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Cutting Edge: The HLA-A*0101-Restricted HY Minor Histocompatibility Antigen Originates from *DFFRY* and Contains a Cysteinylated Cysteine Residue as Identified by a Novel Mass Spectrometric Technique¹

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In this report, we describe the use of novel mass spectrometry instrumentation to identify a male-specific minor histocompatibility Ag restricted by HLA-A*0101 (A1-HY). This Ag has the sequence IVDC*LTEMY, where C* represents a cysteine disulfide bonded to a second cysteine residue. The core peptide sequence is found in the protein product of *DFFRY*, a Y chromosome gene not previously identified as the source of an HY Ag. The male-specific form of the peptide differs from its X chromosomal counterpart by the substitution of serine for the C* residue. Both peptides are expressed on the cell surface at 30 or fewer copies per cell. However, A1-HY-specific CTL recognize the *DFFRY*-derived peptide at a 1500-fold lower dose than the female homologue. Thus, these studies have identified a new source of HY epitopes and provide additional information about the influence of posttranslational modifications of class I-associated peptides on T cell recognition. *The Journal of Immunology*, 1999, 163: 6360–6364.

Minor histocompatibility Ags (mHag)⁴ are key factors in the rejection of solid organ allografts and in the development of graft-vs-host disease (GVHD) following bone marrow transplantation in animal models. Likewise, there is evidence that human mHag provoke transplantation immunity and function as targets of GVHD in bone marrow transplants in HLA-identical donor/recipient pairs (1, 2). Although >50 different mHag loci have been defined among inbred strains of mice (3), the number in humans is unknown. The genetics and tissue distribu-

tion of several human mHag have been studied using specific T cells isolated from patients suffering from GVHD as a consequence of bone marrow transplantation (4–7). mHag are peptides derived from cellular proteins that are presented by class I MHC molecules (4, 8). However, because of the difficulty in characterizing such antigenic peptides, the chemical structures for mHag in both humans and mice remain largely unknown.

Peptide Ags displayed by class I MHC molecules have been successfully identified either by the construction and screening of cDNA libraries (reviewed in Ref. 9) or the analysis of class I-associated peptides extracted directly from the cell (10, 11). Both approaches employ cytotoxic T cells to screen either transfected cells or peptide fractions, and both have been successfully used to identify mHag (reviewed in Refs. 4 and 8). By using the direct peptide extraction approach in conjunction with HPLC fractionation and mass spectrometry (MS), our groups have successfully identified four human classical mHag (12–15). Two of these peptides are human male-specific mHag restricted by HLA-B*0702 and HLA-A*0201 and are derived from the product of *SMCY*, a

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² R.A.P., E.D.F., and J.M.M.d.H. made equal contributions to this work and the order of their listing should be considered arbitrary.

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⁴ Abbreviations used in this paper: mHag, minor histocompatibility Ag; A1-HY, HLA-A*0101-restricted HY Ag; GVHD, graft-vs-host disease; HFBA, heptafluorobutyric acid; MS, mass spectrometry; FTMS, Fourier transformation mass spectrometer; LCQ, ion trap mass spectrometer; CAD, collision activated dissociation; ESI, electrospray ionization; C*, cysteinylated cysteine residue; M^{ox}, oxidized methionine residue.

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gene on the Y chromosome. Simpson and coworkers used the cDNA cloning strategy to identify two murine HY Ags, one originating from *Smcy* (16) and the second derived from another Y chromosomal gene *Uty* (17). Collectively, these results raise the question of whether additional Y chromosomal genes give rise to HY T cell epitopes.

To address this issue, we have implemented the combination of nanoflow liquid chromatography with electrospray ionization (ESI) on a Fourier transform mass spectrometer (FTMS). This allows detection of peptides at levels as low as 2–10 amol with mass measurement accuracy in the millimass range (18). With the inherent resolution of this instrument, assignment of the charge state to ions is easily accomplished, and coeluting peptides of similar mass are readily resolved (18). In this manuscript, we describe the use of this novel analytical system for identification of an HY Ag restricted by HLA-A*0101 (A1-HY).

Materials and Methods

Cell culture

The CD8⁺ CTL clone A42 has been shown to specifically recognize HLA-A*0101-positive male cells (19). It was maintained as described previously (19), or by using a rapid expansion protocol (20). The HLA-A*0101⁺ male (Rp) and female (C1R-A1) B lymphoblastoid cell lines were grown in RPMI 1640 containing 10% FCS and 3 mM L-glutamine.

