transfected cells were labeled with [^{32}P]orthophosphate (1 mCi/ml; ICN) in phosphate-free media for 3 to 4 hours before shake-off. Cells were lysed with RIPA buffer [150 mM NaCl, 50 mM tris-HCl (pH 7.5), 0.5% deoxycholate, 1.0% NP-40, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride, leupeptin, aproptinin, and pepstatin (each 1 μg/ml), and phosphatase inhibitors] and clarified by centrifugation at 15,000*g* for 10 min at 4°C. mAb 12CA5 to HA (Babco) was added to the supernatant (1:500) and incubated for 2 hours at 4°C. Antibody to mouse immunoglobulin G (IgG) coupled to agarose (Sigma) was then added to precipitate the immune complex. After the pellet had been washed, samples were boiled in SDS–sample buffer, separated by SDS-PAGE, transferred to Immobilon-P (Millipore), and probed with a rabbit polyclonal antibody (1:500 dilution) to CENP-E^{COOH368}. Bound primary antibodies were detected with ^{125}I-labeled protein A (ICN). For phosphatase treatment, precipitated beads were washed twice with RIPA and twice with alkaline phosphatase buffer and incubated with 50 U of calf intestinal phosphatase (Gibco) at 37°C for 30 min. For immunofluorescence staining, transfected cells grown on glass cover slips were fixed directly in −20°C methanol, rehydrated in PBS containing 0.1% bovine serum albumin, and incubated with either monoclonal antibody 12CA5 or antibody to CENP-E (pAb1) (*2*) followed by fluoroscein isothiocyanate–conjugated antibody to mouse or rabbit IgG (Gibco). Staining was visualized with a 100X PlanNeofluor objective using a Zeiss Axiophot microscope.

15. Phosphoamino acid analysis was done by hydrolyzing gel-purified ^{32}P-labeled protein in 6 N HCl at 110°C and separating the hydrolysate in the presence of unlabeled phosphoamino acid standards by 2D thin-layer chromatography. After electrophoresis at pH 3.5, ascending chromatography in 62.5% (v/v) isobutyric acid was used to separate the phosphoamino acids. Standards were visualized by spraying the plates with ninhydrin and then exposing the plate to film. Phosphopeptide maps were generated by digestion of ^{32}P-labeled protein with 20 μg of trypsin in 50 mM ammonium bicarbonate (pH 8.05) at 37°C overnight. Separation was carried out by electrophoresis (at pH 3.5) at 1 kV for 1.5 hours. Ascending chromatography was carried out in solvent consisting of acetic acid, pyridine, butanol, and water (1:4.9:3.2:4). Labeled peptides were detected by autoradiography and quantitated with a Fujix Bio-Imaging Analyzer (Fuji Photo).
16. MT-binding assays were done by addition of 20 μg of purified tubulin and 20 μM taxol (final concentration) to S100 extracts prepared by hypotonic lysis [10 mM tris-HCl (pH 7.0), 2 mM $MgCl_2$, and 10 mM NaCl, including protease inhibitors] of transfected cells. After incubation at 37°C for 30 min, the reaction was centrifuged and the supernatant and pellet fractions were examined for the presence of the HA-tagged CENP-E^{COOH368} by immunoprecipitation and immunoblotting as described above.
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21. We are indebted to L. Cohen and J. Hittle for providing clam extracts and for advice on kinase reactions. We also thank D. Murphy and E. Holzbaur for advice on purification of brain tubulin, L. Cassemiris for providing tubulin monoclonal antibodies and for advice on making subtilisin microtubules, and B. T. Schaar for helpful discussions and suggestions. T.J.Y. is a Lucille P. Markey Scholar. Supported in part by NIH grant GM-44762-02, USPHS core grant CA-06927, the Lucille P. Markey Charitable Trust, the American Cancer Society CB-1, and an appropriation from the Commonwealth of Pennsylvania.

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Importance of Peptide Amino and Carboxyl Termini to the Stability of MHC Class I Molecules

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An influenza virus matrix peptide in which either the charged amino or carboxyl terminus was substituted by methyl groups promoted folding of the class I human histocompatibility antigen (HLA-A2). A peptide modified at both termini did not promote stable folding. The thermal stability of HLA-A2 complexed with peptides that did not have either terminus was ~22°C lower than that of the control peptide, whereas matrix peptide in which both anchor positions were substituted by alanines had its stability decreased by only 5.5°C. Thus, the conserved major histocompatibility complex class I residues at both ends of the peptide binding site form energetically important sites for binding the termini of short peptides.

The three-dimensional structure determination of the human major histocompatibility complex (MHC) class I molecule HLA-A2 shows that both ends of the peptide binding site are composed of polar residues conserved among all human and murine class I sequences (*1–3*). The conserved MHC residues form hydrogen bonds with polar main chain atoms of the peptide NH_2- and COOH-termini (Fig. 1) (*2, 3*). These hydrogen bonds were revealed in x-ray structures of class I molecules complexed with single viral peptide 8-mers (*4, 5*), 9-mers (*5–8*), and 10-mers (*6*) and with a mixture of endogenous peptides of various lengths (*9*).

