that determine the folding of a linear polypeptide chain into a predetermined tertiary structure, we have proposed the concept of TASP (Mutter, 1988a,b; Mutter et al., 1988). Over the past few years we have been able to establish this approach by the design, total chemical synthesis, and structural investigation of several four- α -helix bundle TASP molecules (Mutter et al., 1988, 1989, 1992; Mutter & Tuchscherer, 1988; Ernest et al., 1990). Very recently, attempts to incorporate some functional properties, e.g., catalytic activity (Hahn et al., 1990), ligand binding (Sasaki & Kaiser, 1990), or membrane pore formation (Montal et al., 1990), by applying similar strategies have been described. In the field of immunology, the design of peptide-based

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Abbreviations: TASP, template-assembled synthetic protein; MHC, major histocompatibility complex; LDI-MS, laser desorption/ionization mass spectrometry; HLA-A2, human lymphocyte antigen A2; T-LCL, T-lymphoblastoid cell line; MA2.1, monoclonal antibody against the HLA-A2 molecule; FCS, fetal calf serum; HS, human serum; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; TFE, 2,2,2-trifluoroethanol; SPPS, solid-phase peptide synthesis; TcR, T-cell receptor; HPLC, high-performance liquid chromatography; MBHA, methylbenzhydrylamine; EDT, ethanedithiol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEAP, triethyl ammonium phosphate; ELISA, enzyme-linked immunosorbent assay; CD, circular dichroism; BOP, benzotriazol-1-yl-oxybis(dimethylamino)phosphonium hexafluorophosphate; HOBT, 1-hydroxybenzotriazole.

antigens has attracted considerable interest in view of the potential for development of synthetic vaccines (Van Regenmortel, 1987; Milich, 1989; Loleit et al., 1990). The use of a branched-chain architecture (Tam, 1988; Kobs-Conrad et al., 1992) to enhance the antigenicity of small peptide antigens can be considered as a conceptually related approach to our TASP concept. However, the stabilization of the native structure in synthetic models as described by Kaumaya et al. (1990, 1992a,b), as aimed in the TASP approach, seems to represent a prerequisite for obtaining highly specific antibodies able to recognize the native molecule (Laver et al., 1990; Vuilleumier & Mutter, 1992).

In order to test this hypothesis, we have recently focused on the design of TASP molecules of four- α -helix bundle topology, in which antigenic helical segments of protein surface domains are assembled on suitable templates (Mutter & Tuchscherer, 1988; Ernest et al., 1990; Vuilleumier & Mutter, 1992). The MHC class I molecule can be regarded as an interesting candidate of considerable immunological relevance for modeling according to the TASP concept. Most notably, the three-dimensional structure of human class I MHC (Bjorkman et al., 1987a; Garrett et al., 1989; Madden et al., 1991) reveals a characteristic folding topology that can serve as a structural motif in protein de novo design. For example, the extracellular portion of the heavy chain is composed of three domains α_1 , α_2 , and α_3 , each composed of about 90 amino acid residues; the α_1 and α_2 domains form a deep groove of about 25 Å long and 10 Å wide, considered to be the binding site for antigenic peptides (Bjorkman et al., 1985).

The construction of TASP molecules exhibiting some of the characteristic structural features could contribute to elucidation of the functional relevance of the individual building blocks of MHC and serve as a synthetic antigen for obtaining MHC-specific antibodies. As the first example in these ongoing studies, we describe the design, chemical synthesis, and properties of a four- α -helix bundle TASP molecule, in which a segment of the α_1 -helix is attached to a cyclic template molecule (MHC-TASP) (Fig. 1). We will show that antibodies can be raised against the TASP molecule and that these anti-TASP antibodies specifically recognize the native MHC class I molecule on the surface of HLA-A2-positive cells.

