Chemical Identification of a Low Abundance Lysozyme Peptide Family Bound to I-A^{k} Histocompatibility Molecules*

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The processing by antigen-presenting cells (APC) of the protein hen egg-white lysozyme (HEL) results in the selection of a number of peptide families by the class II major histocompatibility complex (MHC) molecule, I-A^{k}. Some of these families are expressed in very small amounts, in the order of a few picomoles/10^{6} APC. We detected these peptides from an extract of class II MHC molecules by using monoclonal anti-peptide antibodies to capture the MHC-bound peptides prior to their examination by HPLC tandem mass spectrometry. Here, we have identified several members of a family of peptides encompassing residues 20–35, which represent less than 1% of the total HEL peptides. Binding analysis indicated that the core segment of the family was represented by residues 24–32 (SLGNWVCAA). Asn-27 (shown in boldface) is the main MHC-binding residue, mapped as interacting with the P4 pocket of the I-A^{k} molecule. Analysis of several T cell hybridomas indicated that three residues contacted the T cell receptor: Tyr-23 (P–1), Leu-25 (P3), and Trp-28 (P5). The HEL peptides isolated from the APC extract were sulfated on Tyr-23, but further analysis showed that this modification did not occur physiologically but took place during the peptide isolation.

To understand the nature of the CD4 T cell response to peptides in a protein antigen, it is important to explain the basis for peptide selection during processing. It requires, therefore, the identification and quantification of the peptides that are selected by the class II MHC1 molecules of the antigen-presenting cells (APC). There are two major difficulties with the identification of the class II MHC-bound peptides. The first is that peptides are selected as families, which comprise many members; a family is characterized by having a core sequence that interacts with the P1 to P9 anchoring sites of the MHC binding groove and flanking residues (1–7). The flanking residues vary greatly in length and determine the number of members in a family. The flanking residues contribute to the affinity of binding to class II molecules and to the half-life of the peptide-MHC complex on the surface of APC (7–10). Additionally, flanking residues can have a marked effect on the T cell recognition (11, 12). The length of the peptides and their size heterogeneity, however, make it difficult to purify them for analysis as well as to predict binding motifs. A second difficulty with the analysis of class II-bound peptides is that their amounts vary greatly among the various families, by as much as 250-fold (13). Thus, characterization of these heterogeneous peptide families is particularly difficult.

Studying the HEL protein as an antigen, we identified most of the segments selected for presentation by the I-A^{k} molecule (14). Most of the HEL-specific T cell response was directed to the HEL epitopes: HEL-(18–33), HEL-(31–47), HEL-(48–63), and HEL-(115–129) (14). In a previous study, we identified the naturally processed peptides of the HEL-(48–63) family (DGSTDYGILQINSRWW) (4), the most abundant epitope, occupying up to 10–20% of the total class II MHC molecules (15). However, we had to develop a peptide immunoaffinity capture technique to isolate and characterize the lower abundance HEL peptide families (9). This approach consisted of purifying and concentrating the HEL peptides from a class II MHC peptide extract by using an anti-HEL peptide-specific monoclonal antibody (mAb) followed by their identification by HPLC/electrospray tandem mass spectrometry (ESI-MS). This method allowed the identification and quantification of the HEL-(91–47) peptide family (AAKFSNFNTQATNRNT) (9). The anti-HEL peptide-specific antibodies were also used by applying ELISA methods to quantitate the peptides in the class II MHC peptide extract (13).

In this study we report our studies of a second HEL peptide family that stimulates T cells, although it is represented in very low amounts on the APC class II molecules, i.e. ~100 copies of 20–35-mer peptide/10^{6} I-A^{k} molecules (13). Despite the 20–35-family occurring in extremely small amounts, we were able to characterize five members by using the antipeptide capture technique.

