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Immunology

Identification of the naturally processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule I-A^k

(histocompatibility/antigen presentation/antigen processing/antigenic determinant/antigenicity)

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ABSTRACT **A murine B-cell lymphoma bearing the class II major histocompatibility complex molecule I-A^k was cultured with the protein antigen hen egg white lysozyme (HEL). The I-A^k molecules were purified, and their associated peptides were extracted for characterization. Five HEL peptides were identified. Four contained the 10 amino acid residues HEL 52–61 (DYGILQINSR) but were heterogeneous in length and flanking residues. This core sequence is known to confer a high binding affinity for I-A^k. One additional peptide contained the amino acid residues HEL 48–60. These data demonstrate that the HEL epitope containing residues 52–61 is the most abundant HEL epitope presented on the major histocompatibility complex of the antigen-presenting cells and consequently explains its immunodominance.**

T cells recognize linear sequences of amino acid residues in peptides derived from proteins biochemically processed within antigen-presenting cells (APC). These peptides are recognized on the surface of the APC in association with molecules of the major histocompatibility complex (MHC) (reviewed in refs. 1 and 2). Through an uncharacterized process of determinant selection, first identified by Rosenthal *et al.* (3) while studying the response to insulin in guinea pigs, only a limited set of epitopes from complex proteins are recognized by CD4 T cells in the context of class II MHC molecules. One possible explanation for determinant selection could be that the selected, or immunodominant, peptides exhibit a relatively high affinity for the MHC molecule (4, 5). However, the preferential presentation of high-affinity peptides may not be the sole basis for determinant selection—the chemistry of the degradative processing, as well as other intracellular events, could contribute to the selection of a limited number of peptide sequences from the pool of catabolic products (6, 7).

An understanding of the mechanisms involved in determinant selection might be gained from an examination of the end products of antigen processing. Toward this end, the biochemical characterization of physiologically processed antigen in the form of peptides eluted from affinity-purified class I (8–10) and class II (11–14) MHC molecules has been initiated. The eluted peptides were found to be of low molecular weight and to have limited heterogeneity. This small size was in agreement with that of synthetic peptides, which substitute for processed antigen (15, 16). The naturally processed peptides isolated from class I molecules were 8 or 9 amino acids in length with fixed amino- and carboxyl-terminal ends and were derived from proteins synthesized in the cell cytosol. In contrast, peptides extracted from class II molecules were from 13 to 17 amino acids in length with variable carboxyl-terminal ends, and most were derived from cellular transmembrane glycoproteins. One peptide was recovered from bovine serum albumin, a protein found in the culture medium used to grow the cells (13).

Studies in our laboratory have used hen egg white lysozyme (HEL) as a model antigen for processing and presentation by APC. HEL, a soluble nonglycosylated protein 129 amino acids in length, contains four intrachain disulfide bonds and requires intracellular processing for T-cell recognition. Analysis of the T-cell response to HEL in several mouse strains revealed that some regions were favored over others (17, 18). We previously showed that the major component of the CD4 T-cell response to HEL in H-2^k mice was directed toward the 10 amino acid residues 52–61 (19, 20). These residues lie partially buried inside the native molecule in a β-sheet conformation near the active site of the enzyme. Peptides containing residues 52–61 of HEL bind with high affinity to I-A^k (5). The HEL peptide 52–61 contains amino acid residues that contact the I-A^k molecule interspersed with amino acid residues that contact the T-cell receptor (20).

We are now using this well-characterized model antigen to investigate determinant selection and here report the sequences of physiologically presented HEL peptides eluted from I-A^k after processing by an APC line.

