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Immunology

Covalent HLA-B27/peptide complex induced by specific recognition of an aziridine mimic of arginine

GREGORY A. WEISS*, ROBERT J. VALENTEKOVICH*†, EDWARD J. COLLINS‡§, DAVID N. GARBOCZI‡, WILLIAM S. LANE¶, STUART L. SCHREIBER*‡‖**, AND DON C. WILEY*‡‖**

Departments of *Chemistry and Chemical Biology and ‡Molecular and Cellular Biology, ¶Harvard Microchemistry Facility, and ‖Howard Hughes Medical Institute, Harvard University, Cambridge, MA 02138

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ABSTRACT **The class I major histocompatibility complex (MHC) glycoprotein HLA-B27 binds short peptides containing arginine at peptide position 2 (P2). The HLA-B27/peptide complex is recognized by T cells both as part of the development of the repertoire of T cells in the cellular immune system and during activation of cytotoxic T cells. Based on the three-dimensional structure of HLA-B27, we have synthesized a ligand with an aziridine-containing side chain designed to mimic arginine and to bind covalently in the arginine-specific P2 pocket of HLA-B27. Using tryptic digestion followed by mass spectrometry and amino acid sequencing, the aziridine-containing ligand is shown to alkylate specifically cysteine 67 of HLA-B27. Neither free cysteine in solution nor an exposed cysteine on a class II MHC molecule can be alkylated, showing that specific recognition between the anchor side-chain pocket of an MHC class I protein and the designed ligand (propinquity) is necessary to induce the selective covalent reaction with the MHC class I molecule.**

Cytotoxic T-lymphocytes recognize antigens as short peptides bound to major histocompatibility complex (MHC) class I molecules that consist of a heavy chain and β_2-microglobulin (β_2m) (reviewed in ref. 1). HLA-B27, a MHC class I protein, has been implicated in the autoimmune disorder ankylosing spondylitis (2–4). The molecular basis for this linkage is unclear, but may involve binding of an autoantigen peptide by HLA-B27. Endogenous and viral peptides that bind to HLA-B27 have an arginine in position 2 (P2) (5). The δ-guanidinium group of the arginine is bound at the end of a deep pocket where four polymorphic residues project toward it (6, 7, 24). His-9, Thr-24, Glu-45, and a bound water form a hydrogen bond network with the guanidinium group (Fig. 1*A*). The γ-sulfhydryl of Cys-67 is located directly above the plane of the guanidinium group (ref. 7; Fig. 1*A*). This arrangement of protein side chains suggested that it might be possible to induce the pocket to act as an enzyme active site by designing a suitable substrate. We designed a peptide intended to form a covalent bond with HLA-B27, spontaneously and selectively, by synthesizing an aziridine-containing amino acid mimic of arginine (Fig. 1*B*). The positively charged secondary amine of the aziridine ring is expected to form a hydrogen-bonded charged-pair with Glu-45 in the arginine-specific pocket, positioning the η carbon of the aziridine ring for an in-line, ring-opening attack by the thiolate of Cys-67 (Fig. 1*C*). Selectivity is expected from the polarizability of the aziridine by Glu-45 and the propinquity (8) of the Cys-67 thiol to the bound aziridine ring.

MATERIALS AND METHODS

Folding of HLA-B27. Each complex was folded by incubation at 10°C of HLA-B27 heavy chain (1 μM) and β_2m (2 μM) in a $N_{2(g)}$ sparged solution of 20 mM (*N*-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid) (TES; pH 8.0), 0.2 M L-arginine, 2 mM Na_2EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 20 μM ligand (9). Yields of the complex were sometimes enhanced with rigorously anaerobic folding conditions.