MATERIALS AND METHODS

Cell Lines—The murine B cell lymphoma lines M12.C3.F6 (M12-A^{k}) (16) expressing class II I-A^{k} molecules and the M12.C3.F6 cell line transfected with a membrane form of HEL (M12-A^{k} mHEL) were used (17). Three other C3F6 cell lines expressing mutations of HEL were generated as described previously (18). The lines contained alamime substitutions for Ser-24, Asn-27, or Cys-30. All cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% heat-

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inactivated normal calf serum and were tested for levels of I-A<sup>+</sup> and HEL before their analysis.  

**Synthesis of Peptides**—Unmodified HEL peptides and phosphorylated peptides were synthesized by Fmoc chemistry (N-9-fluorenyl-methoxycarbonyl; model 432A; Applied Biosystems, Foster City, CA) and purified through HPLC (Macherey-Nagel, Milford, MA). The sequences of all peptides were confirmed subsequently by mass spectrometry. HEL-(20–35) peptides sulfated at either Tyr-20 or Tyr-23 were purchased from Synpep (Dublin, CA).

**Production of mAb**—A mAb (15B7–Ib5, IgG<sub>1</sub>) that recognizes the HEL-(18–33) epitope (DNYRGYSLGNWVCAAK) was generated as described previously (13). HEL-(18–33) had been identified previously with a mAb used as one segment that stimulated T cell reactivity. This mAb recognizes the free HEL peptide in solution or bound to an ELISA plate. Briefly, the anti-HEL-(18–33) peptide mAb was generated by repeated immunizations of CB.17 mice with synthetic HEL-(18–33) peptide coupled to OVA protein using bromoacetyl succinimide (Sigma). A cysteine was added to the carboxyl terminus of the peptide to facilitate coupling to OVA, and a serine was substituted for cysteine at detergents (Sigma) in phosphate-buffered saline in the presence of inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetadetergents (Sigma) in phosphate-buffered saline in the presence of 10 column volumes of Milli-Q water. After the washes, the 40 F reading of the blank solution; the excess salt in the column was removed with distilled water. After the washes, 280 nm and 214 nm of the washing solution were equal to the OD.

**Peptide Binding Assays**—I-A<sup>+</sup> molecules expressed in baculovirus were used in a binding competition assay with a standard radiolabeled peptide (20). I-A<sup>+</sup> molecules were expressed containing a peptide from the invariant chain, the CLIP peptide, tethered to a linker segment having a thymbin-sensitive sequence. The purified molecules were treated with thrombin at the same time that a standard T-125 radiolabeled HEL peptide was added. (The peptide was HEL-(52–61) with an added tyrosine at the amino terminus: YDYGILQNSR). The time of incubation was 18–24 h; all reactions were carried out at pH 5.5. The amounts of peptide bound about 20–30% of input cpm. Variable amounts of nonlabeled peptides were tested in the reactions, and the bands that indicated the binding of the standard radiolabeled peptide to I-A<sup>+</sup> were estimated.

**Studies on Tyrosine Sulfation of HEL Peptides**—For a control on the finding of sulfation of the peptide, I-A<sup>+</sup> molecules containing a synthetic peptide were added to a control cell lysate from M12-A<sup>+</sup> cells. I-A<sup>+</sup> molecules expressed in baculovirus (20) or isolated from APC (21) were used to make the HEL-(20–35) peptide-I-A<sup>+</sup> complex in vitro. Similar results were obtained with both I-A<sup>+</sup> preparations. I-A<sup>+</sup> molecules (300 μg) were incubated with 2×10<sup>5</sup> ml of saline, 100 μl of HEL-(20–35) peptide in the presence of 50 μl of 0.5% of phosphate buffered saline and 1 ml 2-mercaptoethanol sulfonic acid at pH 5.5. The mix reaction was incubated for 7 days at room temperature. The HEL-(20–35)I-A<sup>+</sup> complex was added into a control M12-A<sup>+</sup> cellular lysate obtained from ~10<sup>7</sup> HEL-(20–35) cell lysed with 100 ml of MEGA 8/MEGA 9 solubilization buffer (10<sup>6</sup> cell equivalents/1 ml of lysate). The isolation of the HEL-(20–35) peptide and its MS analysis was performed as described above.
molecules were purified from 1.7 × 10^10 M12.A^k mHEL cells, and the peptides bound to I-A^k molecules were isolated using the anti-HEL-(18–33) peptide mAb column. The mass spectrum of the naturally processed HEL peptides shows several doubly charged [M + 2H] ions with an m/z of 932.5, 990.0, and 1047.1 corresponding to peptides HEL-(20–35), HEL-(19–35), and HEL-(18–35), respectively. In addition to those peptides, the mass spectrum shows another three ions with 40 mass units higher than the HEL peptides.