MATERIALS AND METHODS

Isolation of I-A^k-Associated Peptides. Peptide-loaded MHC class II molecules were purified from the I-A^k-expressing B-lymphoma line M12.C3.F6. This line was produced by DNA-mediated cotransfection of genomic clones for the A^k α and β chains into M12.C3, an Ia-negative B-lymphoma line (21). M12.C3.F6 cells were grown in roller flasks containing Dulbecco's minimal essential medium supplemented with 2.5% (vol/vol) fetal calf serum to a density of 5×10^5 cells per ml. For the last 12 hr of growth, cells were cultured either in medium alone or in HEL (grade I; Sigma) at 1 mg/ml. The cells were harvested, washed, and lysed at a concentration of 5×10^7 cells per ml in phosphate-buffered saline containing 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 20 μg/ml, and the detergents octanoyl-*N*-methylglucamide (MEGA-8) and nonanoyl-*N*-methylglucamide (MEGA-9) (22) each at 40 mM. The lysates were cleared of nuclei and debris by centrifugation at 10,000 $\times$ *g* for 30 min. The I-A^k–peptide complexes were purified from the lysate by affinity chromatography using the monoclonal antibody 10-3.6.2 (23) coupled to Sepharose 4B beads. The detergent-solubilized membranes were incubated with the beads under mild agitation at 4°C overnight. The beads were then washed with phosphate-buffered saline containing 20 mM MEGA-8

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Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cell(s); HEL, hen egg white lysozyme; MEGA-8, octanoyl-*N*-methylglucamide; MEGA-9, nonanoyl-*N*-methylglucamide; IL-2, interleukin 2.

and 20 mM MEGA-9 until the OD_{280} of the eluate reached background, usually with about 10 column volumes. Complexes were eluted at pH 10.5 (24) with 0.05 M diethylamine in 0.15 M NaCl containing 20 mM MEGA-8 and 20 mM MEGA-9. One-tenth volume of 1 M Tris in 0.15 M NaCl at pH 6.8 was added to reduce the pH of the eluate to about 7.5. The volume of the purified material was reduced from 30 ml to 2 ml in a stirred-cell concentrator (model 8050; Amicon) over a YM30 ultrafiltration membrane. I-A^k–peptide complexes were precipitated from the concentrate by incubation on ice for 60 min after the addition of 7.5 volumes of acetonitrile, followed by centrifugation for 30 min at 1000 × *g*. Peptides were released from this precipitate by incubation in 2.5 M acetic acid at 37°C for 30 min, collected by centrifugation through a Centricon 10 filter (Amicon), and loaded directly onto a Waters 600E multisolvent delivery system for reverse-phase HPLC fractionation.

The separation employed a Vydac 218TP54 C_{18} column (5-μm particle size; 4.6 mm × 25 cm; 300-Å pore diameter) with an acetonitrile gradient of solvent A (0.06% trifluoroacetic acid in water) in solvent B (0.052% triflouroacetic acid in 80% acetonitrile). The gradient (as described in ref. 13) consisted of, briefly, from 2% to 37.5% solvent B for the first 60 min, from 37.5% to 75% solvent B for the next 25 min, and from 75% to 98% solvent B for the last 15 min. Sample fractions were collected every 0.5 min at a flow rate of 0.5 ml/min. The absorbance was measured at both 214 nm and 280 nm by a Waters 490E multiwavelength detector. Four independent preparations of HEL-fed M12.C3.F6 cells were examined with essentially identical results.

It is possible for the M12.C3.F6 cell line to produce H-2 chimeric A^d α-chain–A^k β-chain dimers, which would bind to 10-3.6.2 (25). However such pairing is greatly unfavored when the A^k α chain is present. Moreover, the A^d α-chain–A^k β-chain dimer does not bind the HEL 46–61 epitope (26); therefore, we feel the presence of A^d α-chain–A^k β-chain dimers does not affect the interpretation of this data.

Sequence Analysis. The amino acid sequence of the HPLC-fractionated material was determined by automated Edman degradation on an Applied Biosystems 470A gas-phase protein sequencer. In some cases, fractions were sequenced several times to determine the composition and length of the peptides.

Presentation Assay. HPLC fractions were tested for their ability to stimulate 3A9 (27), an I-A^k-restricted T-cell hybridoma specific for residues 52–61 of HEL. Briefly, 50 μl (20%) of each HPLC fraction was dried, resuspended in Dulbecco's minimal essential medium, and placed in a single well of a 96-well microtiter plate in a total volume of 200 μl containing 5×10^4 live M12.C3.F6 APC and 1×10^5 3A9 T-hybridoma cells. After 18 hr, culture supernatants were assayed for interleukin 2 (IL-2) content by their ability to maintain the growth of an IL-2-dependent T-cell line (the line CTLL), measured as [^{3}H]thymidine incorporation (28).

