Isolation and Quantitation of a Minor Determinant of Hen Egg White Lysozyme Bound to I-A<sup>k</sup> by Using Peptide-Specific Immunoaffinity

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*J Immunol* 1998; 161:6074-6083; [http://www.jimmunol.org/content/161/11/6074](http://www.jimmunol.org/content/161/11/6074)
Isolation and Quantitation of a Minor Determinant of Hen Egg White Lysozyme Bound to I-A\(^k\) by Using Peptide-Specific Immunoaffinity

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We report here the identification and quantitation of a minor epitope from hen egg white lysozyme (HEL) isolated from the class II MHC molecule I-A\(^k\) of APCs. We isolated and concentrated the peptides from the I-A\(^k\) extracts by a peptide-specific mAb, followed by their examination by electrospray mass spectrometry. This initial step improved the isolation, recovery, and quantitation and allowed us to identify 13 different minor peptides using the Ab specific for the HEL tryptic fragment 34–45. The HEL peptides varied on both the amino and carboxy termini. The shortest peptide was a 19-mer (residues 31–49), the two most abundant were 31–47 (1.3 pmol) and 31–46 (1 pmol), while the least abundant were 31–45 (40 fmol) and 32–45 (4 fmol). Only 0.3% of the total class II molecules were occupied by this family of HEL peptides. The amount of the 31–47 peptide, the predominant member of this series, was 22 times lower than that of 48–62, the major epitope of HEL. The 31–47 peptide bound about 20-fold weaker to I-A\(^k\) compared with the dominant 48–62 peptide. Thus, the lower abundance of the minor epitope correlated with its weaker binding strength.


Several approaches have been taken to analyze the peptides selected by class II MHC proteins during the intracellular processing by APC. Among them, examples of frequently employed strategies are to determine the T cell reactivity to banks of overlapping synthetic peptides that encompass the whole protein or to phage display libraries (1–5). The approach undertaken by our laboratory is to isolate and examine biochemically peptides extracted from the MHC class II molecules of the APC when exposed to foreign protein. This leads us to determine the spectrum of peptides presented by APC, their precise natural sequence, and their relative abundance. Using hen egg white lysozyme (HEL)\(^2\) as a model Ag, we previously established that T cells were reactive to the tryptic fragment encompassing residues 46–61 (6). The T cells were reactive to the core sequence 52–61 (7, 8), which bound with a relatively high affinity to I-A\(^\lambda\) molecules (7, 9). When the peptides were isolated from APC exposed to HEL, we found a relatively large representation of peptides bearing the 52–61 epitope (10–12). These peptides were found as a nested set of long peptides centered on the 52–61 sequence, with variation on both the amino and carboxy termini (10, 11). These terminal extensions have since been shown to be of considerable importance; they contribute to the higher affinity binding of the peptide to the I-A\(^\lambda\)

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Received for publication April 10, 1998. Accepted for publication July 22, 1998.

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\(^2\)Abbreviations used in this paper: HEL, hen egg white lysozyme; M12-A\(^\lambda\), HEL; M12.C3.F6 cell line transfected with a membrane form of hen egg white lysozyme; M12-A\(^\lambda\), M12.C3.F6 cell line expressing class II I-A\(^\lambda\); TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; m/z, mass-to-charge ratio; RIC\(^-\), relative inhibitory capacity.

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Recently, the combined strategy of immunoaffinity chromatography and mass spectrometry analysis was employed for the characterization of peptide fragments from proteins (24–26) and for the isolation of limiting amounts of protein from complex biologic material (27, 28). We have now adapted the principle of immunoaffinity capture and developed it as a strategy to isolate low abundance class II-associated peptides. Using a monoclonal anti-peptide Ab, we recovered picomole-to-femtomole levels of HEL peptides, which were previously identified in the trypptic fragment 34–45 (29).

Materials and Methods

Cell lines

We used the murine B cell lymphoma line M12.C3.F6 transfected with a membrane form of HEL (M12-Ak mHEL) (11). The HEL fusion protein was engineered with site-directed mutagenesis by using a procedure previously described by Brooks et al. (30). Our control cell line, M12.C3.F6, expressing class II I-Ak (M12-Ak) (31), and M12-Ak mHEL were cultured in DMEM supplemented with 5% FCS. The M12-Ak mHEL process and present HEL peptides equally to M12-Ak given exogenous HEL (11).

Production of mAbs

mAbs to HEL peptides were produced by standard hybridoma procedures. Briefly, the synthetic HEL peptide 34–45 was coupled to the carrier protein keyhole limpet hemocyanin using bromoacetyl succinimide (Sigma, St. Louis, MO). CAF.1/J mice (The Jackson Laboratories, Bar Harbor, ME) were first injected i.p. with peptide-hemocyanin in CFA (Sigma), followed by three i.p. injections in incomplete CFA (Sigma) at 4–6-wk intervals. Finally, 3 days before B cell fusion, the mice were boosted i.v. with 25 μg of peptide-hemocyanin in sterile PBS. Splenocytes were fused with the myeloma fusion partner P3X63.Ag8 at a ratio of 5:1. Cell fusion was induced with polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, IN) by using the standard fusion protocol.