Results

Design of the MHC-TASP molecule

Previous CD and NMR studies showed that template-assembled four- α -helix bundle molecules indeed adopt the proposed folding pattern in aqueous solutions (Mutter, 1988a,b; Mutter & Tuchscherer, 1988; Ernest et al., 1990; Mutter et al., 1992). In contrast to the native linear MHC class I polypeptide, the TASP molecule is char-

Fig. 1. Schematic representation of the modeled four- α -helical bundle TASP molecule (Mutter et al., 1992) designated as $T_4-(4\alpha_{17})$ (MHC-TASP). T_4 , cyclic template containing four attachment sites (ϵ -NH₂ groups of the lysine [K] side chains); Ac- α_{17} , 17-mer peptide (see Fig. 2). The helical peptides Ac- α_{17} are covalently linked to the template via amide bond formation as described in Figure 3

acterized by a branched-chain architecture, in which the template is designed to enhance intramolecular folding of the covalently attached amphiphilic helices. As previously reported, a cyclic decapeptide of the sequence

can be used to induce a four- α -helix bundle formation of the attached peptide blocks. Molecular dynamics calculations suggest that this molecule adopts an antiparallel β -sheet connected by a β -turn type II (P-G) and cyclized via an S-S bond (Flögel & Mutter, 1992), as schematically depicted in Figure 1. In this low-energy conformation, the four attachment sites of the helices (ϵ -amino groups of K) are oriented on the same side of the template plane, enforcing helix bundle formation. Starting from the native sequence of the α_1 heavy chain domain 58–84 of HLA-A2 (Bjorkman et al., 1987a), we decided to split this helical sequence in two shorter, overlapping fragments (designated as α_{17} and α_{17}' , respectively), each comprising 17 amino acid residues (Fig. 2).

This strategy was chosen for two reasons: First, the critical chain length for helix formation in TASP molecules has previously been determined to be in the range of 13–15 residues. Consequently, the chain length of 17

Fig. 2. Helical wheel presentation (Schiffer & Edmundson, 1967) of the overlapping 17-mer peptides α_{17}' (C-terminal sequence, outer ring) and α_{17} (N-terminal sequence, inner ring) of HLA-A2 α_1 (58–85) (overlapping region in the native sequence: residues 69–74). Both peptides α_{17} and α_{17}' have an identical hydrophobic core (pointed area of the helical wheel). Bold letters in the native sequence 58–85 represent residues that are exchanged in the 17-mer peptides in order to increase the helical stability (see text).

residues is well beyond this lower limit. Thus, the conservation of the native conformation of this partial sequence of MHC α_1 should be guaranteed. Moreover, the length of the resulting helix corresponds well with the length of the putative MHC class I peptide binding site (25 Å). Second, due to synthetic limitations, the structural reliability of the target molecule dramatically decreases in stepwise solid-phase synthesis beyond this size of the molecule (Mutter et al., 1992). The redesign of the helical peptides was performed under the following considerations: (1) Residues that were not thought to be involved in the interaction of the MHC class I molecule with the TcR (Bjorkman et al., 1987b) were replaced by helix-forming residues (Ala, Leu). (2) In order to enhance hydrophobic interactions in the postulated four- α -helical bundle, Leu residues were inserted in the hydrophobic side of the helical wheel (Schiffer & Edmundson, 1967) thus increasing the amphiphilic character and the propensity for packing into a hydrophobic core of the helical sequences as described recently in a strategy to design a topographical determinant to the protein antigen LDH-C4 (Kaumaya et al., 1990). (3) Amino acids that may raise problems in the stepwise solid-phase synthesis, such as Trp, His, and Arg, are replaced by Leu or Ala residues as far as these substitutions are consistent with (1) and (2).

The resulting sequences of the two overlapping 17-peptides (N-terminal sequence, α_{17} and C-terminal sequence, α_{17}') are depicted in Figure 2. The resulting helical wheel presentation (Fig. 2) indicates that the modeled helices

show a clear segregation into a hydrophilic and a hydrophobic part; the present design results in a remarkable increase in hydrophobicity, allowing the formation of a hydrophobic core as a major driving force for the onset of a four- α -helix bundle conformation. Due to the fact that both peptides α_{17} and α_{17}' have an identical hydrophobic part (pointed area in the helical wheel presentation in Fig. 2), the resulting TASP molecules T_4 -(4 α_{17}) and T_4 -(4 α_{17}') should exhibit similar thermodynamic stabilities.