Purification of HLA-B27. Gel filtration HPLC with a running buffer of 150 mM NaCl and 50 mM Tris (pH 8.0) was used to purify folded HLA-B27 complexes from aggregates and excess β_2m. Folded complexes had a retention time consistent with their molecular weight of 44 kDa. Lower yields were consistently obtained with the aziridine ligand; typical yields, confirmed by amino acid analysis, were ≈60% or less than yields obtained with the control peptide (GRIDKPILK). The lower yields might result from less efficient hydrogen bonding to the aziridine compared with the planar guanidinium group, or decreased reactivity of one diastereomer of the aziridine (kinetic resolution).

SDS/PAGE of HLA-B27. In all preparations of the MHC class I complex based on the aziridine-containing ligand, there was an additional SDS/PAGE band corresponding to unmodified heavy chain. This band became more prominent with decreasing concentrations of the aziridine-containing ligand in the folding buffer and low yields of MHC class I molecules, suggesting that the band is due to empty MHC class I molecules.

Tryptic Digestion and Microsequencing. The carboxyamidomethylated tryptic peptides were separated by narrow-bore HPLC using a Vydac C18 2.1 × 150 mm reverse phase column on a Hewlett–Packard model 1090 HPLC/1040 diode array detector. Putative difference peaks from the chromatogram were chosen based on differential UV absorbance at 210, 277, and 292 nm. These were further screened by matrix-assisted laser desorption time-of-flight spectrometry (MALDI-MS) on a Finnigan (Hemel, U.K.) Lasermat 2000, and submitted to Edman microsequencing on an Applied Biosystems model 477A microsequencer. Details of strategies for the selection of peptide fractions and their microsequencing have been described (10). Microcapillary HPLC/electrospray ionization/tandem mass spectrometry on a Finnigan (San Jose, CA) model TSQ7000 triple quadruplole mass spectrometer was described in ref. 11.

Incubation with Free Thiols. To 950 μl of HLA-B27 folding buffer, the unprotected amino acids cysteine, threonine, and glutamic acid were added each to a final concentration of 20 mM. The mesylate precursor (220 μg) to the aziridine-containing ligand was made basic in 50 μl methanol by the addition of 2 ml 1 M NaOH. After 15 min, the solution contained approximately equimolar distributions of mesylate

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Abbreviations: MHC, major histocompatibility complex; P2, position 2; β_2m, β_2-microglobulin.

†Present address: Department of Chemistry, University of California, Irvine, CA 92717.
§Present address: Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27599.
**To whom reprint requests should be addressed.