To evaluate the tyrosylprotein sulfotransferase (TPST) activity in the M12.A^k cellular lysate, we used as a high-affinity substrate for TPST, the acidic random polymer poly(Glu-Ala-Tyr) (EAY 6:3:1, M_r 43,000 (Sigma)) (22) and as sulfate donor, radioactive 3’-phosphoadenosine 5’-phosphosulfate ([35S]PAPS; PerkinElmer Life Sciences). The assay mixture contained, in a final volume of 100 μl, 10 μl of M12.A^k cellular lysate (6 × 10^6 cells/ml of solubilization buffer), 20 μl [35S]PAPS, and 100 μl EAY. The reaction was incubated at 4 °C overnight and stopped with 2× SDS-sample buffer. Samples were analyzed by SDS-PAGE. Alternatively, the samples were precipitated with 20% trichloroacetic acid, and the incorporated radioactivity in the samples was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

To isolate and quantify the naturally processed forms of the HEL-(18–33) epitope, we used the 15B7–1B5 specific to the HEL peptide sequence from residues 18–33: DNYRGYSGLNWVCAAK (13). This mAb recognizes the HEL-(18–33) peptide both in solution and bound to an ELISA plate and had been used to determine the abundance of this HEL peptide on I-A^k molecules isolated from an APC line that expresses a membrane form of HEL, M12.A^k mHEL. The HEL-(18–33) epitope (13) was expressed in low abundance on the M12.A^k mHEL APC, i.e. about 9 pmol of peptide/5.6 × 10^6 APC. In this study, we isolated the natural sequence, represented mostly by the 20–35 peptide, and identified the amino acid residues responsible for anchoring the peptide to the I-A^k molecules as well as those residues that interact with the T cell receptor (TCR). We call attention to the finding that the peptide was found sulfated, but this change took place following its isolation.

Identification of Naturally Processed Forms of the HEL-(18–33) Epitope—We first extracted all of the peptides bound to class II molecule I-A^k of the M12.A^k mHEL line. Then the members of the HEL-(18–33) peptide family were isolated and concentrated by immunoaffinity chromatography using the 15B7–1B5 mAb specific to the peptide-(18–33). The eluted peptides were analyzed by mass spectrometry. In the mass chromatogram of the purified peptides, we observed several peptides eluting at retention times between 28 and 30 min. The molecular ions were doubly charged ions [M + 2H]^{2+} with an m/z of 932.5, 990.0, and 1047.1. These corresponded to the peptides HEL-(20–35) (DNYRGYSGLNWVCAAKFE), HEL-(19–35) (NYRGYSGLNWVCAAKFE), and HEL-(18–35) (DNYRGYSGLNWVCAAKFE), respectively (Fig. 1). The most abundant member of this HEL family was peptide-(20–35). The amino acid sequence of the HEL-(20–35) peptide was determined by MS/MS analysis; a search conducted in the protein sequence data base, using the SEQUEST program, identified this sequence as corresponding to the HEL peptide.

In addition to these HEL peptide masses, we observed three doubly charged ions with 40 m/z units higher that the HEL peptides and with identical retention times. The m/z values of these ions were 972.5^{2+}, 1030.5^{2+}, and 1087.5^{2+} (Fig. 1). This m/z difference indicates that the HEL peptides were likely

FIG. 1. Identification of the naturally processed HEL-(20–35) peptide family on M12.A^k mHEL cells. Class II molecules were purified from 1.7 × 10^{10} M12.A^k mHEL cells, and the peptides bound to I-A^k molecules were isolated using the anti-HEL-(18–33) peptide mAb column. The mass spectrum of the naturally processed HEL peptides shows several doubly charged [M + 2H] ions with an m/z of 932.5, 990.0, and 1047.1 corresponding to peptides HEL-(20–35), HEL-(19–35), and HEL-(18–35), respectively. In addition to those peptides, the mass spectrum shows another three ions with 40 mass units higher than the HEL peptides.