Preliminary studies showed that the TASP molecule containing the C-terminal sequence (T_4 -(4 α_{17}')) exhibits very unfavorable solubility properties, causing difficulties in the purification and structural characterization of the molecule; in contrast, TASP T_4 -(4 α_{17}) was readily soluble in polar solvents including water (see below). Consequently, we focus in this present communication on the investigation of the TASP molecule containing the N-terminal helical segment.

Peptide synthesis and characterization

The strategy for the synthesis of the TASP molecule T_4 -(4 α_{17}) follows the principles of the Boc/Fmoc-based protection techniques of solid-phase peptide synthesis (Barany & Merrifield, 1980) as described in detail in Materials and methods (Fig. 3). Subsequent to the completion of the linear template molecule on an MBHA resin and removal of the N ϵ -protecting groups for the lysine side chains, the α -helical segments α_{17} were built up in a

Fig. 3. Schematic representation of the solid-phase synthesis of the MHC-TASP T₄-(4α₁₇) (see text).

parallel fashion as depicted in Figure 3. The complete peptides were simultaneously deprotected and cleaved from the resin by treatment with liquid HF. The crude material was directly cyclized with K₃(Fe(CN)₆) under conditions of infinite solution. Figure 3 shows a schematic representation of the individual synthesis steps leading to the TASP molecule T₄-(4α₁₇).

After cyclization of the crude T₄-(4α₁₇) and subsequent concentration on a cation-exchange column, an analytical reversed-phase HPLC shows a major peak that was isolated by preparative HPLC. In the final purification step a 0.1% TFA buffer/CH₃CN solvent was used on the same column. A conservative estimate based on amino acid analysis, analytical HPLC, and mass spectrometry (Fig. 4) indicates a purity for the target molecule T₄-(4α₁₇) of >90%.

Fig. 4. LDI mass spectrum of T₄-(4α₁₇) after purification by reversed-phase HPLC (expected molecular weight, 8,306 Da). Accelerating voltage, 10 kV; CsI ion gun voltage, 25 kV; postaccelerating voltage, 20 kV.

Conformational studies

As depicted by the CD spectra in Figure 5, the TASP T₄-(4α₁₇) exhibits a pronounced tendency for α-helix formation in different solvents. The CD spectra in the helix-promoting solvent TFE (mimicking a more hydrophobic environment) is characterized by two negative cotton effects at 207 nm (π-π* transition) and at 222 nm (n-π*), typical for the onset of a helical structure (Fig. 5).

As expected, the degree of helicity decreases by addition of an aqueous buffer solution (50 mM phosphate). For example, the θ₂₂₂-value in an aqueous buffer solution containing 20% TFE (Fig. 5) is consistent with a helical degree of about 50%, whereas the CD spectra in pure aqueous phosphate buffer solution (Fig. 5) points to a helical degree of only about 20%.

Fig. 5. CD spectra of T₄-(4α₁₇) in 50 mM phosphate buffer, pH 7.0 (—▲—); TFE/50 mM phosphate buffer (20/80), pH 7.0 (—△—); and TFE (—■—). Concentrations of peptide were 1 mg/mL.

Similar to previous findings, the CD spectra of the free peptide (not shown here) exhibits no significant (<5%) helical content under identical conditions. As a major general feature of the TASP approach, the covalent attachment of potentially α -helix-forming peptide blocks to a topological template molecule results in a substantial increase in the helical structure compared to the free peptide. This has also been shown for a number of partial sequences of proteins as well as model peptides of comparable structure (Mutter, 1988a; Mutter et al., 1988, 1989; Mutter & Tuchscherer, 1988).