The hybridoma supernatants were screened by an ELISA assay in which 96-well Maxisorp plates (NUNC, Roskilde, Denmark) were coated with 5 μg/ml of denatured HEL (Sigma). The positive wells were then cloned. Six Abs were found to be specific for the HEL peptide 34–45, of which VAL-3 (isotype IgG1) was used. The epitope of this Ab is on the amino terminus spanning from residues 34–38 (FESNF). VAL-3 hybridoma cells were injected into pristane (Sigma)-treated SCID mice, and the IgG1 Ab was purified from ascites using protein A-Sepharose (Sigma).

Synthesis of synthetic peptides

Peptides were synthesized by F-moc chemistry (model 432A; Applied Biosystems, Foster City, CA) and purified by reverse-phase HPLC (600E; Waters, Milford, MA). The sequences of all peptides were subsequently confirmed by mass spectrometry. For binding studies, peptides were radiiodinated (125I) on Tyr residues using the chloramine-T method (32). Each peptide was iodinated to a specific activity of 0.5 mCi/1.5 nmol of peptide.

Isolation of I-Ak-associated HEL peptides

I-Ak molecules were isolated from M12-Ak mHEL B lymphoma cells (10) and grown to a cell density of 2 × 10^5 cells/ml to yield a total of 10^9 cells. These cells were lysed in the presence of MEGA 8/MEGA 9 detergent (Sigma) and enzyme inhibitors (PMSF, iodoacetamide, and leupeptin) as described (10). The I-Ak molecules were isolated by immunoaffinity chromatography using the anti I-Ak Ab 40F (33). The peptides were subsequently released by acid treatment. In the experiment shown in Fig. 1, each peptide was separated by conventional reverse-phase HPLC (600E; Waters). Each 250-μl fraction was tested in a T cell bioassay using either the T cell hybridoma A6A2, while at 72 and 78 min, five fractions containing HEL peptides with a core region of 52–61 stimulated the 3A9 T cell hybridoma. B, Fractions eliciting an A6A2 T cell response (shown in A) were analyzed by MALDI-TOF mass spectrometry. Compared with the theoretical masses of 1812.86 and 1913.91, weak signals were observed at masses of 1814.58 and 1916.33, which corresponded to the HEL peptides 31–46 and 31–47, respectively. The data are representative of two experiments.

**FIGURE 1.** Recognition of a second HEL determinant by the T cell hybridoma A6A2. Class II molecules were purified by immunoaffinity chromatography from 10^10 M12-Ak mHEL cells. The peptides were released by acid treatment and subsequently fractionated by reverse-phase HPLC. A, An amount of each 250-μl fraction was screened in a T cell bioassay, and the production of IL-2 was measured by CTLL proliferation. At 48 min, five fractions contained HEL peptides reactive to the T cell hybridoma A6A2, while at 72 and 78 min, five fractions containing HEL peptides with a core region of 52–61 stimulated the 3A9 T cell hybridoma. B, Fractions eliciting an A6A2 T cell response (shown in A) were analyzed by MALDI-TOF mass spectrometry. Compared with the theoretical masses of 1812.86 and 1913.91, weak signals were observed at masses of 1814.58 and 1916.33, which corresponded to the HEL peptides 31–46 and 31–47, respectively. The data are representative of two experiments.

For the isolation of peptides by peptide-specific immunoaffinity, we used lysates from 10^9 M12-Ak mHEL cells. The procedure consisted of five consecutive steps: 1) capture of the I-Ak molecules using 40F Ab (40F binds to all I-Ak molecules), 2) release of peptides bound to I-Ak, 3) capture of the peptide by the Ab to 34–45, 4) release of the peptide, and 5) analysis of the peptide by mass spectrometry. Specifically, the cell lysate was incubated for 2 h at 4°C with the Ab 40F coupled to cyanogen bromide-activated Sepharose beads (Sigma). Next, the material was loaded into a disposable 14-cm Polyprep chromatography column (Bio-Rad, Hercules, CA) and washed with at least 20 column volumes of wash buffer (20 mM MEGA 8/MEGA 9 in PBS). The I-Ak peptide complexes were subsequently eluted with 0.1% TFA, causing the simultaneous dissociation of this complex. The free peptide mixture was neutralized with 1 M Tris, pH 7.4, and then passed through a Centricron-10 concentrator (Amicon, Beverly, MA) to exclude the high molecular mass material. In a second chromatography step, this mixture was incubated with VAL-3 coupled to cyanogen bromide-activated Sepharose beads (Sigma). Next, the material was washed in and analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) or tandem mass spectrometry.

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was dried down; reconstituted in a total volume of 80 μl of a 2% acetonitrile, 0.6% acetic acid solution; and then pooled. The sample was subsequently subjected to tandem mass spectrometry.