The network of hydrogen bonds between conserved MHC class I residues and peptide NH_2- and COOH-termini appears to provide a peptide sequence–independent mode of binding for short peptides (*8, 10*). Interactions between polymorphic MHC residues in pockets along the binding site and a few peptide anchor residues provide a peptide sequence–dependent mode of binding (*3–9, 11, 12*). The two or three peptide anchor residues define class I allele–specific sequence motifs (*13–16*). Estimates of binding energies contributed by peptide sequence–specific interactions have been made for several alleles by structural comparisons of three allele-specific pockets (*12*) and by residue substitutions at anchor and non-anchor positions (*13, 14, 17*). However, similar estimates that compare the relative energetic contributions of the peptide termini and of the side chains at anchor positions have not been made. Here, we assess the importance of the NH_2- and COOH-termini of an influenza virus matrix peptide by substituting the charged groups by CH_3 groups and compare the effect of these modifications on the thermal stability of HLA-A2 complexes with those of substituting anchor positions 2 and 9 by alanines.

Because a substitution in the influenza virus matrix peptide (WT), GILGFVFTL (*18–20*), of Leu by Ala at position 9 (L9A) was found to have a negligible effect on the thermal stability of the complex (Table 1), the desired modifications at the peptide NH_2- and COOH-termini were made to L9A, rather than to WT peptide, for synthetic reasons (*21*). At the NH_2-terminus of L9A, the NH_2 group of Gly (Fig. 1, large blue atom) was substituted by a CH_3 group, G1-Ndel (Table 1), which was predicted to eliminate two hydrogen bonds (Fig. 1) to the conserved MHC residues Tyr^7 and Tyr^{171}. At the COOH-terminus of L9A, the COOH group of Ala (Fig. 1, large red atoms) was substituted by a CH_3 group, A9-Cdel (Table 1), which was expected to eliminate four hydrogen bonds (Fig. 1) to MHC residues. Peptide modifications done at either terminus retained the hydrogen and methyl side chains of Gly and Ala, respectively (Table 1). Peptide G1-Ndel+A9-Cdel combined both terminal modifications (Table 1). The optimal residues Leu and Val (*15*), found at anchor positions 2 and 9, respectively, in the HLA-A2–restricted peptides, were substituted in the WT peptide individually (I2L and L9V) and in combination (I2L+L9V) (Table 1). Alanine substitutions at both anchor positions were also made (I2A+L9A) (Table 1).

HLA-A2 complexes were reconstituted from human heavy chain and β_2-microglobulin (β_2M) expressed in *Escherichia coli* in the presence of excess peptide and purified by gel filtration chromatography (Fig. 2A) (*22*). Peptides modified at either terminus (G1-Ndel and A9-Cdel) promoted folding of HLA-A2, but a peptide modified at both termini (G1-Ndel+A9-Cdel) did not promote stable folding and was indistinguishable from the no-peptide control (Fig. 2A). A peptide having both anchor positions substi-

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tuted by alanines (I2A+L9A) also promoted folding of HLA-A2 (Fig. 2A). SDS–polyacrylamide gel electrophoresis (PAGE) of purified complexes confirmed the presence of heavy chain and β_2M (Fig. 2B). Four observations indicated that the purified complexes contained peptides and were not empty HLA-A2 molecules: (i) peptide was required for folding (for example, compare the no-peptide control to I2A+L9A in Fig. 2A); (ii) peptide was found in purified complexes by fast atom bombardment (FAB) mass spectrometry (Fig. 2C); (iii) electron density from x-ray crystallography of three of the complexes shown in Fig. 2A corresponded to the correct peptides (*23*); and (iv) thermal denaturation curves for these complexes (Fig. 3A) lacked the complexity (three separate denaturation transitions) observed for a mixture of empty and peptide-filled class I H-2K^d molecules (*24*).

Thermal denaturation curves were obtained by monitoring the change in circular dichroism (CD) signal at 218 nm (Fig. 3A) (*24, 25*). The observed thermal unfolding transition was largely reversible (Fig. 3, B and C) (*25, 26*). Theoretical lines through the data points in Fig. 3A were generated from a nonlinear least squares analysis based on a two-state unfolding process (*27*). Values for the midpoint of the thermal unfolding transition, T_m (melting temperature), and the calculated temperature shifts, ΔT_m, with respect to the L9A and WT peptides are listed in Table 1.