FIG. 2. The naturally processed HEL-(20–35) peptide is modified by a sulfated group. A, naturally processed HEL-(20–35) peptide family was isolated from M12.A^k mHEL APC, and the HEL-(20–35) peptide (the most abundant member of this family) was submitted to CID in the Finnigan LCQ ion trap mass spectrometer. The resulting product ion spectrum was compared with the CID spectra of the naturally processed HEL-(20–35) peptide family isolated from M12.A^k mHEL APC. The HEL-(20–35) peptide, the most abundant member of this family, was submitted to CID in the Finnigan LCQ ion trap mass spectrometer. The resulting product ion spectrum was compared with the CID spectra of the naturally processed HEL-(20–35) peptide. The CID spectra of the naturally processed HEL peptide and the synthetically sulfated HEL-(20–35) peptide are very similar.
post-translationally modified by either a phosphate or sulfate group, either of which would add a mass of 80 Da to the peptide or protein (for doubly charged ions these modifications change the \( m/z \) of peptides by 40). Although we used larger \( m/z \) mass ranges for many of our experiments (i.e. \( m/z \) 700–1400) than that shown in Fig. 1, we found no signals of greater intensity than those of the putative sulfated peptides and the peptides themselves.

The HEL-(20–35) Epitope Is Modified by a Sulfate Group—The amino acid sequence of the HEL-(20–35) epitope (YRGYS-LGNWCAAKFE) contains three potential residues to be modified: the tyrosines at positions 20 and 23, which could be modified by either sulfation or phosphorylation, and a serine residue at position 24, which could be phosphorylated. We ruled out the phosphorylation at serine 24 as a possible modification because the recognition of the anti-HEL-(18–33) mAb (15B7–18B5) used to isolate this HEL peptide family depends on an unmodified serine 24 (data not shown). Therefore, the only two possible modification sites on the HEL-(20–35) epitope are the two tyrosine residues at positions 20 and 23.

FIG. 3. Tyrosine sulfation of the HEL-(20–35) epitope is generated during the peptide isolation procedure. Unmodified HEL-(20–35) peptide-I-A\(^{a}\) complex was generated in vitro and added into a control M12-A\(^{a}\) cellular lysate. A, mass spectrum of the HEL-(20–35) peptide isolated from the unsulfated HEL-(20–35) peptide-I-A\(^{a}\) complex before being added into the control cellular lysate. Note the presence of unsulfated HEL-(20–35) peptide, mass ion 932.9. B, mass spectrum of the HEL-(20–35) peptide isolated from the unsulfated HEL-(20–35) peptide-I-A\(^{a}\) complex after added into the control cellular lysate. The HEL peptide/I-A\(^{a}\) complex was incubated overnight at 4 °C. Note the presence of sulfated HEL-(20–35) peptide, mass ion 972.5.

FIG. 4. Presence of TPST activity in the M12-A\(^{a}\) cellular lysate. The TPST activity in the cellular M12-A\(^{a}\) lysate was measured using the EAY polymer (poly(Glu-Ala-Tyr)) as a substrate for TPST. EAY (100 \( \mu M \)) was incubated with 10 \( \mu l \) of M12-A\(^{a}\) cellular lysate (5 \( \times 10^{5} \) cell equivalent/ml of solubilization buffer) in the presence of radioactive ([\( ^{35}S \)]PAPS (20 \( \mu M \)) and cold PAP (100 \( \mu M \)). The total volume of the assay mixture was 100 \( \mu l \). The TPST activity is represented as the incorporated radioactivity in dpm.