**Analysis by mass spectrometry**

For MALDI-TOF mass spectrometry analysis, 0.5 μl of the sample (from the standard method) was mixed with 0.5 μl of the MALDI matrix solution 4-hydroxycyanocinnamic acid (Hewlett-Packard, Chicago, IL) on the matrix plate. MALDI-TOF analysis was performed in the linear mode on a PerSeptive Voyager-SPR Reflection instrument (PerSeptive Diagnostics, Framingham, MA) using the Voyager and GRAMS/386 software. The accelerating voltage was 25–30 kV, and the ions were desorbed by an N2 laser at 1 = 337 nm with a 3-nsec pulse width.

Samples from many of the experiments were subjected to capillary reverse-phase HPLC mass spectrometric analysis. Using the Rheodyne 7125 injector, we injected 15 μl of the prepared sample into the reverse-phase HPLC, which consisted of a Zorbax C18, 0.3-mm × 25-cm column (MicroTech, Sunnyvale, CA). Conditions of the gradient were as follows: solvent A (6% acetic acid in water) was kept constant at 10%; solvent B (acetonitrile) was kept constant at 2% for the first 5 min, increased from 2 to 15% in the next 5 min, and then increased from 15 to 90% at a rate of 0.9%/min; and solvent C (water) maintained the mixture at 100% while the eluent flow rate was maintained at 4.5 μl/min. The HPLC pump was a Waters 600MS equipped with pulse damping (silk board). The flow from the pump was split by using the LC packing accurate splitter with a 0.3-mm-wide column calibrator (LC Packing, San Francisco, CA). The total column effluent was then directed at the mass spectrometer.

Peptides were identified by mass and sequenced on a Finnigan liquid chromatography quadrupole ion-trap mass spectrometer (Finnegan, San Jose, CA) (i.e., in the MS and MS/MS modes, respectively). For MS mode, the scan range was at a mass-to-charge ratio (m/z) of 600-1300 in the profile mode, in which every three microscans were averaged to one scan. Acquisition was started 10–15 min after the commencement of the LCQ run. For MS/MS mode, the scan range was m/z 250-1850, again in profile mode, in which every three microscans were averaged to one scan. The parent ion was isolated with a mass window that was 2 m/z wide, and the collisional energy was 25% of the maximum energy. Sequence analysis was performed by comparing the experimental ion mass with the calculated ion mass or by an automated protein database sequencing program (SEQUEST; John Yates, University of Washington, Seattle, WA) on an ICIS workstation (Finnegan).

**Peptide-I-Aκ binding strength assay**

Detergent-solubilized I-Aκ, purified from the T2-Aκ cell line (provided by Dr. P. Cresswell, Yale University School of Medicine New Haven, CT) by affinity chromatography, was incubated with a radioactively labeled reference peptide (IκA) using the Voyager and GRAMS/386 software, a high affinity binder to I-Aκ (35). The amount of peptide required to compete out 50% of this reference peptide was determined by adding known amounts of unlabeled test peptide. Each reaction contained 10 μl of test peptide at different dilutions, 0.25 pmol of 125I-reference peptide, and 25 pmol of purified I-Aκ. After incubating at room temperature for 72 h, the peptide-Aκ complex was separated from free peptide by spinning the material through a Bio-Spin P6 gel filtration column (Bio-Rad). The excluded material was counted using an N2 laser at 1 = 337 nm with a 3-nsec pulse width.

**SDS stability of peptide I-Aκ complexes**

The strength of a peptide-MHC complex was also measured by its resistance to denaturation to SDS when run in SDS-PAGE (36, 37). Briefly, the peptides were radioactively labeled (125I) and incubated with purified I-Aκ for 48 h at room temperature. The complex was recovered by immunoprecipitation using the 40F Ab at a final concentration of 25 μg/ml. The samples were resolved on a 12% SDS polyacrylamide gel. As observed by autoradiography, the peptides remaining bound to the 40F dimer migrated to the top of the gel (SDS-stable peptides), while the dissociated peptides were found at the lower segment of the gel (SDS-unstable peptides). These images were scanned by a PhosphorImager (425E, Molecular Dynamics, Sunnyvale, CA) to determine the relative proportion of SDS-stable complex.

**Results**

**A second HEL determinant from I-Aκ molecules**

Previous studies from our laboratory identified T cells directed to a tryptic fragment of HEL encompassing residues 34–45 (29). The sequence of peptide 34–45 is Phe-Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg. The T cell response to this epitope in HEL-immunized mice was weaker, in comparison with the response of the dominant epitope 48–62. Moreover, peptide 34–45 was a weaker binder to I-Aκ (29). Therefore, our initial impression was that the 34–45 epitope was subdominant or minor.

Using our standard method for peptide isolation, we found the natural form of the 34–45 peptide difficult to isolate from APC exposed to HEL. (The standard method consists of an analysis of peptides extracted from I-Aκ by reverse-phase HPLC. Fractions that score positive in the T cell assay are then examined by mass spectrometry.) Earlier experiments that had been successful in isolating and characterizing the major epitope 48–62 failed to detect the 34–45 peptide (10). Two of three recent experiments revealed weak activity for the 34–45 HEL determinant. These results now are described so that a comparison can be made with the peptide immunosolation method.