The denaturation curves (Fig. 3A) for HLA-A2 complexed with the WT and L9A peptides show a single unfolding transition at $T_m \cong 65$°C (Table 1). Because free β_2M also unfolded at 65.0°C (Table 1 and Fig. 3A), the dissociation of peptide and the unfolding of heavy chain and β_2M is likely to have occurred simultaneously, as also observed for thermal denaturation of peptide-filled class I H-2K^d molecules (*24*). The denaturation curves (Fig. 3A) for HLA-A2 complexed with peptides modified at either terminus (G1-Ndel and A9-Cdel) show a major transition at $T_m \cong 44$°C (Table 1) that was well before the unfolding transition for free β_2M and that was possibly associated with the dissociation of peptide and the unfolding of heavy chain. The downward slope in curves 3 and 4 at high temperatures is likely to be caused by the independent unfolding of dissociated β_2M. A similar profile was obtained for thermal denaturation of empty class I H-2K^d heavy chain complexed with human β_2M (*24*).

The denaturation curves (Fig. 3A) for HLA-A2 complexed with peptides having optimal residues at the anchor positions (I2L, L9V, and I2L+L9V) show a single unfolding transition at melting temperatures ranging between 71.9° and 75.5°C (Table 1), beyond the unfolding transition for free β_2M. Thus, the thermal stability of β_2M can be increased by the presence of a peptide-stabilized heavy chain (*28*). However, because the HLA-A2–I2L+L9V complex appeared less stable than expected on the basis of the independent contribution of each anchor residue (Table 1), there might be a limit to the extent to which a peptide-stabilized heavy chain can increase the thermal stability of β_2M. The denaturation curve (Fig. 3A) for HLA-A2 complexed with peptide having alanine substitutions at both anchor positions (I2A+L9A) shows a single unfolding transition at 59.4°C (Table 1), only 5.5°C lower than the T_m of the WT peptide and 16.1°C below that of the optimized I2L+L9V peptide (Table 1).

To confirm the relative thermal stabilities of HLA-A2 complexes and to observe directly the separate denaturation of heavy chain and β_2M, we compared the sensitivity of some HLA-A2 complexes to thermolysin digestion at different temperatures. Because thermolysin is active over a broad range of temperature and digests mostly denatured proteins, thermolysin can be used to approximate T_m values (*29*) and to reveal differences in the thermal stability of heavy chain and β_2M. We used SDS-PAGE analysis of products of thermolysin digestion (*30*) for HLA-A2 complexed with

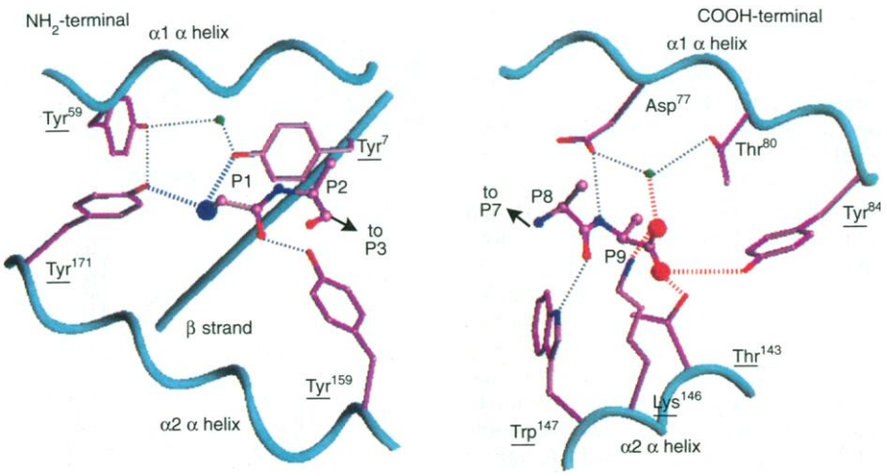

Fig. 1. Conserved hydrogen bonds between the charged NH_2- and COOH-termini of influenza virus matrix peptide [positions (P) P1, P2, P8, and P9] and HLA-A2 residues. Side chains at P2 (Ile), P8 (Thr), and P9 (Leu) are shown to the β carbon. After substituting P9 with Ala, the peptide NH_2- (large blue atom) or COOH- (large red atoms) termini were substituted by CH_3 groups. Both α helices and a β strand of HLA-A2 are shown in turquoise with conserved residues underlined to eliminate two (dashed blue lines) or four (dashed red lines) hydrogen bonds, respectively. Atom colors: magenta, carbon; blue, nitrogen; red, oxygen; and green spheres, bound water molecules. The figure was generated with the computer program RIBBONS (*38*).

Table 1. Midpoint temperatures of unfolding transitions for HLA-A2 complexed with various peptides (*20*). Values for T_m were generated from a nonlinear least squares analysis of thermal denaturation curves (Fig. 3A) (*27*). The error in T_m is estimated to be ±0.5°C. All values for ΔT_m were calculated relative to the L9A peptide (top of table) or to the WT peptide (bottom of table).