To identify the nature of the modification present on the naturally processed HEL-(20–35) epitope, we isolated from the M12-A\(^{a}\)mHEL line the HEL-(20–35) peptide family. The most abundant member of this family (HEL-(20–35) peptide) was submitted to CID in the ion trap. The resulting product-ion spectrum was compared with those of synthetic sulfated or phosphorylated HEL-(20–35) peptides. The product-ion spectra of the naturally processed HEL peptide and the synthetic modified peptides are very similar (Fig. 2). In contrast, the CID spectrum of the synthetic phosphorylated HEL-(20–35) peptide is significantly different. This observation strongly indicates that the HEL-(20–35) peptide isolated from APC was sulfated (not phosphorylated) on tyrosine. In subsequent MS experiments, we identified the tyrosine at position 23 as the amino acid modified by the sulfate group. Synthetic peptides with sulfated tyrosine at either residue 20 or 23 were added to the natural peptide and submitted for analysis; the addition of the Tyr-20 sulfated peptide resulted in two peaks (retention times of 28.6 and 29.5 min) that were base line-resolved, whereas addition of the Tyr-23 sulfated peptide only yielded one peak (29.5 min).

Interestingly, when we analyzed the mass spectrum of the synthetic HEL-(20–35) peptide that was sulfated at Tyr-23, we observed two ions with \( m/z \) values of 932.5\(^{2+}\) and 972.5\(^{2+}\). Those \( m/z \) values correspond to the unsulfated and sulfated forms of the HEL-(20–35) peptide, respectively. This facile loss of sulfur trioxide that accompanies electrospray ionization of a sulfated peptide was described previously (23, 24) and it is due to the weak bond between the sulfate group and peptide (25) and the thermochemical stability of SO\(_3\). Furthermore, there were a considerable number of fragment ions generated in the CID of the synthetic phosphorylated peptide, which were not present either in the product-ion spectrum of the naturally processed peptides or in that of the synthetically sulfated peptides (Fig. 2). The loss of SO\(_3\) from sulfated peptides occurs so readily that the typical fragmentation of the peptide chain to give b and y ions, for example, is not competitive. The stronger linkage to phosphate, however, allows the other fragmentations to compete with the loss of the phosphate group. Others have pointed out this contrast between sulfated and phospho-
rylated peptides (23–25), and the difference appears to be general.

The loss of 98 atomic mass units (either H3PO4 or HPO3 and H2O) that is observed for the phosphorylated standard is often viewed as difficult or impossible from peptides that contain phosphorylated Tyr, as exemplified in a recent report (26). Nevertheless, there is ample precedent for this loss from singly charged phosphopeptides, beginning with some of the earliest reports of matrix-assisted laser desorption ionization (MALDI)/MS of these materials (27–29). The precedent that is most relevant to our observations is that reported by DeGnore and Qin (30), who showed that doubly charged 20-mer peptides phosphorylated on Tyr can lose 98 Da in CID to produce abundant product ions. It is likely that the loss takes place in two steps, the first being that of HPO3 and the second that of H2O. This loss of 98 Da, however, is not general for peptides that contain phosphotyrosine, and the rules governing its occurrence are not known.

We emphasize that the mass spectrum of the naturally processed HEL-(20–35) peptide family also showed the sulfated and unsulfated versions of the HEL-(20–35), HEL-(19–35), and HEL-(18–35) peptides (Fig. 1). All of these sulfated and unsulfated forms showed identical retention times in the HPLC preceding the MS, indicating that the signal for the unsulfated HEL peptides in this mass spectrum was because of the loss of the sulfur trioxide from the sulfated peptide during the ionization process in the mass spectrometer and not the presence of both sulfated and unsulfated HEL peptides in the original sample.

In subsequent MS experiments, we showed that the peptide remaining after loss of SO3 is indeed HEL-(20–35) by comparing its product-ion spectrum (MS/MS) with that of the unknown. The spectrum of the synthetic standard HEL-(20–35) gave an abundant but incomplete series of b ions and a less abundant and incomplete series of y ions (relative abundance is shown in parentheses): b15 (100%), b14 (5%), b13 (55%), b12 (20%), b11 (15%), b10 (35%), b9 (15%), b8 (10%), b7 (5%), and b4 (5%); y14 (5%), y13 (5%), y12 (5%), y11 (5%), and y4 (5%).