Class II I-Aκ molecules were purified from 1010 M12-Aκ mHEL cells. By acid treatment, the complex mixture of peptides was released and then fractionated by reverse-phase HPLC. Each fraction was screened by the T-cell hybridoma 3A9 to identify the HEL peptides consisting of the core region 52–61. These peptides were identified at retention times of 72 and 78 min (Fig. 1A). In accordance with our previous observations, analysis by MALDI-TOF mass spectrometry revealed two prominent peptides, 48–62 and 48–63 (data not shown); these peptides were shown to have relative yields of 32 and 56%, respectively (10–12). In addition, each HPLC fraction was screened with the T cell hybridoma A6A2 that recognizes an epitope in the tryptic fragment 34–45. Five fractions with a retention time of 48 min showed detectable levels of IL-2, thus providing evidence for a second HEL determinant (Fig. 1A). It is important to note that we required 36% (or 90 μl) of each fraction to detect significant levels of IL-2 for this epitope, whereas only 4% (10 μl) was required to detect the major HEL epitope 48–62.

MALDI-TOF mass spectrometry analysis of the A6A2-positive fractions revealed no prominent peaks. Two weak signals with masses of 1814.6 and 1916.3 were comparable with the theoretical peptide masses, 1813.4 and 1914.4, corresponding to the HEL peptides 31–46 and 31–47, respectively (Fig. 1B). Clearly, the signals of the two peptides were too weak to incur any confidence in these masses.

We adjusted our approach by subjecting the T cell-positive fractions to the reverse-phase HPLC online and, this time, to the electrospray mass spectrometer, to improve the resolution of the peptide samples. Doubly charged ions [M + 2H]2+ were observed for the dominant HEL peptides that triggered the 3A9 T cell. Fig. 2A shows the m/z of 863 and 913, which corresponded to two members of this family, 48–62 and 47–62, respectively. In addition, the [M + 2H]2+ ions of 907.92+ and 957.72+ represented the minor HEL peptides 31–46 and 31–47, which stimulated the A6A2 T cell hybridoma. Ions to other minor HEL peptides were evident, such as the 1043.32+ ion (residues 31–49) (Fig. 2A). Thus, our initial MALDI-TOF results were confirmed. However, the abundance of these ions was still too weak among prominent contaminating peptides and thus difficult to analyze.
Using this last approach, we compared the recovery of the 31–47 peptide to the dominant HEL peptide 48–62. The abundances of these two naturally processed peptides were determined by electrospray mass spectrometry, comparing the amount recovered with known concentrations of synthetic peptide that were injected into the electrospray mass spectrometer. Fig. 3 shows a linear calibration curve of the 48–62 and 31–47 standard synthetic peptides. By determining the area of each ion chromatogram for the naturally processed HEL peptides 48–62 and 31–47 (Fig. 2B), the concentration of the recovered peptide was interpolated from the calibration curve. In the same experiment, the quantity of 48–62 peptide was 22.1 pmol compared with 1.01 pmol for the 31–47 peptide, indicating that the display of the 31–47 peptide was at least 22-fold lower than that of the 48–62.

**FIGURE 2.** Identification of the minor HEL peptides by electrospray mass spectrometry. The A6A2- and 3A9-positive fractions (shown in Fig. 1A) were dried down and reconstituted in 100 μl of 5% acetonitrile, 0.5% TFA. Fifteen percent of this sample was injected into the electrospray mass spectrometer, and after a 10-min delay, mass spectra were recorded continuously. A, [M + 2H]2+ ions with an m/z of 863.3 and 913.9 correspond to the HEL peptides 48–62 (m/z 863.4) and 47–62 (m/z 914.0) (top). The three observed ions, 907.97+, 957.77+, and 1043.32+ represent the minor HEL peptides 31–46 (m/z 907.5), 31–47 (m/z 958.0), and 31–49 (m/z 1044.0) (bottom). B, Peptides eluted from the online HPLC are shown as the total ion chromatogram (TIC; top). The naturally processed HEL peptides, 31–47 and 48–62, eluted at 17 and 25 min, respectively. Reconstructed ion chromatograms were derived for these two peptides at an m/z range of 862.0–865.0 (middle) and 956.6–960 (bottom). The area under the 48–62 chromatogram was calculated at 327 × 10^6, and the area under the 31–47 chromatogram was 17.4 × 10^6. A broad ion chromatogram was observed for the 48–62 peptide due to overloading of the sample on the capillary reverse-phase HPLC column. (This could not be avoided because of the relatively low abundance of the 31–47 peptide.) The recovery for each peptide was derived from the calibration curve in Fig. 3.