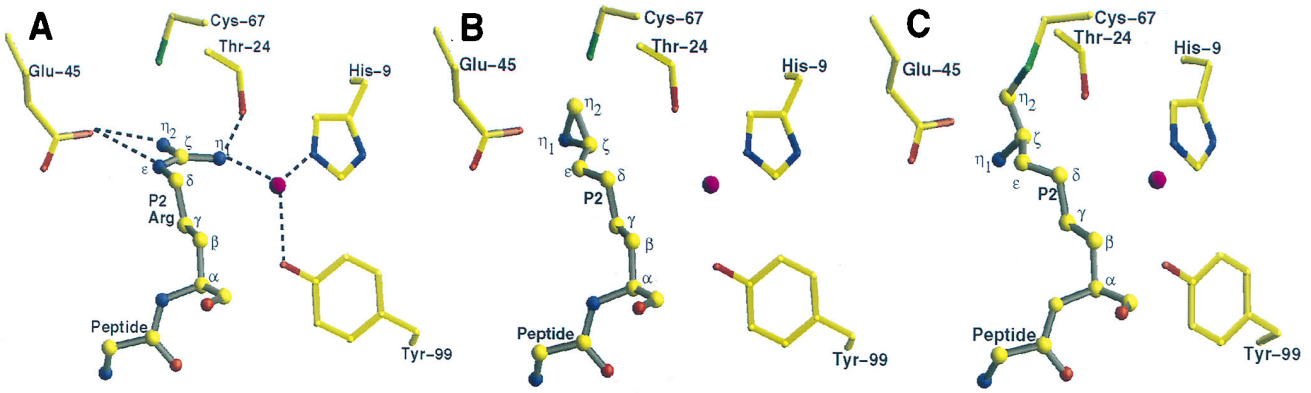

FIG. 1. The P2 pocket of HLA-B27 binding arginine and a model of the aziridine-containing ligand. (*A*) The P2 pocket of HLA-B27 forms a planar network of hydrogen bonds to the guanidinium group of arginine at P2 of a bound peptide. Perpendicular to this planar network of hydrogen bonds, the free thiol of Cys-67 is separated from the guanidinium group by 3.7 Å (7). (*B*) The aziridine-containing ligand was designed to bind in place of the guanidinium group with the aziridine amine forming a hydrogen-bonded salt bridge to Glu-45. In this model with one diastereomer of the aziridine depicted, the free thiol of Cys-67 is poised for in-line attack. (*C*) The expected geometry of the covalent complex between Cys-45 and the aziridine-containing peptide.

precursor and aziridine-containing ligand (verified by reverse phase-HPLC), and the solution was added to the folding buffer (both precursor and aziridine-containing ligand have a final concentration of 0.1 mM in the folding buffer), which was incubated at 10°C. After half an hour and approximately every 12 h later, 100 μl of the folding buffer was removed and subjected to reverse phase-HPLC, running a gradient of 100:0 to 40:60 (0.1% trifluoroacetic acid in water: CH_3CN) over 30 min and monitoring at 214 nm.

Incubation with HLA-DR1. Following standard peptide loading procedures for class II MHC molecules (12), empty, folded HLA-DR1 was incubated at 37°C with an 18-fold molar excess of the aziridine-containing peptide, G(az)IDKPILK. Aliquots were removed daily for 3 days, and denatured in 6 M guanidine hydrochloride, before being tested for the presence of free thiol using Ellman's reagent.

Circular Dichroism Monitored Thermal Stability of HLA-B27. An Aviv Associates model 62DS (Lakewood, NJ) equipped with thermoelectric temperature controller was used to obtain thermal denaturation curves in triplicate, from 25 to 95°C (13). The addition of 50 mM NaCl to the typical 10 mM Mops (pH 8.0) buffer was found to prevent aggregation of HLA-B27. The additional salt had no effect on the measured melting temperature (T_m) of the HLA-A2 complex formed with a hepatitis B-derived peptide (data not shown).

RESULTS

Synthesis of Aziridine-Containing Peptides. Synthesis of an aziridine-containing dipeptide followed methods developed by Vederas and coworkers (14, 15) (Fig. 2). Nonamer peptides containing the aziridine were synthesized by coupling the dipeptide to synthetic heptamers corresponding to the seven C terminal amino acids of two peptides known to bind to HLA-B27, GRIDKPILK and GRAFVTIGK (5). The aziridine within a peptide was cyclized from the mesylate (Fig. 2) in solution before addition to the folding buffer containing recombinantly expressed, purified HLA-B27 heavy chain and β_2m.

Folding of Covalent MHC Molecules. As in previous folding studies of recombinant class I MHC molecules (9), the folding of HLA-B27 was dependent upon a peptide ligand, here containing aziridine (data not shown). HLA-A2, a MHC molecule lacking specificity for arginine at P2, failed to fold in the presence of the aziridine-containing peptide, as expected (data not shown).

Characterization of Covalent MHC–Peptide Bond. The aziridine-containing ligands were shown to form covalent bonds with HLA-B27 by a shift in mobility of the heavy chain on SDS/PAGE; reaction with Cys-67 was shown by tryptic digestion, mass spectrometry (MS), and Edman microsequencing. SDS/PAGE of HLA-B27 complexed with aziridine-peptides revealed a mobility shift of the heavy chain of the complex due to addition of the aziridine-containing ligand (Fig. 3, lane 1), when compared with heavy chain from complexes with arginine in the P2 position (Fig. 3, lane 2). The mobility of β_2m remained unchanged. To identify the alkylated amino acid, the aziridine-modified complex was digested with trypsin and the fragments separated by reverse phase-HPLC. The chromatogram of the proteolyzed fragments was compared with a tryptic digest of the MHC class I protein formed with the arginine-containing parent peptide (GRIDKPILK).