Peptide	Position									T_m (°C)	ΔT_m (°C)
	1	2	3	4	5	6	7	8	9		
Modifications at NH_2- and COOH-termini											
L9A	G	I	L	G	F	V	F	T	A	65.9	0
G1-Ndel	CH_3CH_2CO	I	L	G	F	V	F	T	A	44.9	−21.0
A9-Cdel	G	I	L	G	F	V	F	T	$NHCH(CH_3)_2$	42.8	−23.1
G1-Ndel+A9-Cdel	CH_3CH_2CO	I	L	G	F	V	F	T	$NHCH(CH_3)_2$	--*	
Substitutions at anchor positions 2 and 9											
WT	G	I	L	G	F	V	F	T	L	64.9	0
I2L	G	L	L	G	F	V	F	T	L	73.7	8.8
L9V	G	I	L	G	F	V	F	T	V	71.9	7.0
I2L+L9V	G	L	L	G	F	V	F	T	V	75.5	10.6
I2A+L9A	G	A	L	G	F	V	F	T	A	59.4	−5.5
											(−16.1)†
Free β_2M										65.0	

*Indicates that peptide did not promote stable folding of HLA-A2 (Fig. 2A). †Calculated from T_m(I2A+L9A) − T_m(I2L+L9V).

L9A, G1-Ndel, free β_2M, and I2L+L9V, respectively (Fig. 4). The temperature at which the HLA-A2–L9A complex (Fig. 4A) was digested by thermolysin was between 63° and 66°C, where both heavy chain and β_2M bands disappeared simultaneously, as suggested from the profile of denaturation curve 2 (Fig. 3A). Results for the HLA-A2–G1-Ndel complex (Fig. 4B) show that heavy chain becomes sensitive to thermolysin between 34° and 39°C. At such temperatures, however, β_2M was not digested by thermolysin, as indicated by the stronger band for β_2M that disappears at ~55°C (as observed for free β_2M) (Fig. 4C). The weak band for heavy chain in Fig. 4B persisting past the melting temperature determined by CD (T_m = 44.9°C) (Table 1) suggests that a small fraction of heavy chain remains associated with β_2M and disappears only as β_2M is digested by thermolysin. Similar results were obtained for the HLA-A2–A9-Cdel complex. These results are consistent with the profile of denaturation curves 3 and 4 (Fig. 3A) and confirm the independent unfolding of both heavy chain and β_2M. The temperature at which the HLA-A2–I2L+L9V complex (Fig. 4D) was digested by thermolysin was between 69° and 73°C, where both heavy chain and β_2M bands disappeared simultaneously. Thus, β_2M was more resistant to denaturation in the presence of a stable peptide–heavy chain subunit (compare Figs. 4C and 4D), as suggested from the profile of denaturation curve 7 (Fig. 3A).

Preliminary x-ray crystallographic studies of HLA-A2 complexed with L9A, G1-Ndel, and A9-Cdel (*23*) reveal peptides bound in the same conformation as seen in the structure of HLA-A2–WT complex (*6*). For the G1-Ndel peptide, the CH_3 group that substituted for the NH_2-terminal group rotated (by means of a 110° change in ψ) into the position expected of an Ala side chain, leaving a cavity at the NH_2-terminal binding site. To determine the effect on thermal stability of a CH_3 group at the position 1 side chain binding site, we assembled the HLA-A2–G1A+L9A complex and measured a T_m of 67.5°C, a value comparable to that of the HLA-A2–L9A complex (T_m = 65.9°C) (Table 1).

Our strategy of using synthetic peptides in which the hydrogen bonding ability of the NH_2- or COOH-terminus was abolished allowed us to assess the importance of the conserved hydrogen bonds between peptide and MHC residues without the addition of any potential steric clashes and without modifications to the protein structure. Previous studies of the importance of peptide termini reveal a strong length dependence for binding (*13, 18, 19, 31*). It was difficult to attribute this effect to the peptide-charged termini by acetylating the NH_2-terminus or amidating the COOH-terminus (*18, 32*) because the bulky *N*-acetyl group is sterically hindered from fitting into the NH_2-terminal binding site and because the C-amido group retains the ability to hydrogen-bond to MHC residues at the COOH-terminal binding site. Similarly, when two conserved HLA-A2 residues at each end of the peptide binding site were mutated, the resulting HLA-A2–WT complexes showed an effect in T cell recognition assays only for mutations done at the NH_2-terminus and not at the COOH-terminus (*33*). However, such assays could not distinguish a change in the protein structure caused by the mutation of two conserved MHC residues from an alteration in peptide binding or peptide-MHC complex stability.