**FIGURE 3.** Quantitation of the naturally processed HEL peptides 48–62 and 31–47. A linear calibration curve was acquired by injecting 15 μl of known concentrations (5, 10, 50, 100, and 500 fmol/μl) of synthetic 48–62 and 31–47 peptide into the electrospray mass spectrometer (□). The area of the ion chromatogram was determined and plotted against each peptide concentration. Using the same conditions, we injected 15 μl of the 100-μl peptide sample and obtained ion chromatograms for the naturally processed 48–62 and 31–47 peptides (shown in Fig. 2B). The peptide concentration of 48–62 (168 fmol/μl) and 31–47 (8 fmol/μl), shown in dark symbols, was interpolated from the respective calibration curves. The total amount of peptide 48–62 was 20.1 pmol compared with 1.01 pmol for the 31–47 peptide.
FIGURE 4. Identification of a minor HEL epitope by peptide-specific immunoaffinity. Class II I-Ak molecules were purified from 10^9 B lymphoma cells. The associated peptides were released with 0.1% TFA, and HEL peptides containing the epitope FESNF were concentrated with the mAb VAL-3. The HEL peptides were injected into the reverse-phase HPLC online to the electrospray mass spectrometer, and mass spectra were acquired after 10 min. A. The m/z range of 600-1300 revealed no detectable HEL peptides from M12-Ak cells. B. In M12-Ak mHEL, doubly charged [M + 2H]^2+ ions ranging from 779.1 to 1043.8^2+ were detected. Thirteen of these ions corresponded to HEL peptides (Table I), of which the two most abundant ions, 907^2+ and 957^2+, corresponded to 31–46 and 31–47, respectively. Total ion chromatograms (TIC) are shown for the peptides eluted from M12-Ak (C) and M12-Ak mHEL (D) (note scale change). HEL peptides are eluted at 17.7, 18.0, and 18.2 min. Reconstructed chromatograms for the [M + 2H]^2+ ions 850.1^2+, 907.2^2+, 957.7^2+, 1015.1^2+, and 1043.8^2+ correspond to the 31 series of HEL peptides.
Isolation of the 31–47 HEL determinant by peptide-specific immunoaffinity

To improve the analysis of the 31–47 HEL epitopes, we developed an approach involving peptide-specific immunoaffinity chromatography. Class II I-A\(^k\) molecules were purified from the lysates of M12-A\(^k\) mHEL cells and from control M12-A\(^k\) cells. The peptides associated with I-A\(^k\) molecules were released by acid treatment, and HEL peptides were isolated by immunoaffinity capture using the Ab VAL-3 directed to the 34–45 peptide. These fractions were pooled and injected into the reverse-phase HPLC online to the electrospray mass spectrometer. Analysis of the peptide material isolated from the B lymphoma line expressing I-A\(^k\) showed no detectable peptides (Fig. 4A). By marked contrast, analysis of the peptides recovered from the M12-A\(^k\) mHEL cell line revealed an array of [M + 2H]\(^+\) ions ranging from a m/z of 779\(^+\) to 1043.8\(^+\) (Fig. 4B); 13 of these ions had masses corresponding to HEL peptides (Table I). The sequences of these peptides were confirmed by MS/MS analysis, which was performed on 10 of 13 peptides. An example of the collision-activated dissociation performed on the parent ions 907.2\(^+\) and 957.7\(^+\) is shown in Fig. 5. Furthermore, a search conducted in the protein sequence database, using the SEQUEST program, identified these sequences as HEL peptides.

Five peptides were identified starting with residue 31 (alanine) but differing at their carboxy terminus, ending at residue 45, 46, 47, 48, or 49. The two most abundant ions, 907.2\(^+\) and 957.7\(^+\), were specific to HEL peptides 31–46 and 31–47 (Fig. 4B). In addition, we identified eight other peptides, four of which started at position 32 (alanine) and four of which started at position 33 (lysine). These peptides had the same variation at the carboxy terminus as those observed with the peptides beginning at position 31. In three experiments, we did not detect the peptides 32–48 or 33–48 (see Discussion). The shortest peptide isolated was a 13-mer (residues 33–45) corresponding to the ion 779.0\(^+\), while the longest peptide was a 19-mer (residues 31–49) with an m/z of 1043.8\(^+\).

Analysis of the total ion chromatogram from M12-A\(^k\) mHEL shows the elution of HEL peptides at 17.7, 18.0, and 18.2 min (Fig. 4D). These elution times are specific for the peptides starting at position 33, 32, and 31, respectively. Based on the m/z of each ion, the total ion chromatogram was reconstructed for each HEL peptide (an example is provided in Fig. 4D). By contrast, the total ion chromatograms of M12-A\(^k\) showed no corresponding HEL peptides (Fig. 4C).

Quantitation of HEL peptides extracted from I-A\(^k\) molecules

We determined the recovery of peptides during the early steps of isolation and then attempted to quantitate them. First, the \(^{125}\)I-labeled HEL peptide 31–47 was incubated with purified class II I-A\(^k\) for 48 h at room temperature. The \(^{125}\)I-labeled 31–47\(^k\) complex was separated from free peptide and added to the cell lysate. The relative amount of peptide recovered after each step is shown in Table II. Thus, we estimated the peptide recovery to be about 50%.