From these data, it can be concluded that the MHC-TASP molecule does not adopt a stable helical bundle conformation in purely aqueous solutions; however, in a more hydrophobic medium or in interaction with a receptor molecule, the helical conformation can easily be induced as indicated by the CD data obtained in TFE-containing solutions.

Antibody responses of mice to the MHC-TASP

The presence of specific antibodies in the sera obtained from mice immunized with the MHC-TASP was detected by ELISA.

Figure 6 shows that antisera obtained from both Balb/c (H-2^d) (Fig. 6A) and C57BL(H-2^b) (Fig. 6B) mice immunized with the MHC-TASP contained specific antibodies able to recognize the MHC-TASP molecule. The graphics show that specific antibodies in both mouse strains recognize the synthetic MHC-TASP molecule in a concentration-dependent fashion (—●—: Balb/c in Fig. 6A; C57BL in Fig. 6B). Yet, the antibodies recognize the cyclic template molecule relatively weakly (—■—: Balb/c in Fig. 6A; C57BL in Fig. 6B). This implies that the antibodies raised against the four-helix bundle arrangement are predominantly directed against the four α -helices, which constitute about 87% of the entire MHC-TASP molecule.

As a control to exclude unspecific antibody response to the MHC-TASP and/or the cyclic template molecule we tested the preimmune sera from the same mice and showed that they did not recognize the MHC-TASP (—○—: Balb/c in Fig. 6A; C57BL in Fig. 6B) or the template (—□—: Balb/c in Fig. 6A; C57BL in Fig. 6B). The titer of the anti-MHC-TASP antibodies increased substantially after the second boost as shown in Figure 7.

Contrary to most immunization protocols, where peptides conjugated to large carrier proteins, e.g., KLH or BSA (Mitchison, 1971; Arnon, 1984; Walker et al., 1985), are used as immunogens, we succeeded in raising specific antibodies to the TASP molecule without prior conjugation to a carrier (Figs. 6, 7). It is likely that the cyclic decapeptide used as the template or the helical segment modeled from the α_1 domain of the A2 molecule is a T-helper epitope itself.

Fig. 6. Detection of mouse anti-MHC-TASP antibodies by ELISA. **A:** Sera from Balb/c mice immunized with the MHC-TASP were incubated on plates coated with the MHC-TASP (—●—) or with the template (—■—). Preimmune sera were also incubated on plates coated with the MHC-TASP (—○—) or with the template (—□—). **B:** Sera from C57BL mice immunized with the MHC-TASP were incubated on plates coated with the MHC-TASP (—●—) or with the template (—■—). Preimmune sera were also incubated on plates coated with the MHC-TASP (—○—) or with the template (—□—).

Fig. 7. Detection of anti-MHC-TASP antibodies in sera of Balb/c mice obtained after different immunizations with MHC-TASP. First immunization, day 0 (—○—); second immunization, 4 weeks after priming (—●—); third immunization, 8 weeks after priming (—■—).

Flow cytometry analysis of A2-positive cells using anti-MHC-TASP antibodies

We examined the binding specificity of the anti-MHC-TASP antibodies by immunofluorescence on the T2 T-LCL, which expresses the HLA-A2 molecule. As can be seen on Figure 8, the anti-A2-TASP antibodies but not the preimmune sera, recognized a molecule on the surface of T2, an A2-expressing cell line, but not on KT2, which does not express this molecule. The A9 peptide 58–68 of the influenza matrix protein (where the Phe 62 is substituted by a Lys) was used as a means to enhance A2 expression on the surface of T2 cells (Shimojo et al., 1989; Townsend et al., 1989). The increased expression of A2 molecule on the surface of T2 cells due to exposure to the influenza matrix protein peptide A9 is detected by the anti-A2 monoclonal antibody as well as by the anti-MHC-TASP antibodies as shown in Table 1. The MA2.1 monoclonal antibody recognizes a conformational epitope on

Table 1. Fluorescence staining of T2 cells after induction of the HLA-A2 molecule with the A9 peptide^a

Antibody	Mean fluorescence
FITC	0.55
MA2.1	97.21
Immune serum ^b	81.59
Preimmune serum ^b	35.03

^a The A9 peptide was used at a concentration of 100 μ M as described in Materials and methods.