Our results show that the conserved hydrogen bonds between a peptide NH_2- or COOH-terminus and MHC residues contribute ~22°C to the thermal stability of the peptide-MHC complex, whereas both anchor positions 2 and 9 contribute only 5.5°C to the thermal stability of the HLA-A2–WT complex, or 16.1°C when compared with the stability of the HLA-A2–I2L+L9V complex (Table 1). Thus, for stable binding to MHC class I molecules peptides need to fit either terminus into the ends of the binding site, but no optimal

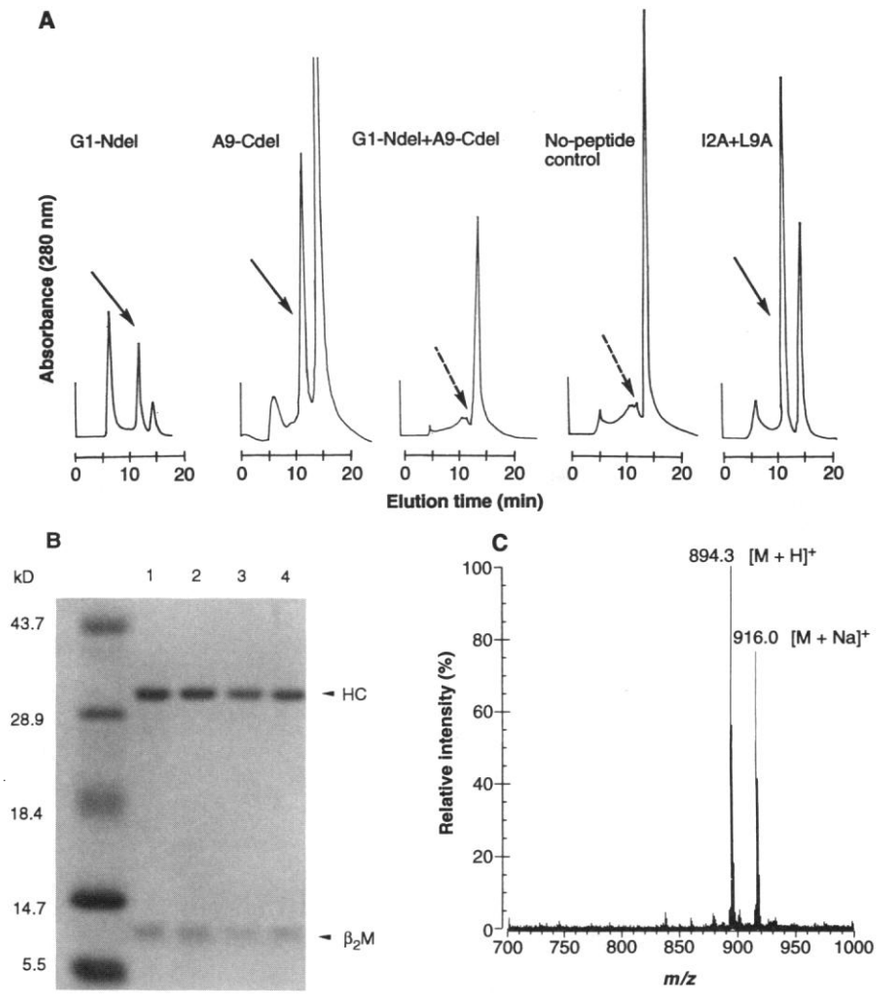

Fig. 2. (**A**) Gel filtration chromatograms of HLA-A2 complexed with various peptides. Complexes eluted as single peaks, indicated by solid arrows, at the expected time (11 min); adjacent peaks are aggregated heavy chain (6 min) and β_2M (14 min). Similar chromatograms (not shown) were obtained for all complexes listed in Table 1, except for G1-Ndel+A9-Cdel, where no complex peak (dashed arrows) was detected, even at peptide concentrations of 100 μM. The control chromatogram corresponds to a reconstitution experiment in which no peptide was present. Reconstitution yields varied among peptides from 5 to 13%. (**B**) SDS-PAGE (15%) of purified complexes, showing heavy chain (HC) and β_2M. Lanes 1 through 4, HLA-A2 complexed with WT, L9A, G1-Ndel, and A9-Cdel, respectively. The molecular sizes of standard proteins are indicated at left. (**C**) Typical FAB mass spectrum (*m/z*, mass-to-charge ratio) of purified HLA-A2, here complexed with A9-Cdel, showing the expected peptide ion peaks: $[M + H]^+$ = 894.3 (calculated = 894) and $[M + Na]^+$ = 916.0 (calculated = 916).

anchor residues are strictly required [for example, I2A+L9A (*13*, *14*)]. Our observations that marginally stable complexes, with a $T_m \cong 44°C$, are obtained if either peptide terminus forms hydrogen bonds with conserved MHC residues is consistent with the formation of complexes in which peptides may extend out at either end of the binding site (*34*) or in which short peptides are impaired from reaching both ends of the binding site (*35*).

Values of ΔT_m (Table 1) can be used to estimate the binding free energy contributed by peptide-MHC complex interactions. Using a number of assumptions frequently made in thermal denaturation studies of proteins (*36*), we estimate that the loss of hydrogen bonds mediated by the peptide-charged NH_2- or COOH-terminus gives a value of $\Delta\Delta G = -4.6$ kcal/mol, and the loss of side chains at both anchor positions gives a value of $\Delta\Delta G = -1.2$ kcal/mol in comparison to that of the WT peptide or of $\Delta\Delta G = -3.0$ kcal/mol in comparison to that of the optimized I2L+L9V peptide (*37*). Together, results from reconstitution experiments (Fig. 2A) and thermal denaturation studies (Fig. 3A and Table 1) show that for influenza virus matrix peptide, the relative contribution of either terminus to the stability of the HLA-A2 complex is greater than the combined effects of side chains at both anchor positions. These results suggest that MHC class I molecules have evolved important, conserved sites at both ends of the peptide binding site to act as universal sites for binding the charged termini of short peptides.