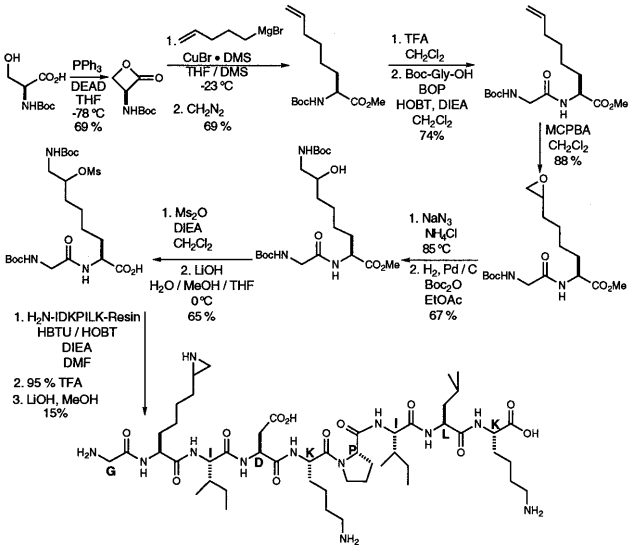

FIG. 2. Synthesis and structure of the aziridine-containing ligand. The peptide sequence of the ligand shown is based upon a ribosomal peptide (GRIDKPILK) eluted from HLA-B27 by Jardetzky *et al.* (5). Diastereomers of the aziridine were unresolved. All reactions followed standard laboratory practices. All intermediates and products were characterized by ^{1}H NMR, ^{13}C NMR, IR, and fast atom bombardment (FAB) MS. Boc, butoxycarbonyl; Ph, phenyl; DEAD, diethyl azodicarboxylate; THF, tetrahydrofuran; DMS, dimethyl sulfide; TFA, trifluoroacetic acid; Gly, glycine; BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; HOBT, 1-hydroxybenzotriazole; DIEA, diisopropyl ethylamine; MCPBA, *m*-chloroperoxybenzoic acid; EtOAc, ethyl acetate; Ms, mesyl; Me, methyl; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DMF, dimethylformamide.

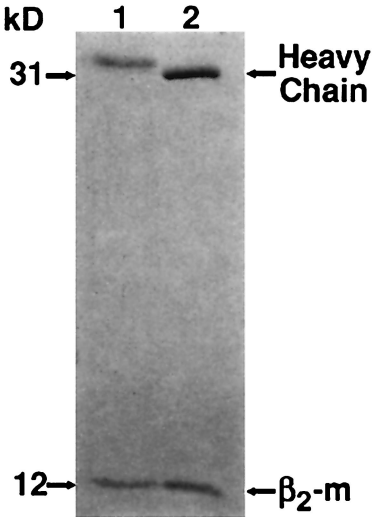

FIG. 3. SDS/PAGE (15%) of HLA-B27 complexed with the aziridine-containing peptide [G(az)IDKPILK] (lane 1) and the control peptide (GRIDKPILK) (lane 2).

Matrix-assisted laser desorption time-of-flight (MALDI) MS was used to screen rapidly putative difference peaks, which were deficient in UV absorbance at 277 and 292 nm (lacking tyrosine and tryptophan). A fraction was found with a singly charged ion (M^{H+}) of the expected mass (m/z = 1759) of the tryptic fragment resulting from alkylation of Cys-67 by the aziridine-containing ligand. Edman microsequencing revealed this fraction to contain the peptide ligand in equimolar amounts with the HLA-B27 heavy chain fragment containing Cys-67. Consistent with the specific alkylation of Cys-67 by the aziridine, no phenylthiohydantoin derivatives were observed at either the sequence position of the aziridine-amino acid in the peptide or Cys-67 in the heavy chain. Microcapillary LC electrospray mass spectrometry (LC-ESIMS) (10, 11) further confirmed the expected mass of the aziridine-modified fragment (m/z = 879.8 M^{2H++}).