Extraction and HPLC fractionation of HLA-A*0101-associated peptides

HLA-A*0101 molecules were immunoaffinity purified from male Rp cells (HLA-A*0101, A*0201, B8, B27), and their associated peptides were extracted as previously described (11–15). Iodoacetamide was omitted from the protease inhibitor mixture to avoid potential amidocarboxymethylation of free cysteine residues. HLA-A*0101 was purified by first removing the HLA-B and -C molecules with the mAb B1.23.2 (21), then depleting the HLA-A2 molecules with mAb BB7.2 (22), and finally isolating the HLA-A*0101 molecules with mAb W6/32 (23). Peptides were separated from class I H chains and β_2 -microglobulin by elution in 10% acetic acid and passage through a 5-kDa cutoff filter. One Rp extract was separated as previously described (15). A second Rp extract was fractionated on a HAI-SIL C18 column (2.1 × 40 mm, 5 μ m particles, 300 Å pore size) (Higgins, Winter Park, FL) using a linear gradient of 15–60% solvent B for 50 min. Solvent A was 0.1% TFA (HPLC grade; Applied Biosystems, Foster City, CA) in NANOpure water (Barnstead, Dubuque, IA), and solvent B was 0.085% TFA in 60% acetonitrile (HPLC grade; Mallinckrodt, Paris, KY). Fractions were collected every 40 s at a flow rate of 200 μ l/min. Active fractions were pooled and run through a second round of chromatography with the identical column and gradient, but using heptafluorobutyric acid (HFBA) as the ion-pairing agent.

Epitope reconstitution assays

Aliquots of each HPLC fraction were incubated with 2000 ⁵¹Cr-labeled C1R-A1 target cells for 30 min at 37°C and then tested for their recognition by A42 CTL in a standard chromium release assay (13). Synthetic peptides were assayed using the same protocol, except that HBSS containing 1% BSA and 50 mM HEPES was employed as the medium to avoid cysteinylolation of free cysteine residues (15).

Peptide analysis using an on-line effluent splitter and an FTMS

Biologically active second dimension HPLC fractions were analyzed by ESI on an FTMS equipped with nanoflow liquid chromatography and a modified on-line effluent splitter (11, 18, 24). Samples were fractionated using an on-line microcapillary HPLC column at a total flow rate of 825 nl/min. Thirteen-fourteenths of the effluent were deposited into wells of a microtiter plate containing 50 μ l of 0.1% acetic acid and reserved for epitope reconstitution assays. The remaining one-fourteenth was directed to the FTMS and analyzed by ESI MS.

Sequence analysis of candidate Ags

Collision-activated dissociation (CAD) mass spectra were recorded on selected peptide candidates using a Finnigan ion trap mass spectrometer (LCQ) equipped with sheathless nanoflow HPLC ESI as previously described (18). Data were acquired by manually switching from MS-only

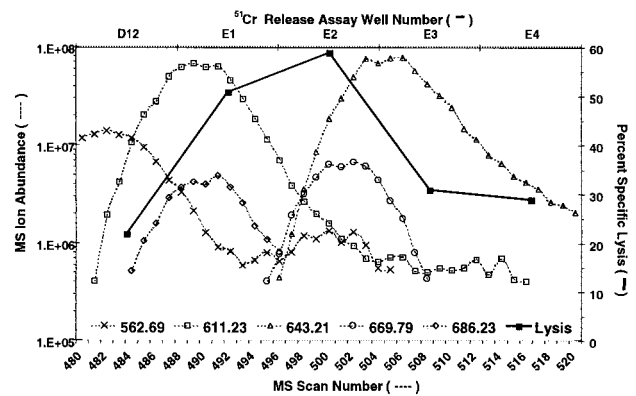


FIGURE 1. Determination of A1-HY candidate peptides. Aliquots of each splitter fraction (1.2×10^9 cell equivalents) were tested for their ability to reconstitute epitope activity as described in *Materials and Methods*. Ion abundances of candidate masses were determined using the FTMS.

mode to MS/MS mode after the chromatographic elution of a marker peptide. In MS/MS mode, the ion of interest was isolated using a 3.0 atomic mass unit isolation window and fragmented using 35% collision energy.

Synthetic peptides

Peptides were synthesized and cysteinylated as described (15), except the cysteinylolation time was 45 min. Oxidation of Met residues to the corresponding sulfoxide was accomplished by incubation with performic acid for 1 min at room temperature. The reaction mixture was diluted with 0.1% acetic acid and purified by reverse phase HPLC. Sequences of all synthetic peptides were confirmed by MS.