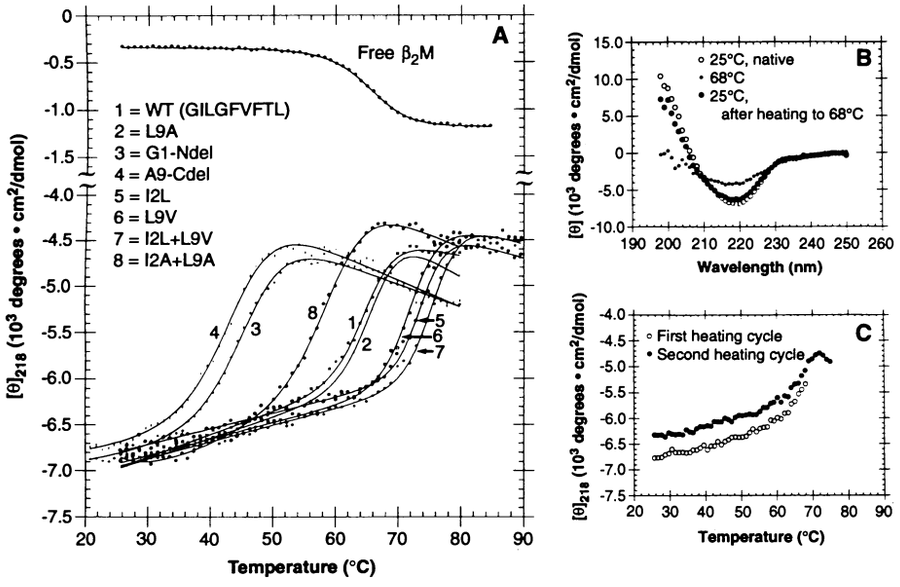

Fig. 3. (**A**) Thermal denaturation curves for HLA-A2 complexed with various peptides. Curves were obtained by monitoring the change in CD signal at 218 nm in the range of 20° to 90°C with a scan rate of 0.7°C per minute. Each point represents the average of three to five experiments with a complex from two independent reconstitution experiments. The curve for free β_2M was divided by 4 to represent its proportionate contribution to the molar residue ellipticity of the HLA-A2 complex. (**B**) CD spectra (250 to 198 nm) of the HLA-A2–WT complex; native protein, open dots; unfolded protein at 68°C, small closed dots; and renatured protein, closed dots (*26*). (**C**) Thermal denaturation curves for the HLA-A2–WT complex obtained as in (A). After the first heating cycle (open dots) between 25° and 68°C, the solution was immediately cooled to 25°C and at equilibrium (2 hours) a second heating cycle (closed dots) to 75°C was recorded (*26*). The shift seen along the *y* axis reflects the difference at 218 nm between the spectra of the native and the renatured protein in (B).

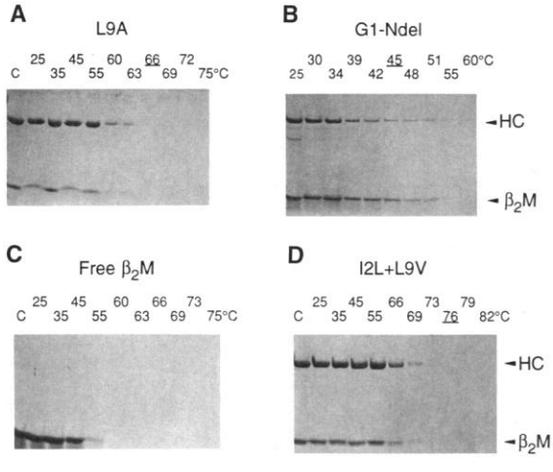

Fig. 4. SDS-PAGE analysis of products of thermolysin digestion of HLA-A2 complexed with L9A (**A**), G1-Ndel (**B**), free β_2M (**C**), and I2L+L9V (**D**), respectively. Each gel shows bands for heavy chain (HC) and β_2M at the indicated digestion temperatures. Values for T_m (Table 1) are underlined. Control lanes (marked with C) represent HLA-A2 complexes without thermolysin.