The naturally processed peptide, which we found in lesser quantities than the standard, gave a spectrum that agrees well with that of the standard (nd indicates “not detected”): b15 (100%), b14 (5%), b13 (35%), b12 (20%), b11 (10%), b10 (20%), b9 (10%), b8 (nd), b7 (nd), and b4 (nd); y14 (3%), y13 (5%), y12 (5%), y11 (5%), and y4 (nd).

Some of the low abundance ions could not be detected because of the (chemical) noise level in certain m/z regions of the naturally processed species’ spectra was poorer than that of the standard. Nevertheless, the agreement between spectra and

**Table I**

| T cell contact residues of the HEL-(20–35) epitope |
|--------------------------------------------------|
| This table summarizes the data shown in Fig. 7, n, no response or very poor response at the highest peptide concentration tested (10 μM). ND, not determined. |

| T cell | Y20A | R21A | G22 | Y23A | S24A | L25A | G26 | N27A | W28A | V29A | C30A | Ala-31 | Ala-32 | K33A | F34A | E35A |
|-------|------|------|-----|------|------|------|-----|------|------|------|------|--------|--------|------|------|------|
| NIH18 | +    | +    | +   | +    | +    | -    | -   | +    | +    | +    | +    | +      | +      | +    | +    | +    |
| S8F   | +    | +    | +   | +    | -    | +    | -   | -    | +    | +    | +    | +      | +      | +    | +    | +    |
| HC49  | +    | +    | +   | -    | +    | -    | -   | +    | +    | +    | +    | +      | +      | +    | +    | +    |
| I3F12 | +    | +    | +   | +    | +    | +    | +   | +    | +    | +    | +    | +      | +      | +    | +    | +    |
| I1H9  | +    | +    | +   | -    | -    | -    | -   | -    | +    | +    | +    | +      | +      | +    | +    | +    |
| I2E4  | +    | +    | +   | +    | -    | +    | -   | +    | +    | +    | +    | +      | +      | +    | +    | +    |
| I2H12 | +    | +    | +   | +    | +    | +    | +   | +    | +    | +    | +    | +      | +      | +    | +    | +    |
| I3H10 | +    | +    | +   | +    | +    | +    | +   | +    | +    | +    | +    | +      | +      | +    | +    | +    |
| II5D1 | +    | +    | +   | +    | +    | +    | +   | +    | +    | +    | +    | +      | +      | +    | +    | +    |
| II5C6 | +    | +    | +   | +    | +    | +    | +   | +    | +    | +    | +    | +      | +      | +    | +    | +    |
| II1D7 | +    | +    | +   | +    | +    | +    | +   | +    | +    | +    | +    | +      | +      | +    | +    | +    |
|       | +    | -    | +   | +    | +    | +    | +   | +    | +    | +    | +    | +      | +      | +    | +    | +    |

*The T cell hybridomas tested were generated and selected using the HEL-(18–33) peptide. Therefore, the recognition of the Ala-substituted peptides at positions 34 and 35 was possible as expected. To know whether these two amino acids (Phe-34 and Asp-35) play a role in the T cell recognition of peptide-(20–35), we generated T cell hybridomas specific to HEL-(20–35) peptide, all of them (10 of 10) recognized the Ala-substituted peptides-(20–35) Ala-34 and Ala-35, indicating that these two amino acids are not T cell contact residues.*

**Table II**

**Summary of HEL peptides**

This table indicates the three major members of the peptide families studied thus far. The P1 to P9 sequence is underlined, and the main residues responsible for binding are in bold type. Residues that are underlined are those identified as TCR contacts. Binding studies are found in Refs. 3 and 8. Quantitation studies were reported in Ref. 13.

| Peptide | Peptide segment | Binding IC50 | Amounts |
|---------|----------------|--------------|---------|
| 48–63   | DGSTDYGILQINSRW | μM | pMol/106 |
| 31–47   | AKFESNFINTQATNRNT | 0.9 | 7 |
| 20–35   | YRGYSNLGWVCAAKFE | 1.4 | 2 |
the sequence coverage add confidence that the naturally processed peptide is sulfated HEL-(10–35).