RESULTS

Identification and Characterization of HEL-Derived Peptides. I-A^k binding peptides, purified from HEL-fed M12.C3.F6 B-lymphoma cells, were fractionated by reverse-phase HPLC on a C_{18} column to yield the simultaneous 280-nm and 214-nm absorbance profiles shown (Fig. 1 *A* and *B*). The 214-nm absorbance profile of peptides from HEL-fed cells, when compared with the 214-nm absorbance profile of peptides purified from M12.C3.F6 cells grown in medium alone (Fig. 1*C*), contained five additional prominent peaks (numbered 1–5, Fig. 1*B*). The amino acid sequence of the material in these fractions was determined by Edman degradation (Table 1). Each fraction contained a HEL-derived fragment that overlapped the same region of lysozyme, amino

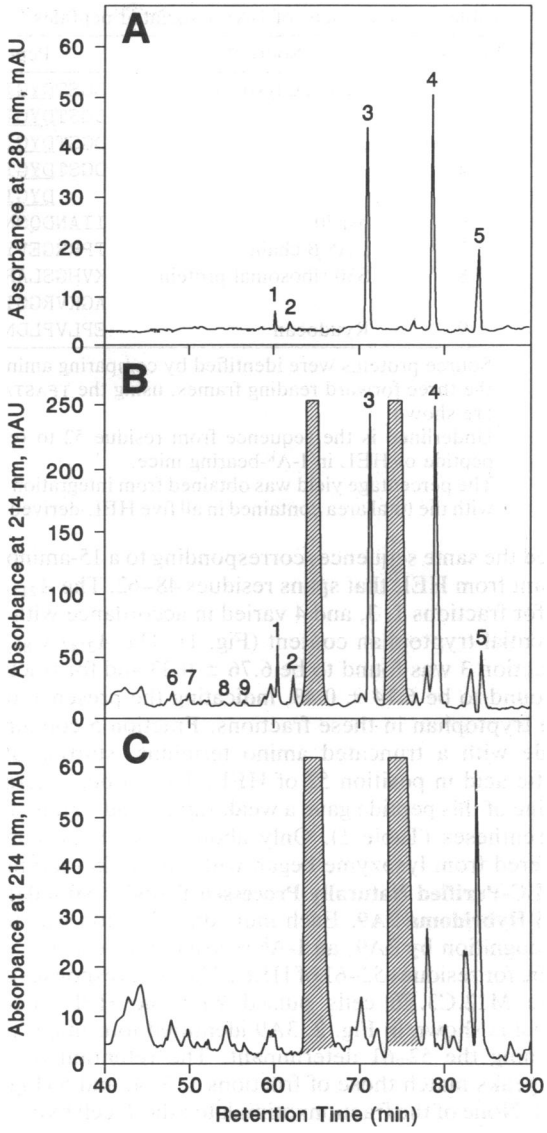

FIG. 1. Chromatograms of peptides eluted from purified I-A^k, fractionated by reverse-phase HPLC on a C_{18} column. The trace of acid-eluted peptides recovered from 80 liters of M12.C3.F6 cells cultured in HEL at 1 mg/ml is shown at an absorbance of 280 nm (*A*) and at an absorbance of 214 nm (*B*). (*C*) The trace of acid-eluted peptides recovered from 40 liters of M12.C3.F6 cells cultured in medium alone is shown at an absorbance of 214 nm. The shaded peaks in the 214-nm profiles are an artifact caused by detergents used in the purification. Sequence data were obtained for the numbered peaks (Table 1). The average A_{214}/A_{280} ratios for fractions 1, 3, and 4 obtained from four independent experiments were 18.83 ± 1.83, 6.76 ± 0.33, and 6.14 ± 0.43, respectively. Synthetic peptide sequences 48–61, 48–62, and 48–63 gave A_{214}/A_{280} ratios of 15.1, 6.44, and 4.29, respectively. The number of tryptophan residues in our unknown HEL peptides were determined by comparison with these reference values. The synthetic peptides corresponding to the sequences 48–61, 48–60, and 48–62 comigrated with the materials in fractions 1, 2, and 3, respectively.

acid positions 52–61 (Table 1). Fraction 1 corresponded to amino acid residues 48–61 and represented ≈8% of the total lysozyme material recovered as calculated by integration of the 214-nm absorbance profile. Fraction 2 contained about 3% of the recovered lysozyme-derived material and had a carboxyl-terminal serine based on the absence of a predicted arginine residue from the next cycle of the amino acid sequencer. Fractions 3 and 4 together contained about 72% of the material recovered from lysozyme. These two fractions