Specificity of the Aziridine-Containing Ligand for HLA-B27. The selectivity of the aziridine-containing peptide for the free thiol on HLA-B27 was demonstrated in three ways. First, neither the aziridine-peptide nor its mesylate precursor reacted with a 200 molar equivalent excess of the amino acid components of the P2 binding pocket: the free amino acids cysteine, threonine, or glutamic acid. This was accessed in a time course experiment over 3 days (Fig. 4), which also established the stability of these ligands in aqueous solution over that time period [although a small fraction of the mesylate gradually converted to the aziridine (Fig. 4)]. Second, the aziridine-containing peptide also failed to alkylate the free cysteine residue in the peptide-binding groove of the class II MHC molecule HLA-DR1 (12). The portion of HLA-DR1 containing the only free cysteine, Cys-30, does not bind arginine and lacks the glutamate and threonine of the arginine binding pocket in HLA-B27 (16); nevertheless it represents a free thiol accessible on the surface of a protein. The free thiol of Cys-30 remained unalkylated and its concentration was constant throughout the course of the experiment. As a control, the addition of one molar equivalent of iodoacetic acid to the denatured HLA-DR1, after 2 h, was shown to decrease measured amounts of free thiol by >50%, demonstrating that the response to Ellman's reagent was due to the presence of a free thiol. Third, the aziridine ligand alkylates Cys-67 during the folding of recombinant HLA-B27 despite a 5000 molar equivalent excess of cysteine in the glutathione of the refolding buffer.

Stability of Covalent MHC–Peptide Complexes. The thermodynamic stability of the class I MHC molecules from mouse and human have been shown to depend on the sequence of the bound peptide (13, 17, 18). We measured the thermal transition of the HLA-B27 complexes using circular dichroism. The covalent complexes of HLA-B27 with two aziridine-containing peptides, G(az)IDKPILK and G(az)AFVTIGK, both denature at approximately the same temperature, 62°C (Fig. 5). The noncovalent complexes of HLA-B27 with the cognate peptides (GRIDKPILK and GRAFVTIGK) melted at 72°C and 60°C, respectively (Fig. 5). These data indicate that covalently bound peptides with an aziridine replacing arginine stabilize HLA molecules approximately like noncovalently bound peptides, in one case stabilizing slightly more and in one less. Although the stability to thermal denaturation is comparable for noncovalent and covalent complexes, the covalently bound peptides should resist dissociating at 37°C for much longer than the noncovalently bound peptides.

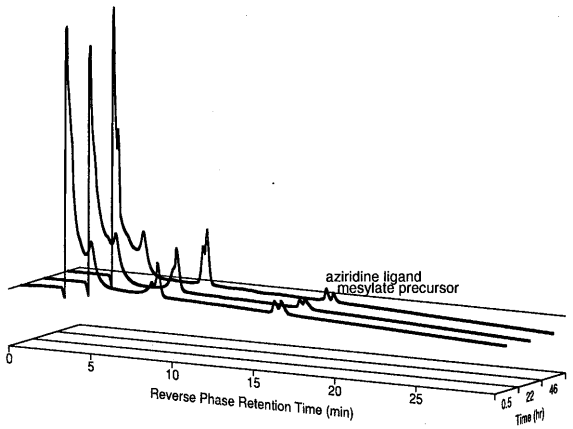

FIG. 4. Stability of the aziridine to free thiols. The aziridine-nonameric-peptide [G(az)IDKPILK] and its mesylate precursor are stable and do not react with free cysteine, threonine, or glutamic acid. Elution profiles of reverse phase-HPLC after designated incubation times show constant levels of the aziridine and mesylate containing peptides (labeled). The unprotected amino acids cysteine, threonine, and glutamic acid (each present in 200-fold molar excess) were incubated with equal molar amounts of the aziridine-containing ligand and its mesylate precursor in the HLA-B27 folding buffer containing reduced glutathione (50-fold molar excess of free thiol). Over 3 days, the sum of the mesylate precursor and aziridine-containing ligand remained approximately the same. No degradation or reaction products of the aziridine or mesylate were observed. Peaks at 15.63 and 16.05 min correspond to the aziridine-containing ligand and its mesylate precursor, respectively; other peaks belong to the folding buffer. Peaks were assigned by injection of standards or fast atom bombardment MS. The ordinate is proportional to absorbance with arbitrary units.