Class I MHC peptide binding assays

Quantitative, cell-free peptide binding assays were performed essentially as described (15), except that we used HLA-A*0101 molecules purified from the B lymphoblastoid cell line HAR and the iodinated indicator peptide used had the sequence YTAVVPLVY (25).

Results

Mass spectrometric identification of the A1-HY epitope

HLA-A*0101-associated peptides were purified from male Rp cells and fractionated by reverse phase HPLC using HFBA as the ion-pairing reagent. Epitope reconstitution was performed by adding aliquots of these fractions to the HLA-A*0101⁺ female target C1R-A1 and assaying with the A1-HY-specific CTL clone A42. A single peak of reconstituting activity was observed (data not shown). Active fractions were pooled and rechromatographed using TFA in place of HFBA, and, again, a single peak of reconstituting activity was seen (data not shown). Candidate masses for the HY Ag were identified by an on-line effluent splitter analysis of these active fractions as previously described (11–14), except that an FTMS was employed in place of a triple quadrupole mass spectrometer instrument. Identification of candidate peptides was made after plotting the abundances of ions observed in spectra recorded on peptides from wells that showed epitope reconstitution activity. The abundances of only five ions were considered to be similar to the CTL lysis profile (Fig. 1). Two of these (m/z 669.786⁺² and 611.232⁺²) were analyzed by CAD on the LCQ instrument, and their sequences were determined to be M^{ox}TEXTYDYPKY (data not shown) and XVDC*XTM^{ox}Y (Fig. 2A), respectively. X represents either Ile or Leu, which cannot be differentiated by the instrument; C* represents a Cys residue covalently modified by the attachment of a Cys via a disulfide linkage; M^{ox} represents Met in which the sulfur has been oxidized to a sulfoxide. When incubated

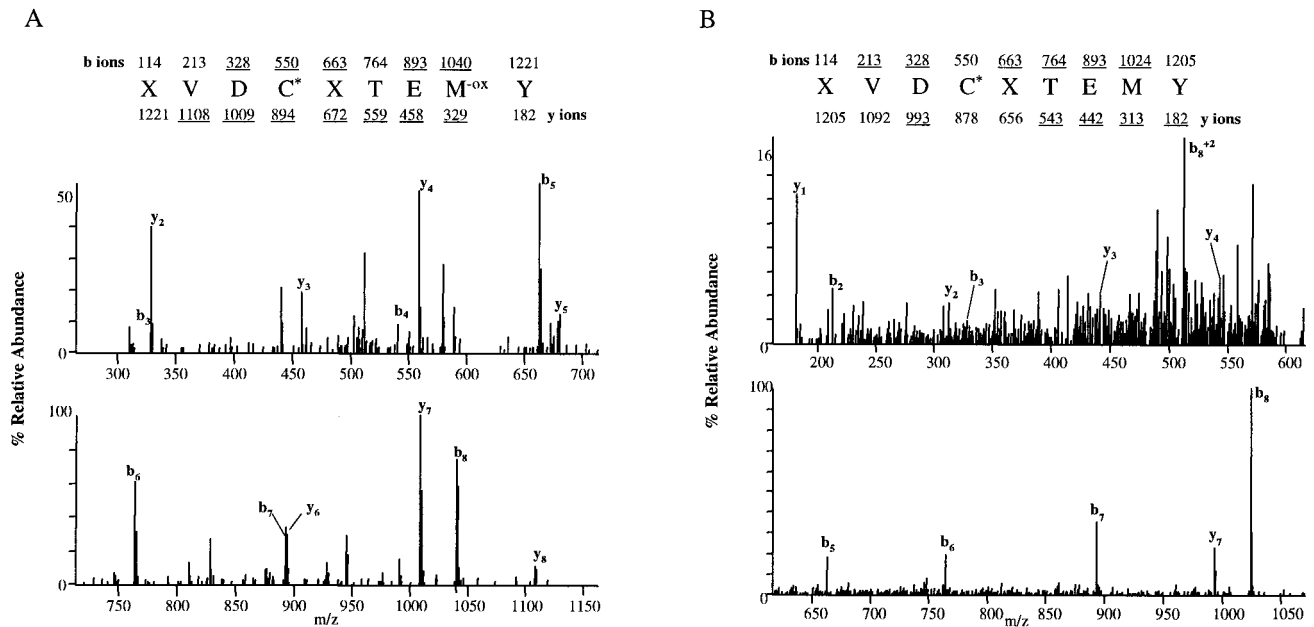


FIGURE 2. CAD mass spectra of A1-HY candidate peptide ions. Mass spectra were recorded as described in *Materials and Methods*. Ions observed in the spectrum are underlined. X represents isoleucine or leucine. *A*, Candidate peptide ($M + 2H$)²⁺ ion with monoisotopic m/z of 611.23. *B*, Candidate peptide ($M + 2H$)²⁺ ion with monoisotopic m/z of 603.25.