^b The polyclonal sera were used at 1/100 dilution in RPMI/3% FCS as described in Materials and methods.

folded A2 molecules associated with β_2 -microglobulin (McMichael et al., 1980) and recognizes a polymorphic determinant shared by HLA-A2 and B17 molecules (Ways et al., 1986). The fact that the anti-A2-TASP antibodies stain the T2 cells to the same extent as MA2.1 indicates that the antibodies obtained recognize the native molecule on the surface of A2-positive cells.

Discussion

In this report we have demonstrated that TASP molecules are suitable for mimicking some of the structural and immunological features of a native class I MHC molecule by the assembly of a modeled partial sequence of the α_1 heavy chain domain of HLA-A2 on a cyclic template molecule.

According to the general strategy of TASP design, the modeled MHC- α_1 helix was covalently attached by the C-terminal amino acid to the ϵ -NH₂ group of the template lysines via amide bond formation, resulting in a four-helix bundle architecture as depicted in Figure 1. The total synthesis of the four- α -helix bundle TASP molecule applying solid-phase methods and orthogonal protection techniques resulted after extensive HPLC purification in the desired target molecule of high chemical purity as proved by analytical HPLC, amino acid analysis, and mass spectrometry. As shown previously (Mutter & Tuchscherer, 1988; Mutter et al., 1992) the use of tailor-made template molecules reinforces the onset of secondary and tertiary structures of the assembled amphiphilic peptide blocks as shown by the CD data; consequently, in addition to increasing the molecular weight (and hence the antigenicity) of the peptide antigen as realized by the attachment of peptide chains to multifunctional carrier molecules (Tam, 1988; Kobs-Conrad et al., 1992), the TASP approach allows for the stabilization of the native conformation in the antigenic peptide fragment. Most importantly, the stabilization of the native conformation in the fully synthetic TASP molecule gives access to the presentation of conformational epitopes and results in antibodies of high specificity. Interestingly, by

Fig. 8. Flow cytometry analysis of anti-MHC-TASP antibodies on (A) T2 (HLA-A2-positive) cells and (B) KT2 (HLA-A2-negative) cells.

applying multivalent peptide carriers designed to adopt a β -sheet conformation, Kobs-Conrad et al. (1992) were able to raise antibodies against these synthetic multi-epitope-containing molecules, and their data indicate that these anti-peptide antibodies specifically recognize the native molecule.

Contrary to immunizing with peptides conjugated to protein carriers, the approach of using TASP molecules as immunogens provides several advantages: First, the template-assembled peptide remains in its original form; modifications of amino or carboxylic side chains, which can occur during conjugation, may alter the antigenic epitope. Second, chemical conjugation of peptides to carrier proteins has been shown to influence protein structure and thus immunogenicity (Van Regenmortel, 1987).

As shown in this article, the resulting TASP molecule proved to be highly immunogenic in two different mouse strains (Balb/c, H-2^d; and C57BL, H-2^b). Most notably, we succeeded in raising antibodies against the MHC-TASP molecule without prior conjugation to a carrier molecule. The antibodies to the MHC-TASP molecule were detected by ELISA. Both Balb/c and C57BL mice produced peptide-specific antibodies with high antibody titers obtained 3 weeks after the primary immunization and an increase in antibody response after boosting, indicating a memory response. It is likely that the template or the helical segment modeled from the α_1 domain of the A2 molecule is a T-helper epitope itself.

The anti-TASP antibodies proved to be specific for the native HLA-A2 molecule as indicated by the immunofluorescence experiments on the A2-positive T-lymphoblastoid cell line. The detection of the increased expression of the HLA-A2 molecule by the anti-TASP antibodies due to exposure to the influenza matrix protein peptide A9 indicates further the specificity of the anti-MHC-TASP antibodies for the native HLA-A2 molecule on the cell surface.