DISCUSSION

Covalent complexes of peptides with MHC molecules have been constructed previously by generating recombinant proteins with elongated N termini that extended into the binding grooves of MHC class I (19) and MHC class II (20) molecules. These molecules are active and have been suggested as possible therapeutics to induce T-cell tolerance in cases where the peptide autoantigens can be identified (20, 21).

The strategy we have used to form covalent complexes of peptides with HLA-B27 does not add any new amino acids to the surface of the MHC molecule (that might be immunogenic), but instead forms the covalent attachment deep in a pocket buried by the bound peptide. Although designed to react specifically with the arginine binding pocket of HLA-B27, the aziridine strategy could probably be generalized to other recombinant class I MHC molecules by mutating the P2 pocket residues to those in HLA-B27. The reverse experiment, engineering an HLA-A2 P2 specificity pocket into HLA-B27 by

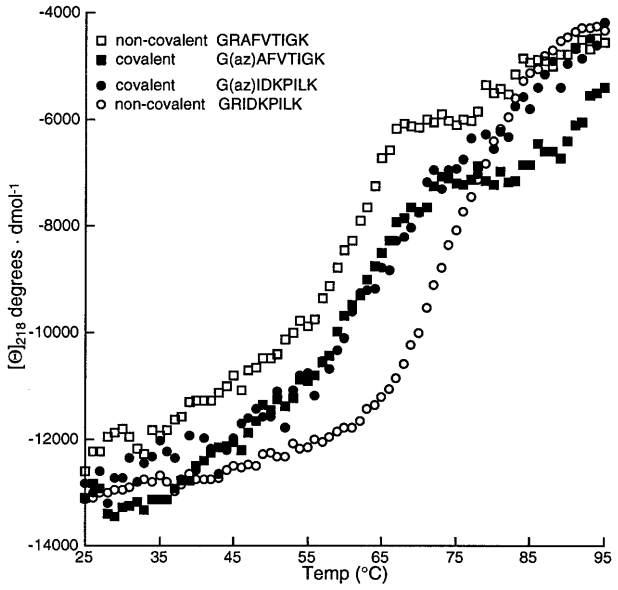

FIG. 5. Thermodynamic stability of HLA-B27 complexed covalently and noncovalently with peptides. Thermal denaturation was performed as described (13). The covalent complex formed from the aziridine-containing peptide [G(az)IDKPILK] and HLA-B27 melted at 62°C, the noncovalent complex formed from the control peptide (GRIDKPILK) and HLA-B27 at 72°C. The covalent complex formed from the G(az)AFVTIGK peptide melted at 62°C, while the noncovalent complex with GRAFVTIGK melted at 60°C.

mutating residues 9, 24, 45, 66, 67, and 70 has already been reported (22), and had the desired effect of altering the specificity for the P2 position of bound peptide but retaining the same ability to interact with T cells as the unmodified protein. [The success of that reported experiment also suggests that any small adjustments in the local shape of the MHC molecule that might accompany covalent bond formation will not affect T-cell recognition, as the covalent bond formation (Fig. 1) is a much smaller modification than six amino acid substitutions.] Covalent MHC–peptide complexes like that reported here may find use generating anergy if administered alone (20) or activation if administered with coligands for inducing costimulatory signals (23). It may be possible to design a nonpeptide ligand capable of crossing cellular membranes to block selectively HLA-B27, based on the same aziridine specificity.

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