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20. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Substitutions are indicated as follows: $Leu^9 \rightarrow$ Ala for L9A; if two substitutions are in the same peptide, they are indicated as I2L+L9A.
21. Peptides WT, L9A, G1-Ndel, I2L, L9V, I2L+L9V, and I2A+L9A were synthesized on the 4-hydroxymethylphenoxy resin (Applied Biosystems) [G. B. Fields and R. L. Noble, *Int. J. Peptide Protein Res.* **35**, 161 (1990)]. The last coupling step for peptide G1-Ndel was performed with excess propionic anhydride:diisopropylethylamine (DIEA) (1:1) in methylene chloride (DCM) to provide the desired NH_2-terminal propionyl group (Ndel) (Table 1). Peptidyl resins were suspended in trifluoroacetic acid (TFA):water (95:5) for 1 hour, and crude peptides were precipitated by addition of cold ether. Peptides A9-Cdel and G1-Ndel+A9-Cdel were synthesized simultaneously on the Kaiser oxime resin (2 g; 0.37 mmol per gram of resin) (NOVA Biochem, La Jolla, CA) [W. F. De Grado and E. T. Kaiser, *J. Org. Chem.* **47**, 3258 (1982)]. Synthesis was initiated with Boc-Thr(Bzl) and proceeded to Ile, after which the peptidyl resin was divided in two equal parts. Coupling was con-

tinued with either Boc-Gly, for peptide A9-Cdel, or with excess propionic anhydride:DIEA (1:1), for peptide G1-Ndel+A9-Cdel. Peptidyl resin was suspended in DCM, and 10 equivalents of isopropylamine was added followed by 1.2 equivalents of acetic acid (AcOH) [G. Ösapay, M. Bouvier, J. W. Taylor, in *Techniques in Protein Chemistry II*, J. J. Villafranca, Ed. (Academic Press, San Diego, 1991), pp. 221–231]. After shaking for 24 hours, resin was filtered and washed and filtrate was evaporated to a white material. This last cleavage-coupling step provided the desired COOH-terminal isopropylamino group (Cdel) (Table 1). Crude protected peptide (260 mg) was dissolved in TFA:DCM (50:50) (13 ml) and stirred at room temperature. After 1 hour, the mixture was evaporated and this chromatographically purified intermediate (22 mg) was dissolved in *n*-butanol:AcOH:water (1:1:1) (15 ml) and hydrogenated over 10% Pd on carbon (approximately 50 mg). After 20 hours, the mixture was filtered through Celite (Aldrich, Milwaukee, WI) and concentrated. We purified all crude peptides by reverse-phase chromatography (RP-HPLC) on a Vydac C_4 preparative column using linear gradients of acetonitrile in water (containing 20% isopropanol for peptides A9-Cdel and G1-Ndel+A9-Cdel). Purified peptides were characterized by amino acid analysis, FAB mass spectrometry, and analytical RP-HPLC. Stock solutions in dimethylsulfoxide were kept frozen.

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25. Circular dichroism experiments were done in 10 mM MOPS (pH 7.5), HLA-A2 complexes (0.18 mg/ml), and free β_2M (0.36 mg/ml). Concentrations were determined by amino acid analysis with the use of norleucine as an internal standard and spectrophotometrically (94,240 and 19,180 M^{-1} cm^{-1} for HLA-A2 and β_2M, respectively, at 280 nm). Ellipticities are expressed on a molar residue basis. Spectra were recorded with use of a 1-mm cell and an Aviv 62DS spectropolarimeter equipped with a thermoelectric temperature controller.
26. Reversibility of the unfolding transition was demonstrated by a series of independent experiments each including two heating cycles separated by cooling to 25°C. The first heating cycles were scanned from 25°C to various temperatures; such a cycle between 25° and 68°C is shown in Fig. 3C. The CD spectrum of unfolded protein at 68°C, recorded separately, is shown in Fig. 3B. After cooling immediately to 25°C, the CD spectrum recorded at equilibrium (2 hours) (Fig. 3B) is nearly identical to that of the native protein. A second heating cycle to 75°C (Fig. 3C) closely resembled the first cycle, another indication that cooling resulted in correctly renatured HLA-A2. If the sample was maintained at 68°C for short times (15 min) before cooling to 25°C, a decrease in the extent of reversibility was observed, indicating that kinetic factors are responsible for the formation of some irreversibly unfolded HLA-A2. When the first heating cycle was between 25° and 80°C, the longer time above 68°C appeared to be responsible for a slightly reduced reversibility.
27. Denaturation curves were fit by a nonlinear least squares analysis (Kaleidagraph, Synergy Software) to the following relation describing a two-state unfolding process