The results presented here suggest that TASP molecules with a predetermined, stabilized tertiary structure can play an important role in studies of protein structure and antigenicity; they represent an encouraging starting point for the further design of MHC-like molecules according to the strategies delineated in these studies.

The scope of the present work was to demonstrate that TASP molecules can serve as a promising tool in immunological research. Though the present data do not yet prove the ultimate superiority of TASP molecules compared to alternative approaches in vaccine design, the stabilization of potentially secondary structure-forming oligopeptides (and thus, e.g., of conformational epitopes) on topological template molecules can be regarded as a well-established general feature of TASP molecules. Further studies are needed to establish a peptide model for MHC interaction with antigenic peptides based on this preliminary approach.

The design and chemical synthesis of TASP molecules

containing segments of both helical chains (α_1 and α_2) of the HLA-A2 molecule are in progress in our laboratory.

Materials and methods

Synthesis of TASP T₄-(4 α_{17}) (MHC-TASP)

Apparatus

Analytical reversed-phase HPLC was performed on a Hitachi 655A-11 liquid chromatograph equipped with a Hitachi 655A variable wavelength UV detector and a Hitachi 655A-61 data processor chart recorder. Chromatograms were obtained at flow rates of 2 mL/min with UV detection at 210 nm using reversed-phase stainless steel analytical columns (0.46 $\times$ 25 cm) packed with Vydac C18 derivatized silica, 5-mm particle size, 300-Å pore size, purchased from The Sep/a/ra/tions Group (Hesperia, California).

Preparative reversed-phase HPLC was carried out on a PreP LC 3000 system from Waters in combination with a Fisher Recordall series 5000 chart recorder and a Shimadzu SPD-6A UV monitor. The reversed-phase preparative columns were dry packed in our laboratory (Salk Institute) with Vydac C18 derivatized silica (Sep/a/ra/tions Group), 15–20-mm particle size. Empty polyethylene cartridges (30 $\times$ 5 cm) and frits were obtained from Waters. Preparative separations were accomplished at flow rates of 100 mL/min with UV detection at 280 nm.

Amino acid analyses were performed on a Perkin-Elmer HPLC after hydrolysis in 4 N methanesulfonic acid at 110 °C for 24 h using orthophthalaldehyde postcolumn derivatization and fluorescence detection.

CD spectra were recorded on a JASCO J-600. The concentration of the peptide in the different buffer systems was 1 mg/mL.

Peptide synthesis

All amino acids – Boc-Cys(S-p-OMeBzl), Boc-Lys(Fmoc), Boc-Gly, Boc-Pro, Boc-Leu, Boc-Lys(2ClZ), Boc-Ala, Boc-Glu(cHxl), Boc-Asp(Bzl), Boc-Thr, Boc-Arg(Mtr), Boc-Gln, Boc-Tyr(oBzl), and Boc-Val – were purchased from Bachem (Torrance, California) and were used directly without further purification. Solvents were obtained from Aldrich. The synthesis was carried out manually using an MBHA 1% cross-linked polystyrene resin (2.0 g) that was obtained according to published procedures (using *p*-toluoyl chloride instead of benzoyl chloride) with a substitution of 0.45 mmol NH₂/g (Mutter et al., 1992). The synthesis protocol followed established SPPS methodology (Barany & Merrifield, 1980) for Boc protection except for the Fmoc-protected ϵ -amino groups of the four template lysine side chains (Mutter et al., 1992). The linear protected precursor of the cyclic decapeptide template was assembled in a stepwise manner according to the following general procedure: the Boc