In experiments not shown, we also examined HEL peptides from the M12.C3.F6 line cultured in the presence of exogenous HEL. We identified HEL-(20–35), HEL-(19–35), and HEL-(18–35) as observed in the M12.Ak mHEL APCs experiment (Fig. 1). The most abundant member of this HEL peptide family was the peptide HEL-(20–35). We also identified peptide-(20–36) and -(20–37) but in lesser abundance. All of the HEL peptides carried a post-translational modification of 80 atomic mass units, as indicated by the two signals separated by 40 m/z in the mass spectra.

Tyrosine Sulfation of the HEL-(20–35) Epitope is an Artifact Generated during the Peptide Isolation Procedure—Tyrosine sulfation is a post-translational modification of secretory, plasma membrane, and lysosomal proteins occurring in all multicellular eukaryotic organisms (31, 32). This modification is catalyzed by the TPST, an integral membrane glycoprotein residing in the trans-Golgi network (33). TPST uses PAPS as the sulfate donor in the sulfate transfer reaction. Based on the Trans-Golgi localization of TPST, we were surprised by the isolation of sulfated HEL peptides from APC fed with exogenous HEL protein. Therefore, we evaluated whether the tyrosine sulfation of this HEL peptide was occurring physiologically or was generated during the peptide isolation procedure. We performed in vitro the unsulfated HEL-(20–35) peptide-I-Ak complex. When the 20–35 peptide was isolated from this complex and examined by MS, we found the correct mass of the unsulfated peptide to be 932.9\(^{2+}\) (Fig. 3A). We next added the complex into a control M12-Ak cellular lysate, at the cell equivalent concentration used to obtain the previous results. The mass spectrum of the recovered HEL peptide showed the expected mass ion for the sulfated HEL-(20–35) peptide, m/z 972.5 (Fig. 3B). The presence of sulfate group in the HEL peptide was confirmed by MS/MS analysis. Indeed, other studies using radioactive PAPS to evaluate the TPST activity in the M12-Ak cellular lysate indicated the presence of such activity in the extract (Fig. 4). (We also examined, using HPLC/MS, whether the HEL protein isolated from the APCs M12-Ak mHEL or from M12-Ak fed with exogenous HEL was sulfated on tyrosine residues. We did not find any evidence of sulfation on either or both protein samples, indicating that under physiological conditions in APC, the HEL protein was not sulfated.)

Finally, we generated a panel of T cell hybridomas that specifically recognize sulfated peptide. These T hybridomas, developed by immunizing mice with sulfated HEL-(20–35) peptide, were selected in vitro for reactivity to the sulfated HEL peptide. We used these T hybridomas as a read-out system to evaluate whether the sulfated HEL peptides were presented on APC. None of these T cells reacted with APC cultured in HEL or in APCs, which express the HEL protein as a membrane-bound protein, confirming that under physiological conditions epitope-(20–35) was not sulfated. Thus, the tyrosine sulfation of the HEL-(20–35) family of peptides was generated during the peptide isolation procedure, resulting from the presence of TPST in the extract.

**Binding Studies**—The binding of the most abundant peptide, HEL-(20–35), to I-A\(^k\) molecules was tested. In 17 different experiments, the IC\(_{50}\) averaged 1.4 \(\mu\)M. Although the degree of binding varied about 30% depending on the preparation, it was highly consistent within experiments in which various mutant peptides were tested. Fig. 5 summarizes experiments testing HEL-(20–35) peptide in which several residues were changed to alanines in order to see the effects on MHC binding. Notably, changing Asn-27 to Ala resulted in a drop in binding of 200%, an average from six different experiments. No other amino acid when changed to alanine reduced binding significantly. However, a change of Cys-30 to Ala increased binding by 72%.