Table 1. Sequences of I-A^k-associated peptides

Fraction	Source*	Peptide sequence†	Positions	Length	Yield ± SD‡
1	Hen egg lysozyme	DGSTDYGILQINSR	48–61	14	7.7% ± 1.8%
2		DGSTDYGILQINS	48–60	13	3.3% ± 1.0%
3		DGSTDYGILQINSRW	48–62	15	37.8% ± 6.7%
4		DGSTDYGILQINSRW	48–62	15	33.8% ± 5.3%
5		DYGILQINSRWW(C)	52–63 (64)	12 (13)	17.4% ± 8.8%
6	hsp70	IIANDQGNRTTPSY	28–41	14	
7	I-A^k β chain	TPRRGEVYTCHVEHP	165–179	15	
8	S30 ribosomal protein	KVHGSLARAGKVRGQTPKVAKQ	75–96	22	
		AGKVRGQTPKVAKQEKKKKKT	83–103	21	
9	Ryudocan	EPLVPLDNHIPENAQPG	84–100	17	

*Source proteins were identified by comparing amino acid sequences to the GenBank nucleic acid data base, translated in the three forward reading frames, using the TFASTA program (13). Only instances of 100% identity with a known protein are shown.

†Underlined is the sequence from residue 52 to residue 61, previously identified as representing the immunodominant peptide of HEL in I-A^k-bearing mice.

‡The percentage yield was obtained from integration of the 214-nm trace by comparing the area contained in a given fraction with the total area contained in all five HEL-derived fractions. Data represent the average of four independent experiments.

yielded the same sequence, corresponding to a 15-amino acid remnant from HEL that spans residues 48–62. The A_{214}/A_{280} ratio for fractions 1, 3, and 4 varied in accordance with their differential tryptophan content (Fig. 1). The A_{214}/A_{280} ratio for fraction 3 was found to be 6.76 ± 0.33 and for fraction 4 was found to be 6.14 ± 0.43, indicating the presence of the single tryptophan in these fractions. Fraction 5 contained a peptide with a truncated amino terminus, starting at the aspartic acid in position 52 of HEL. The carboxyl-terminal cysteine of this peptide gave a weak signal, and so it is shown in parentheses (Table 1). Only about 17% of the material recovered from lysozyme began with this amino terminus.

HPLC-Purified Naturally Processed Peptides Stimulate the T-Cell Hybridoma 3A9. Each individual fraction was tested for recognition by 3A9, an I-A^k-restricted T-cell hybridoma specific for residues 52–61 of HEL. The IL-2 response of 3A9 to live M12.C3.F6 cells pulsed with the HPLC-purified material is shown in Fig. 2. 3A9 identified four major peaks containing the 52–61 determinant. The retention times of these peaks match those of fractions 1, 3, 4, and 5 (Fig. 1 *A* and *B*). None of the fractions stimulated the T-cell hybridoma A6.A2, which is reactive with residues 34–45 of HEL (29).

Peptides Recovered from Endogenous Proteins. Other fractions were sequenced in the attempt to identify additional HEL-derived material. Four such fractions, peaks 6, 7, 8, and 9 (Fig. 1*C*), yielded sequence with 100% homology to known proteins (Table 1). Fraction 6 contained a 14-amino acid fragment derived from one (or more) of the proteins of the hsp70 heat shock family. A 15-amino acid fragment from the β_2 domain of the murine MHC class II I-A^k β chain was found in fraction 7. Fraction 8 contained two large overlapping peptides, and although the murine sequence is unavailable, these peptides match residues 75–103 of a rat S30 ribosomal protein and therefore probably represent the murine homolog of that protein. Fraction 9 contained a peptide that is at least 17 amino acids long and matches residues 84–100 of murine ryudocan (R. D. Rosenberg, personal communication), an integral membrane proteoglycan (30).