protecting group was removed with 80% TFA and 1% EDT in CH_2Cl_2 (25 min); washes with 1% EDT in 2-propanol, MeOH, and CH_2Cl_2 ; neutralization with 10% $\text{Et}_3\text{N}/\text{CH}_2\text{Cl}_2$, coupling with a twofold excess of activated amino acid derivative using either diisopropylcarbodiimide (DIC) (with or without HOBt as additive) or BOP as activating agents. Depending on the solubility of the Boc amino acid, couplings were performed in CH_2Cl_2 , $\text{CH}_2\text{Cl}_2/\text{DMF}$ or NMP/DMF mixtures. Each coupling step was monitored by the qualitative ninhydrin test, and recouplings were performed if necessary. After completion of the template molecule using Boc-Lys(Fmoc), the Fmoc groups were removed (20% piperidine/ CH_2Cl_2 ; 5 min and 10 min), and the four identical helical building blocks were synthesized in parallel following the strategy for Boc protection. N-terminal acetylations were performed with 10% Ac_2O in 1% pyridine/ CH_2Cl_2 .

Cleavage of the peptide from the resin with anhydrous HF in the presence of anisole (5%) and ethyl methyl sulfide (5%) was carried out using an in-house (Salk Institute)-designed Kel-F line (1.5 g peptide/resin, 0 °C, 75 min). After evaporation of HF, the peptide was precipitated with anhydrous ether, collected by filtration, and redissolved in 60 mL water/acetic acid (80:20, v/v).

For the cyclization process, the obtained crude product was diluted with 3 L of water and then added dropwise to a vigorously stirred solution of $\text{K}_3(\text{Fe}(\text{CN})_6)$ (200 mg) and NH_4OAc (10 g) in 1 L water (total addition time about 4 h). The pH was kept between 6.8 and 6.9 by simultaneous addition of 10% aqueous NH_4OH . The solution was stirred at 4 °C overnight. The pH was adjusted to 5.0 by addition of acetic acid, and a slurry of the anion-exchange resin (10 mL; Bio-Rad anion-exchange resin AG3-X4A [analytical grade, 100–200 mesh], chloride form) was added. The mixture was stirred for 10 min, filtered, and the filtrate applied to an ion-exchange column, filled with AG3-X4A (15 mL gel bed volume). The eluate was subsequently loaded on a cation-exchange column filled with BioRex 70 resin (analytical grade, 100–200 mesh, H^+ form, 30 mL gel bed volume). The peptide that was retained on the column as shown by the absence of any absorbing material by HPLC analysis of the eluate was eluted with 50% acetic acid in water (total volume about 100 mL). After lyophilization the crude T_4 -($4\alpha_{17}$)Ac was purified by preparative HPLC using three different systems to gain maximum purity: (1) buffer A = 0.1% TEAP, pH 2.25; buffer B = 80% acetonitrile/20% A (50% B to 90% B in 50 min); (2) buffer A = 0.1% TEAP, pH 6.25; buffer B = 80% acetonitrile/20% A (50% B to 90% B in 50 min); (3) buffer A = 0.1% TFA; buffer B = 0.1% TFA in 80% acetonitrile/20% H_2O (50% B to 90% B in 20 min).

The amino acid analysis gave the expected amino acid ratios, and mass spectrometric analysis confirmed the calculated molecular weight (Fig. 4) for the T_4 -($4\alpha_{17}$) (MHC-TASP).

The A9 peptide was synthesized by standard solid-phase methods using Fmoc-protected amino acids. The crude peptide was first purified on a Sephadex G25 column and subsequently by reversed-phase HPLC on a Vydac C18 column. Amino acid analysis gave the expected amino acid ratios.

Media and reagents

The medium used was HEPES-buffered (10 mM HEPES) RPMI-1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 50 $\mu\text{g}/\text{mL}$ kanamycin (all obtained from Gibco Laboratories, Paisley, Scotland). The culture medium was supplemented with either 10% fetal calf serum (Gibco) or 5% AB-human serum (HS; Swiss Red Cross).

For T cell growth, RPMI-HS was supplemented with 100 U/mL human rIL-2 (Roche, Nutley, New Jersey). Cultures were incubated at 37 °C, 5% CO_2 in air. The cells were washed in HEPES-buffered (10 mM HEPES) RPMI.