with C1R-A1 targets and CTL clone A42, XVDC*XTEM^{ox}Y reconstituted activity, while M^{ox}TEXTYDYPKY did not (Fig. 3A). These results suggested that XVDC*XTEM^{ox}Y represents the A1-HY epitope.

Modification of the cysteine and methionine residues alters recognition of the A1-HY epitope

To establish the impact of cysteinylolation and methionine oxidation on CTL recognition of the peptide, several peptides with different

combinations of oxidation and cysteinylolation were synthesized and evaluated for their ability to reconstitute the epitope recognized by the A42 CTL clone. The peptide with the highest immunological activity (half-maximal lysis at a concentration of 3.5 pM) contained a cysteinylated Cys, but a nonoxidized Met (Fig. 3B). Oxidation of Met resulted in a 130-fold reduction in immunological activity in three independent experiments (Fig. 3B and data not shown). Interestingly, removal of the cysteinylolation from the peptide with a nonoxidized Met had a much more modest impact, ranging from a 2- to 15-fold reduction in three independent experiments. However, in peptides with an oxidized Met, removal of the cysteinylolation led to an ~400-fold reduction in biological activity (Fig. 3B). These results demonstrate that the structure of the amino acids at P4 and P8 are both important for immunological activity.

The immunological activity results suggested that the oxidation of Met in the peptide we had identified was quite deleterious for recognition by the A42 CTL clone. Consequently, we prepared a second HLA-A*0101 peptide extract from Rp under conditions that minimize the possibility of oxidation during purification. Analysis of immunologically active second dimension fractions by FTMS failed to detect an ion of m/z 611.232⁺² corresponding to the previously identified peptide with an oxidized Met. We then searched for an ion corresponding to the cysteinylated peptide with a nonoxidized Met (calculated m/z = 603.244⁺²) and detected a candidate signal of m/z 603.254⁺². A CAD spectrum recorded on this ion confirmed the expected sequence (Fig. 2B). We also searched for ions corresponding to XVDCXTEMY and XVDCXTEM^{ox}Y, but neither was detected. We conclude that Met oxidation represents an artifact of peptide extraction in one preparation, and that the true A1-HY epitope is represented by the sequence XVDC*XTEMY.

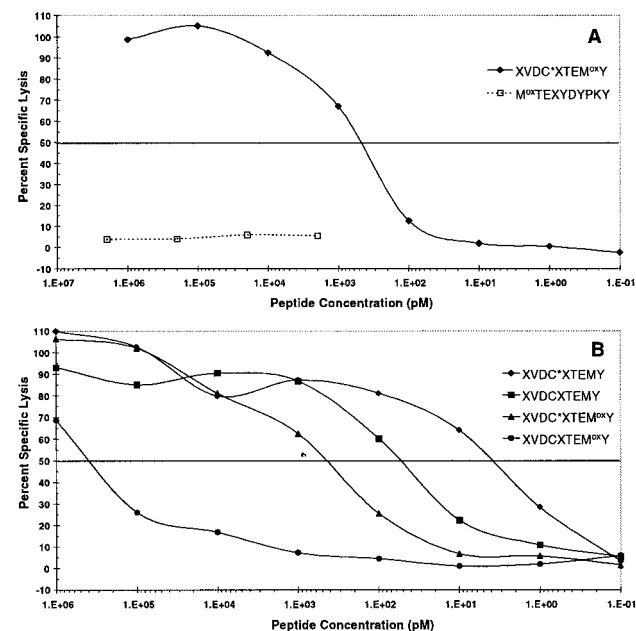


FIGURE 3. A1-HY epitope reconstitution with synthetic peptides. The indicated synthetic peptides were assayed for epitope reconstituting activity as described in *Materials and Methods*. *A*, Candidate peptide ions 611.23⁺² (XVDC*XTEM^{ox}Y) and 669.79⁺² (M^{ox}TEXTYDYPKY). *B*, Effects of posttranslational modification of the core sequence XVDCXTEMY.