The crystal structure of I-A\(^k\) containing the HEL-(48–62) peptide indicated the interactions of the peptide amino acid residues with the various binding sites on I-A\(^k\), as well as the relationship between amino acids that served to anchor the
peptide to I-A<sup>k</sup> with those that stimulated the T cell (34). The central or core sequence occupied the stretch from P1 to P9. An acidic residue at P1, Asp-52, was favored for strong binding as well as for peptide selection. At P4, small hydrophobic residues or asparagines were favored. As with most MHC-displayed peptides, amino acids that contacted the TCR were positioned at P2, P5, and P9.

Extrapolating this information to peptide-(20–35), we mapped the P1 to P9 stretch to residues 24–32 (Tables I and II). As will be noted below, changing Leu-25 and Trp-28 to Ala did not affect binding but reduced entirely the T cell response; these two residues were TCR contact residues. In residues 24–32, Leu-25 is at P2, a TCR contact, Asn-27 at P4, the main MHC contact, and Trp-28 at P5, another TCR contact. P1 is occupied by Ser-24, contributing little to binding energy.

Fig. 6 shows the results of one experiment in which the binding of HEL-(20–35) was compared with HEL-(24–32); there was a drop in binding from 2.1 to 5.6 c.p.m. Testing the HEL-(24–32) peptide, we found that a Ser-24 to Ala change reduced binding, whereas the change of Asn-27 to Ala resulted in a complete lack of interaction.

We also examined the selection of the HEL-(20–35) family from cell lines expressing HEL in which either the Ser-24 or the Asn-27 had been changed to alanines. The cell lines were tested for their presentation of the chemically dominant peptide-(48–63) or -(20–35) (Fig. 7). Very clearly, the presentation of
peptide-(48–63) was not affected by the changes, which was not an unexpected result. However, the changes in either residue 24 or 27 profoundly affected presentation. (Note below, however, that such changes did not affect T cell responses.) Not shown are the results of changing Cys-30 to Ala, which had no effect on presentation.

In summary, these results, shown in Figs. 5–7, indicate the important role of Asn-27 in binding as well as in peptide selection from processing. They also indicate the importance of Ser-24 and, as indicated in Fig. 6, the contribution of residues outside the central core to binding strength. That is to say, peptide-(20–35) bound better than -(24–32). Previously, we had established that residues outside the central core, as in the case of peptide-(52–61), contributed to binding strength, as a result of main chain interactions with residues in the binding site of I-\(\text{A}^k\) (8, 34).

**T Cell Contact Residues of the HEL-(20–35) Epitope**—To identify the TCR contact residues involved in the recognition of the HEL-(20–35) epitope, we evaluated the response of 11 HEL-(20–35)-specific T cell hybridomas to Ala-substituted peptides. The T cell recognition of all the T hybridomas was completely abolished when the Trp at position 28 was changed to Ala. A similar response was observed for the Ala-substituted HEL-(20–35) Ala-25 peptide (Fig. 8 and Table I). On the basis that the HEL-(20–35) Ala-25 and Ala-28 peptides bind to I-\(\text{A}^k\) molecule as well as the HEL-(20–35) peptide, we can state with confidence that residues Leu-25 (P2) and Trp (P5) are obligatory TCR contacts. The Ala substitution of Tyr-23 (P–1) had an effect on T cell recognition; 6 of 11 T cell hybridomas did not respond to the HEL-(20–35) Ala-23 peptide, indicating that residues outside the core peptide (HEL-(24–32)) can influence the T cell recognition. A significant number of T cells (7 of 11) was sensitive to the change of Val to Ala at position 29. This change did not reduce the peptide binding to the class II molecule I-\(\text{A}^k\), suggesting that this amino acid substitution on the anchor residue P6 (Val) can alter the display of the HEL-(20–35) peptide to I-Ak (Fig. 5). Previously, we had established that residues outside the central core, as in the case of peptide-(52–61), contributed to binding strength, as a result of main chain interactions with residues in the binding site of I-\(\text{A}^k\) (8, 34).

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