The MA2.1, an HLA-A2-specific monoclonal antibody (McMichael et al., 1980; Ways et al., 1986), was obtained as supernatant from the B-cell hybridoma line HB54 obtained from the American Type Culture Collection.

The polyclonal anti-HLA-ABC antibody was obtained as supernatant from the B-cell hybridoma line B9.12.1 obtained from the American Type Culture Collection.

Cell lines

The isolation and characterization of the T2 T-lymphoblastoid cell line (Alexander et al., 1989; Hosken & Bevan, 1990) and the KT2 T-cell clone is described elsewhere (Demotz et al., 1989).

Mice and immunization

Female Balb/c or C57BL mice, 7–10 weeks old, purchased from Iffa Credo (St. Germain-sur-l'Arbresle, France) were immunized subcutaneously at the base of the tail (Corradin et al., 1977) with a 100- μL volume containing 50 μg of MHC-TASP emulsified in complete Freund's adjuvant (CFA) 1:1 (v/v). Mice were boosted twice at the base of the tail with 50 μg of MHC-TASP in incomplete Freund's adjuvant 3–4 weeks after priming. Three days after, mice were bled and the serum was tested by ELISA for the presence of specific antibodies.

Detection of antibodies by ELISA

Antibodies to the MHC-TASP used as immunogen were detected by ELISA using the MHC-TASP or the cyclic template itself as solid phase. Briefly, 50 $\mu\text{g}/\text{mL}$ of MHC-TASP or template in 0.1 M borate buffer (pH 9.6) was coated overnight at 4 °C on the wells of a flat-bot-

tom 96-well microtiter plate (50 μ L/well). Unreactive sites were blocked with 5% BSA/PBS for 2 h at room temperature. Dilutions of antisera or preimmune sera were made in 1% BSA/PBS. Duplicate 50- μ L aliquots of the sera dilutions were added to the wells of the plate and allowed to incubate with the immobilized peptide for 2 h at room temperature. The wells were washed five times with wash buffer (PBS/0.05% Tween-20 (w/v); Sigma). Fifty microliters of goat-anti-mouse IgG conjugated to alkaline phosphatase (Sigma) diluted 1:1,000 in 1% BSA/PBS was added to each well, and the plates were incubated for 1 h at room temperature. The wells were subsequently washed five times with wash buffer, and alkaline phosphatase activity was detected by adding 50 μ L of substrate solution (*n*-nitrophenyl phosphate, disodium, Sigma; 1 mg/mL in 50 mM NaHCO₃, pH 9.5) to individual wells and incubating for 1 h at room temperature. Optical density was read by an ELISA reader (Dynatech).

Flow cytometric analysis

For flow cytometric analysis 2×10^5 T2 cells were incubated in medium alone (complete RPMI) or in the presence of the peptide A9 (peptide 55–73 of the influenza matrix protein) at a concentration of 100 μ M for 6 h at 37 °C. The T2 cells were then harvested, washed once with RPMI/3% FCS, and the pellet was resuspended in 100 μ L of MA2.1 supernatant, 100 μ L of the anti-MHC-TASP polyclonal sera, or preimmune sera diluted 1:100 in RPMI/3% FCS or with medium alone as negative control at 4 °C for 30 min. In the case of the KT2 T-cell clone, 2×10^5 cells were washed once and the pellet was resuspended in 100 μ L of B9.12.1 supernatant (anti-HLA-ABC) in addition to the antibodies used for surface staining of the T2 cells. After washing with RPMI/3% FCS, the cells were incubated for a further 30 min at 4 °C with 100 μ L of 1:20 dilution of fluoresceinated goat-anti-mouse antibody (Sigma). After an additional washing, the cells were fixed with 300 μ L 3% paraformaldehyde (Sigma) in phosphate-buffered saline and then analyzed using a Becton and Dickinson flow cytometer.

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