The A1-HY epitope is derived from the DFRY gene encoded on the Y chromosome

A search of the known protein sequence databases generated two matches for the XVDC*XTEMY sequence. These two sequences, IVDCLEMY and IVDSLTEMY, represent amino acid residues

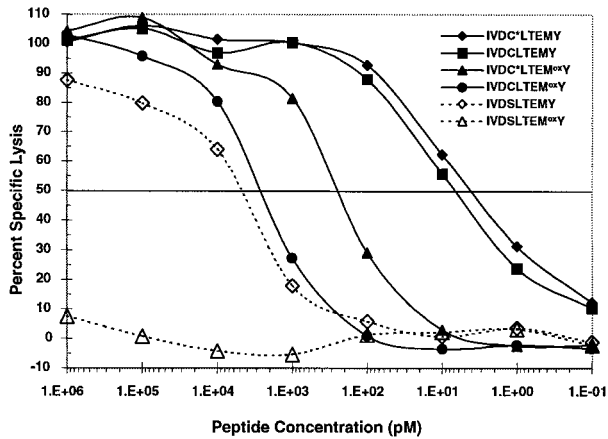


FIGURE 4. Comparative recognition of A1-HY-related peptides derived from *DFFRY* and *DFFRX*. Peptides corresponding to the A1-HY epitope (IVDCLTEMY), its cysteinylated and oxidized derivatives, and the *DFFRX*-encoded homologue (IVDSLTEMY) were assayed for epitope reconstituting activity as described in *Materials and Methods*.

1521–1529 and 1512–1520 of the genes *DFFRY* and *DFFRX* (26, 27), which are located on the Y and X chromosomes, respectively. Allowing for the inability to distinguish Ile and Leu on the mass spectrometer, and the cysteinylated cysteine residue at P4, the peptide identified by MS was an exact match for the sequence from the *DFFRY* gene. Support for this was obtained by synthesizing the four possible variants of the XVDC*XTEMY peptide based on substitution of either Leu or Ile for X. When spiked into an aliquot of the naturally processed peptide extract, only the form containing Ile at P1 and Leu at P5 coeluted with the naturally occurring biological activity (data not shown). The *DFFRX* homologue of the peptide that we had identified differed from the *DFFRY* sequence by the substitution of a Ser residue for the cysteinylated Cys. To provide additional evidence that the sequence we had identified was the A1-HY Ag, both the Y and X chromosomal homologues were evaluated for their ability to reconstitute the epitope for the A42 CTL clone. Recognition of the nonoxidized X homologue required 1500-fold more peptide than did recognition of the nonoxidized Y peptide, regardless of whether the latter was cysteinylated (Fig. 4). These observations support the conclusion that the sequence of the A1-HY epitope is IVDC*LTEMY and is derived from the *DFFRY* gene.

We performed peptide binding assays to determine the relative affinities of the noncysteinylated male, cysteinylated male, and female peptides for HLA-A*0101. Both the female and noncysteinylated male forms inhibited the binding of an iodinated indicator peptide by 50% (IC_{50}) at a concentration of 6–8 nM. The cysteinylated male form bound 10-fold less well (IC_{50} = 70 nM). This suggests that for any given dose in the reconstitution assays in Figs. 3 and 4, the levels of this peptide on the cell surface are 10 times lower than those of the noncysteinylated male form. Thus, the apparently modest difference in CTL recognition of the noncysteinylated and cysteinylated male peptides is due to the fact that the latter binds 10-fold less well. Instead, the A1-HY-specific CTL recognize the male cysteinylated form substantially better than the noncysteinylated form. This result provides additional support for designating the cysteinylated male peptide as the A1-HY epitope.

Both the male and female forms of the A1-HY epitope are present at the cell surface

Male cells generally express the protein products of both X and Y chromosomes (28). Accordingly, the homologue peptide derived

from the *DFFRX* gene was expected to be present on the surface of an HLA-A*0101⁺ male cell. To evaluate this possibility, synthetic IVDSLTEMY was chromatographed under the same conditions as the first dimension separation of the A1-HY⁺ peptide extract, and its elution point corresponded to fraction 31 of the first dimension fractionation. Fractions 29–33 of the Rp peptide extract were screened on the LCQ instrument for charge states corresponding to the mass of the homologous X chromosome peptide. An ion of m/z 535.698⁺² was present in fraction 33, and its amino acid sequence (IVDSLTEMY) was confirmed by CAD analysis (data not shown). Based on coelution of the naturally occurring peptide with a known amount of synthetic IVDSLTEMY, we calculated the female peptide to be present at about 10 copies per cell. Similarly, we calculated that ~30 copies of the male peptide are present per B lymphoblastoid cell line.