$$\theta(T) = \theta_u + [(\theta_f - \theta_u)/1 + \exp(x)]$$

$$x = (-\Delta H_m/R)(1/T - 1/T_m) + (\Delta C_p/R)[(T_m/T - 1) + \ln T/T_m]$$

where $\theta(T)$ is the observed residue ellipticity at T, and θ_f and θ_u are linear functions of temperature for the pre- and post-transition region, respectively. T_m is the midpoint temperature of the unfolding transition, ΔH_m is the enthalpy change at T_m, R is the gas constant, and ΔC_p is the difference in heat capacity between the folded and unfolded states. We obtained initial estimates for T_m and ΔH_m from the van't Hoff equation $\Delta H = RT^2 (\delta \ln K/\delta T)$, using values of K, equilibrium constant, calculated in the narrow temperature range of the transition region from the relation $K = [\theta_f(T) - \theta(T)]/[\theta(T) - \theta_u(T)]$. The van't Hoff plots were fit to a second-order polynomial equation, and ΔH_m was calculated from the first derivative of the equation at $T = T_m$ and $\ln K = 0$. Values of ΔC_p were assumed to be independent of temperature [P. L. Privalov and S. J. Gill, *Adv. Protein Chem.* **39**, 191 (1988)] and were estimated from $\Delta C_p = (\delta \Delta H/\delta T)_p$ with values of ΔH calculated in the narrow temperature range of the transition region.
28. Leu fits better than Val into the binding pocket at position 2 and allows formation of shorter hydrogen bonds between peptide main chain atoms at position 2 and MHC side chains (*6*). When Val occupies the binding pocket at position 9, MHC residues Arg^{97} and Tyr^{116} orient to form favorable intramolecular hydrogen bonds, absent in the x-ray structure of the HLA-A2–WT complex (*6*).
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30. Thermolysin digestions at different temperatures were carried out for 15 min (60 min for G1-Ndel) in 10 mM MOPS (pH 7.5) containing 2 mM $CaCl_2$ at an enzyme:substrate ratio of 1:300 (w/w). Incubations were terminated by addition of EDTA to a concentration of 10 mM and immediate cooling of samples on ice. Products were analyzed by SDS-PAGE (15%).
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34. Peptides likely to extend out at the COOH-terminal binding site have been identified in peptide binding studies [Y. Chen *et al.*, *J. Immunol.* **152**, 2874 (1994); A. C. Olsen *et al.*, *Eur. J. Immunol.* **24**, 385 (1994)], and one example has been observed by x-ray crystallography [E. J. Collins, D. N. Garboczi, D. C. Wiley, unpublished results].
35. Peptides with one residue deleted at the NH_2-terminus have been reported [U. Utz, S. Koenig, J. E. Coligan, W. E. Biddison, *J. Immunol.* **149**, 214 (1992); (*19*)], and a peptide 8-mer too short to reach the NH_2-terminal binding site has been observed by x-ray crystallography to leave that site unoccupied [P. Ghosh and D. C. Wiley, unpublished results].
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37. Values for $\Delta\Delta G$, the difference in Gibb's free energy of unfolding between mutant and control complexes, were calculated at 60°C, a value intermediate among T_m values of HLA-A2 complexes, from the approximation $\Delta\Delta G = \Delta T_m \cdot \Delta S$ (*36*). Values of ΔT_m are equal to T_m (mutant) − T_m (control), where the control is L9A for G1-Ndel and A9-Cdel, and WT or I2L+L9V for I2A+L9A. Values for ΔS at 60°C for L9A, WT, and I2L+L9V are 0.209, 0.214, and 0.186 kcal/mol·*K*, respectively (*27*). An average value of ΔC_p = 3.6 kcal/mol·*K* obtained from nonlinear least squares analysis of denaturation curves (*27*) was used.
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39. We thank D. Garboczi, F. Hughson, T. Jardetzky, D. Madden, and L. Stern for many helpful discussions; P. Bjorkman, D. Garboczi, F. Hughson, D. Madden, and L. Stern for critical reading of the manuscript; H.-C. Guo for preparing Fig. 1; D. Minor for help with CD analysis; and the Mass Spectrometry Facility of the Harvard University Chemistry Department. M.B. is supported by a Cancer Research Institute fellowship. D.C.W. is an Investigator of the Howard Hughes Medical Institute.

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Fibrous Mini-Collagens in *Hydra* Nematocysts

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Nematocysts (cnidocysts) are exocytotic organelles found in all cnidarians. Here, atomic force microscopy and field emission scanning electron microscopy reveal the structure of the nematocyst capsule wall. The outer wall consists of globular proteins of unknown function. The inner wall consists of bundles of collagen-like fibrils having a spacing of 50 to 100 nanometers and cross-striations at intervals of 32 nanometers. The fibrils consist of polymers of "mini-collagens," which are abundant in the nematocysts of *Hydra*. The distinct pattern of mini-collagen fibers in the inner wall can provide the tensile strength necessary to withstand the high osmotic pressure (15 megapascals) in the capsules.

Nematocysts are exocytotic organelles that are characteristic of the phylum Cnidaria. There are at least 25 morphologically different capsule types, which are involved in a variety of functions, including capture of prey, defense, and locomotion (*1*). Capsules have very high internal pressures of up to 15 MPa (*2*), which drive nematocysts' discharge, during which the capsule's internal tube is everted (*3*). High-speed cinematography has shown that the entire process takes about 3 ms and takes place at accelerations of up to 40,000*g* (*4*). The explosive discharge of nematocysts is thus one of the fastest events in biology. The extreme osmotic pressure in resting capsules and the extraordinary speed of

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