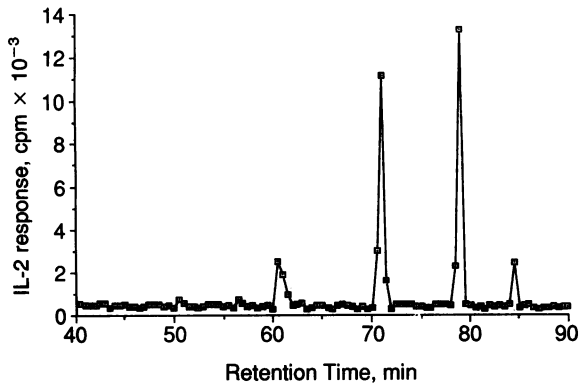

FIG. 2. Stimulation of the T-cell hybridoma 3A9 by HPLC-purified material. On the day of the assay, the plateau response of 3A9 to M12.C3.F6 pulsed with a synthetic peptide (YEDYGILQINSR) was 17,230 ± 860 cpm and occurred at a concentration of 8 μM. The background CTLL incorporation in this assay was 374 ± 42 cpm.

DISCUSSION

There are two major findings from this study. First, the majority of HEL peptides, extracted from I-A^k after processing by an APC line, contained the sequence 52–61. This result explains the immunodominance of the HEL 52–61 epitope and favors the concept that immunodominant epitopes represent those most abundantly presented by the MHC of the APC.

Second, we found that this single determinant is processed into at least five different fragments. This result, that a single minimal epitope would be processed into a set of fragments, was predicted by previous work on unsequenced heterogeneous material eluted from affinity-purified MHC class II molecules by Grey and coworkers (11, 12). In our study, the majority of the peptides, HEL residues 48–62, migrated at two different retention times on reverse-phase HPLC. The phenomenon of two class II-associated peptides having the same sequence and length but different mobilities was also observed in the study by Rudensky *et al.* (13). Whether this difference is due to a modification caused by the purification procedure or by a process within the live cells remains for further study.

Four of the five purified fractions stimulated the production of IL-2 by 3A9, an I-A^k-restricted T-cell hybridoma specific for residues 52–61 of HEL, proving that the recovered material binds the class II molecule and is immunogenic. The material from fraction 2, residues 48–60, did not appear to give a response, probably due to the absence of residue 61. It is possible, however, that peptide 48–60 is immunogenic for another set of T cells.

It is interesting to speculate on the basis for the biochemical selection of the 52–61 sequence with additional residues included on both the amino- and carboxyl-terminal ends. These termini could result from the primary proteolytic event, which for HEL takes place in a lysosomal organelle (31); these ends could also represent the limits of protection

afforded from further processing by the MHC molecule (13, 32); peptides with these ends may represent the most stable fragments; the length of a peptide may be an indication of the amount of time spent by that fragment in the proteolytic compartment before rescue; these sequences could have been selected over the minimal 10-mer sequence because they possess higher affinities for the I-A^k molecule; or these ends may facilitate transport to, or loading of, the I-A^k molecule. Additional studies are needed to examine these issues.

The heterogeneous fragments containing the 52–61 sequence merit discussion with respect to our previous studies. First, by using photoaffinity probes on peptides 46–61 and 52–61, we showed that the amino terminus of the 46–61 peptide, six residues longer than the core peptide, interacted outside the peptide-combining site with the α_2 and β_2 domains of I-A^k (33). It is conceivable that contacts made through the extended ends of these peptides helped in stabilizing the loaded class II molecule. Second, we previously characterized T-cell hybridomas with differential sensitivities to changes in residues outside the minimal antigenic determinant of residues 52–61, and at that time we suggested that HEL peptides bound through residues 52–61 would generate more than one T-cell epitope (34). We obtained similar results in an examination of the response toward HEL residues 34–45 (29).

We were unable to recover any other portion of the HEL molecule. These peptides were missed, either because they are produced in quantities too low to detect or because they have affinities too low to survive our purification. Another possibility is that no other epitope could compete for binding to the class II molecule at the high concentration of antigen used. We favor this last possibility, based on the proven high affinity of residues 52–61 for I-A^k. Preliminary experiments performed at lower antigen concentration, in search of subdominant HEL epitopes, have proven encouraging.

Finally, it is surprising to find peptides from two cytoplasmic proteins (the hsp70 and S30 ribosomal proteins) associated with class II MHC. If specific, it implies that there are mechanisms for delivery of relatively large cytosolic peptides to the class II pathway.

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