Second, we were concerned that the more weakly binding 31–47 peptide might endure a preferential greater loss during the purification of I-A\(^k\). We addressed this issue by contrasting the recovery of \(^{125}\)I-labeled 31–47 peptide to the more strongly binding \(^{125}\)I-labeled 48–61 peptide. The peptides were incubated with purified class II I-A\(^k\) molecules for 48 h, and then the complexes were isolated from free peptide by centrifugation through a BioSpin P6 gel filtration column. The complexes were added into the cell lysate and recovered from an anti-class II immunoaffinity chromatography column. In accordance with the results described above, the eluted material consisted of 42% \(^{125}\)I-labeled 31–47 peptide compared with 31% \(^{125}\)I-labeled 48–61. Thus, during the isolation of I-A\(^k\), there were no preferential losses of the 31–47 more weakly binding peptides.

We also examined the efficiency of our VAL-3 immunoaffinity column. Ten picomoles of synthetic 31–47 peptide in pH-neutralized 0.1% TFA solution was incubated with 500 μl of a 50% VAL-3-cyanogen bromide-activated Sepharose slurry (250-μl bed volume). The peptide was eluted, and the analysis was performed by MALDI-TOF mass spectrometry. A recovery of 77% was quantitated by comparing the abundance of the 31–47 peptide with our reference peptide (AAKFDNSEFNTQASNRRT), which was added at 0.25 pmol/μl. Overall, the peptide recovery by the anti-peptide purification strategy before the mass spectrometry analysis was estimated to be 35%.

Finally, the naturally processed HEL peptides were recovered by peptide-specific immunoaffinity and quantitated by electrospray mass spectrometry. A calibration curve was generated for four sequence-specific synthetic peptides (residues 31–47, 32–47, 33–47, and 34–46) by injecting known amounts into the reverse-phase HPLC online to the electrospray mass spectrometer (Fig. 6). Using the same conditions as those for the standard peptides, we obtained ion chromatograms for each naturally processed HEL peptide (an example is provided in Fig. 4D) and calculated the area under each

### Table I. Recovery of naturally processed peptides from membrane HEL B-lymphoma line

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<td>1741.8</td>
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<td>32–47</td>
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<tr>
<td>9</td>
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<td>1771.8</td>
<td>33–47</td>
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<td>15</td>
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<td>13</td>
<td>972.6</td>
<td>1943.2</td>
<td>1943.9</td>
<td>33–49</td>
<td>KFESNFNTQATNRNTDGD</td>
<td>17</td>
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</tbody>
</table>

* Calculated mass = ([M + 2H]\(^+\)) \times 2 – 2.
FIGURE 5. Sequence analysis of the naturally processed HEL peptides. Collision-activated dissociation was performed on the [M + 2H]^{2+} ions at an m/z of 907.2^{±} (residues 31–46) (A) and 957.7^{±} (residues 31–47) (B) using the Finnigan LCQ ion trap mass spectrometer. The sequence was acquired by comparing the observed masses of the y and b ions with those of the corresponding theoretical ions. Note that the following ions are not shown to scale: b_{16}^{2+} (A) and 958.4^{±} and b_{17}^{2+} (B).
immunodominant HEL 48–61 peptide had an RIC\textsuperscript{2} volume of 80
isolated from the mAb VAL-3 were dried down and reconstituted in a total
Calibration curves (squares) FIGURE 6. Quantitation of the minor HEL peptides presented by I-A\textsuperscript{k}.

Characterization of the subdominant HEL peptide 31–47 to I-A\textsuperscript{k}
Our current binding studies were performed using the most abundant HEL determinant, 31–47, instead of 34–45, which was studied previously (29). Binding strength assays against the reference peptide (RIC\textsuperscript{–1} (1)) revealed the HEL 31–47 peptide to be a 30-fold weaker binder (RIC\textsuperscript{–1} (30)) (Fig. 7A). By comparison, the immunodominant HEL 48–61 peptide had an RIC\textsuperscript{–1} of 1.8, primarily attributed to the Asp\textsuperscript{32} anchoring residue (35, 38). SDS
stability is a measure of the ability of a peptide to bind strongly to the class II \alpha\beta dimer. While the immunodominant HEL peptide 48–61 was 97% SDS stable, the minor 31–47 peptide ran virtually 100% SDS unstable (Fig. 7B).

Discussion
We isolated a family of peptides from HEL, of which the most abundant (residues 31–47) is about 22-fold less abundant than the dominant 48–62 peptides. These peptides also have considerably less binding strength for the I-A\textsuperscript{k}. The analysis of the 31–47 peptides indicates the many problems with the isolation and quantification of class II MHC bound peptides, which can be resolved by the method of peptide immunoaffinity isolation. In brief, the problems with peptide isolation from class II molecules stem from the fact that a wide range of peptides with varying degrees of affinities, lengths, and compositions binds to them. This is in contrast to those that bind class I, in which there appears to be a more restricted set of peptides with regard to all of these parameters. Class I bound peptides tend to consist of 8–10 residues and to be of high affinity and with strict restrictions in their use of anchoring residues. The assays for class II peptide analysis that are now in place, which combine T cell responses and chemical analysis, by necessity introduce a strong bias against low affinity binding peptides. These peptides in complex mixtures are required to be at a higher molar concentration (for the T cell assay) and could be competed

| Table II. Peptide recovery from peptide isolation protocol\textsuperscript{a} |
|-----------------------------|-------------------|-----------------|
| Step                        | Isolation Step    | cpm             |
| Start \textsuperscript{b}   | \textsuperscript{125}I-31-47-A\textsuperscript{k} complex | 1,024,600       |
| 1                           | Peptide-A\textsuperscript{k} bound to anti-class II column | 922,007         |
| 2                           | Eluted peptide-A\textsuperscript{k} complex | 539,055         |
| 3                           | Centricon-10-filtered free peptide | 471,264         |
| % Peptide Recovered         |                   |                 |
| 100                          |                   |                 |
| 90                           |                   |                 |
| 53                           |                   |                 |
| 46                           |                   |                 |