Discussion

In this study, we report that the A1-HY mHag is IVDC*LTEMY. This sequence is represented in only a single known gene, *DFFRY*, which is located on the Y chromosome and encodes a protein of 2555 residues (26). The A1-HY epitope differs by a single substitution from the homologous sequence of the X chromosome counterpart *DFFRX* (27). The assignment of A1-HY to *DFFRY* is supported by the following: 1) the location of *DFFRY* on the Y chromosome is consistent with the expected location for genes that encode HY epitopes; 2) the tissue expression of both *DFFRY* and A1-HY is ubiquitous (5, 26, 29); 3) the amino acid substitution in the homologous peptide from *DFFRX* has a profound influence on immunological activity; 4) the homologous *DFFRX* peptide is also found on the cell surface in similar quantities to A1-HY.

This is the first demonstration that *DFFRY* encodes an HY Ag. Previous studies identified two other Y chromosomal genes, *SMCY* and *Uty*, as sources of HY epitopes. These three are among nine genes in the nonrecombining region of the human Y chromosome that are ubiquitously expressed and have homologous X chromosomal counterparts (28). Furthermore, each of these three genes encodes a product of 1100–2500 amino acids and thus may give rise to numerous additional HY epitopes. It remains to be determined whether any of the six remaining genes in this region also give rise to HY Ags.

The identification of A1-HY was facilitated by the development of new MS technology (11, 13, 14). The previously described effluent splitter (11–14) was modified to deliver a lower flow rate (50 vs 800 nl/min) to the ESI source, and the triple quadrupole mass spectrometer was replaced by the FTMS, which is 1000 times more sensitive (detection limit = 2–10 amol vs 3–5 fmol). Sample consumption is reduced by more than a factor of 10, and masses of Ags present at the single copy per cell level can be determined from $<10^8$ cells. The mass measurement accuracy of the FTMS is in the millimass range; thus, coeluting peptides that differ by several hundredths of a mass unit are easily resolved. This greatly reduces the probability that a mass will be eliminated as a candidate because it coelutes with a second peptide of similar mass. The higher resolution of the FTMS makes it possible to assign charge states to ions based on the observed mass separation between ¹²C and ¹³C isotope peaks and to eliminate candidates whose molecular mass is outside the range expected for class I-associated peptides. Combined with the on-line effluent splitter device, these improvements allowed us to identify the 611.23⁺² ion mass as one of five primary candidates for A1-HY.

The principal advantage of using MS for Ag identification is the direct determination of the structure of the presented peptide. A1-HY was initially found to have a cysteinylated Cys at P4 and

an oxidized Met at P8. Cysteinylation is a common feature of class I-associated peptides, including another HY epitope restricted by HLA-A*0201 (15). In that instance, two different T cell clones showed strong (>100-fold) preferential recognition of either the cysteinylated or noncysteinylated peptides. When relative binding affinities are taken into account, the A1-HY CTL clone used here recognizes the cysteinylated male peptide 10–100 fold better than the noncysteinylated form. Because we have not confirmed the presence of the uncysteinylated IVDCLTEMY peptide, it remains to be determined whether this is present on the cell surface or recognized by other A1-HY-specific CTL.

Oxidation of Met in naturally processed class I-associated peptides has not been previously described. The Met residue at P8 in A1-HY is predicted to be accessible to both solvent and the TCR (25, 30). Consistent with this orientation, oxidation led to a substantial reduction in T cell recognition. Although the exact cause of this oxidation is unclear, our results suggest that it is most likely a result of the peptide extraction procedure. Nevertheless, if such oxidation reactions occur under other conditions in tissue, they may give rise to additional epitopes with the potential to be recognized by the immune system.

In sum, this study of the identification of A1-HY provides further insight into modifications that affect T cell recognition, while its origin establishes a new gene that encodes HY epitopes. Such studies continue to be important in establishing the basis for the existence and recognition of human mHag and to their potential use in improving the outcome of transplants between MHC-identical, mHag-mismatched individuals.

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