\textsuperscript{a} \textsuperscript{125}I-31-47 peptide was incubated with 50 pmol of purified I-A\textsuperscript{k}. The complex was separated by centrifugation through a Bio-spin P6 gel filtration column after 48 h. The I-A\textsuperscript{k} purification protocol was followed. The peptide-A\textsuperscript{k} complex was spiked into 6 ml of cell lysate from the M12-A\textsuperscript{k} B lymphoma line. At each step, samples were counted using the gamma counter to determine the percentage of peptide recovered.

\textsuperscript{b} These peptides in complex mixtures are required to be at a higher molar concentration (for the T cell assay) and could be competed

Table III. Total recovery of naturally processed HEL peptides\textsuperscript{a}

<table>
<thead>
<tr>
<th>Peptide Fragment</th>
<th>Peptide Recovery (fmol)</th>
<th>Total Estimated Content</th>
<th>Relative Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31–45\textsuperscript{1}</td>
<td>40</td>
<td>114</td>
<td>0.96</td>
</tr>
<tr>
<td>31–46\textsuperscript{1}</td>
<td>947</td>
<td>2,705</td>
<td>22.7</td>
</tr>
<tr>
<td>31–47\textsuperscript{**}</td>
<td>1,273</td>
<td>3,637</td>
<td>30.5</td>
</tr>
<tr>
<td>31–48\textsuperscript{**}</td>
<td>74</td>
<td>211</td>
<td>1.8</td>
</tr>
<tr>
<td>31–49\textsuperscript{**}</td>
<td>261</td>
<td>746</td>
<td>6.3</td>
</tr>
<tr>
<td>32–45\textsuperscript{1}</td>
<td>4</td>
<td>11</td>
<td>0.1</td>
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<tr>
<td>32–46\textsuperscript{1}</td>
<td>222</td>
<td>634</td>
<td>5.3</td>
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<tr>
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<td>200</td>
<td>571</td>
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<tr>
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<td>47</td>
<td>134</td>
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<tr>
<td>33–45\textsuperscript{*}</td>
<td>126</td>
<td>360</td>
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<td>418</td>
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<td>33–47\textsuperscript{*}</td>
<td>382</td>
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<td>33–49\textsuperscript{*}</td>
<td>177</td>
<td>506</td>
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<tr>
<td>Total</td>
<td>4,171</td>
<td>11,917</td>
<td>100</td>
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</table>

\textsuperscript{a} Peptides were quantitated by mass spectrometry as described in Results. The amount of each naturally processed peptide was interpolated from four standard synthetic peptides, 31–46, 31–47, 32–47, *33–47. The recovery of each peptide was estimated to be 35%. The amount of class II I-A\textsuperscript{k} purified was determined by the Folin-Ciocalteu protein assay to be 2.4 \times 10\textsuperscript{8} molecules per APC, in accordance with recent direct estimates on cells (41). From this estimate and knowing the content of 11.9 pmol/10\textsuperscript{9} cells, we concluded that 0.3% of the I-A\textsuperscript{k} molecules were occupied by the 31–47 series peptides. The purity of I-A\textsuperscript{k} was estimated to be 90% when resolved on a 12% SDS-polyacrylamide gel.
The binding strength of each peptide was assessed by its ability to inhibit the binding of the radiolabeled standard peptide to I-A\(^k\). The amount of the standard peptide required to inhibit 50% of the labeled peptides binding was 4.8 pmol. By comparison, 145 pmol was required of the 31–47 peptide and only 8.4 pmol for the 48–61 peptide. The RIC\(^1\) of the peptide was determined by dividing these values by the value obtained for the standard peptide. The 31–47 peptide has an RIC\(^1\) of 30, whereas the RIC\(^1\) for 48–61 is 1.8.

The ability of HEL peptides to stabilize the class II \(\alpha\beta\) dimer was analyzed by SDS-PAGE. The 31–47 peptide migrated at the gel front (SDS-unstable). By comparison, the 48–61 peptide migrated at the top of the gel (SDS-stable) bound in the \(\alpha\beta\) dimer. The peptides were synthesized and radioiodinated as described in Materials and Methods. Each labeled peptide was incubated with purified I-A\(^k\) at room temperature for 48 h, and then the complex was recovered by immunoprecipitation using the anti-I-A\(^k\) mAb (40F). The samples were incubated in SDS sample buffer at room temperature and then loaded onto a 12% SDS-polyacrylamide gel. The molecular mass markers are represented in kilodaltons. The data are representative of five experiments.

FIGURE 7. Binding properties of the 31–47 and 48–62 peptides to I-A\(^k\). A, The binding strength of each peptide was assessed by its ability to inhibit the binding of the radiolabeled standard peptide to I-A\(^k\). The amount of the standard peptide required to inhibit 50% of the labeled peptides binding was 4.8 pmol. By comparison, 145 pmol was required of the 31–47 peptide and only 8.4 pmol for the 48–61 peptide. B, The ability of HEL peptides to stabilize the class II \(\alpha\beta\) dimer was analyzed by SDS-PAGE. The 31–47 peptide migrated at the gel front (SDS-unstable). By comparison, the 48–61 peptide migrated at the top of the gel (SDS-stable) bound in the \(\alpha\beta\) dimer. The peptides were synthesized and radioiodinated as described in Materials and Methods. Each labeled peptide was incubated with purified I-A\(^k\) at room temperature for 48 h, and then the complex was recovered by immunoprecipitation using the anti-I-A\(^k\) mAb (40F). The samples were incubated in SDS sample buffer at room temperature and then loaded onto a 12% SDS-polyacrylamide gel. The molecular mass markers are represented in kilodaltons. The data are representative of five experiments.

by other peptides in the mixture. Added to these issues are the loss of peptides during all the steps from class II isolation to the mass spectrometry analysis.

We can state with confidence that the 31–47 peptide family consists of truly minor peptides. The possibility that the peptides were displayed in large amounts but lost, or were not detected by T cell assay, has now been eliminated. There is considerable loss of peptides, as others have noted (39, 40), when we involve a first step of HPLC separation offline from the mass spectrometer (Figs. 1 and 2). For example, the recovery of 31–47 was 1.01 pmol per 10\(^9\) cells (Fig. 2B) compared with the immunofluorescence capture procedure that yielded 12 pmol (in Fig. 6, we estimated a recovery of 1.2 pmol/10\(^9\) cells) (i.e., about 92% of this peptide was lost during the initial analysis). Our estimates of occupancy of M12 C3.F6 (an APC very rich in I-A\(^k\), about 1–2 x 10\(^6\) sites per cell) with the 31–47 series peptide was about 0.3%. In our previous studies we estimated an occupancy with the 48–62 series of 6–9% (41). In summary, the immunofluorescence step bypasses the T cell assay, serves to concentrate the peptides, and permits a more reliable analysis and quantitation. We believe the use of a peptide immunoaffinity procedure will make it considerably easier to identify the whole spectrum of MHC-selected peptides from a known protein.

One factor that may determine the low representation of 31–47 peptides may be their affinity for I-A\(^k\). In our analysis of HEL peptides, the most frequently displayed peptides bound to either I-A\(^k\) or I-E\(^k\) are those with high binding strength (9). Also, in the analysis of self-peptides bound to I-A\(^k\), none is represented to the extent of 48–62, but neither do any of them display the high binding strength of 48–62. Nevertheless, more extensive analysis should tell us how strict the relationship is between extent of peptide display and binding strength for class II molecules. Concerning the 31–47 peptide, in ongoing studies we have been able to identify a major anchoring residue, Asn\(^{39}\), as well as residues that hinder the binding. Whether Asn\(^{30}\) fits into the P1 pocket of I-A\(^k\) (38) is not clear at present. Structural analysis of this peptide-I-A\(^k\) complex is in progress.

The family of 31–47 peptides, like many class II peptides, was displayed as a series varying in amino and carboxy termini. Here, the predominant species (about 53%) started at residue 31, ending at either 46 or 47. Our interpretation of the reasons for these nested peptides lies in the nature of the processing event. Our data lead us to believe that HEL is first denatured, allowing the opened molecule to interact with I-A\(^k\) molecules. I-A\(^k\) molecules select the segments of HEL bearing the higher affinity sequences. Once bound, the segment is protected from catabolism but is then trimmed by amino- and carboxypeptidases. Trimming extends to the edges of the combining site but is somewhat variable, leaving ends of different size. Our data are supported by experiments in which the addition of proline to the flanking residues of HEL-dominant peptide resulted in extensions of the peptide (11). We favor this scenario over other possibilities, such as an initial cutting of the molecule by cathepsins with subsequent selection of peptides. With this in mind, it is likely that presentation of the dominant segment, 48–62, may hinder the presentation of the 31–47 segment. We can conclude that at least 13% of the HEL molecules identified peptide display and binding strength for class II molecules. Concerning the 31–47 peptide, in ongoing studies we have been able to identify a major anchoring residue, Asn\(^{39}\), as well as residues that hinder the binding. Whether Asn\(^{30}\) fits into the P1 pocket of I-A\(^k\) (38) is not clear at present. Structural analysis of this peptide-I-A\(^k\) complex is in progress.

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Acknowledgments

We thank Ms. Shirley Petzold for technical assistance, Ms. Mary Walton for proofreading the manuscript, and Ms. Janet Casmaer for preparation of the manuscript.

References


