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# The HLA-A*0201-Restricted H-Y Antigen Contains a Posttranslationally Modified Cysteine That Significantly Affects T Cell Recognition

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## Summary

**A peptide recognized by two cytotoxic T cell clones specific for the human minor histocompatibility antigen H-Y and restricted by HLA-A*0201 was identified. This peptide originates from SMCY, as do two other H-Y epitopes, supporting the importance of this protein as a major source of H-Y determinants in mice and humans. In naturally processed peptides, T cells only recognize posttranslationally altered forms of this peptide that have undergone modification of a cysteine residue in the seventh position. One of these modifications involves attachment of a second cysteine residue via a disulfide bond. This modification has profound effects on T cell recognition and also occurs in other class I MHC-associated peptides, supporting its general importance as an immunological determinant.**

## Introduction

Class I molecules of the major histocompatibility complex (MHC) bind to peptides derived from intracellular pathogens or from proteins expressed in tumor cells and present them on the cell surface to $CD8^+$ T cells of the host immune system (Morrison et al., 1986; Moore et al., 1988; Townsend and Bodmer, 1989). Class I molecules also present peptides that differ between organ or bone marrow transplant donor and recipient and can act as minor histocompatibility antigens (mHag) that evoke transplant rejection or Graft-versus-Host-Disease (Rotzschke et al., 1990; Goulmy, 1996). The most extensively studied mHag is the male-specific H-Y antigen. This antigen promotes rejection of male-to-female skin grafts in inbred mice strains, whereas transplants made in the other sex combinations always succeed (Eichwald and Silmser, 1955). In humans, the demonstration of H-Y as a transplantation antigen was based on the observation of strong posttransplant cytotoxic T lymphocyte (CTL) responses specific for male donor HLA-matched target cells in the peripheral blood lymphocytes of a female patient who rejected the bone marrow transplant of her HLA-identical brother (Goulmy et al., 1976). It has subsequently been shown that H-Y antigens are ubiquitously expressed on cells of hematopoietic and nonhematopoietic origin (de Bueger et al., 1992b), and H-Y-specific T cell responses can occur following organ transplantation, blood transfusions, and bone marrow transplants (Goulmy, 1988).

Identification of the specific MHC-associated peptides that are recognized by individual T cell epitopes has generally been accomplished by using MHC allele–specific peptide-binding motifs to screen known source proteins and testing the peptides identified for their ability to sensitize appropriate target cells for recognition (reviewed in Engelhard, 1994). However, we have developed a technique for the identification of such peptides without prior knowledge of the source protein. By combining microcapillary liquid chromatography/electrospray ionization mass spectrometry with T cell epitope reconstitution assays, we previously identified peptide antigens recognized by T cells specific for human xeno (Henderson et al., 1993) and alloantigens (W. W. et al., submitted), human melanoma (Cox et al., 1994), a non-sex-linked human mHag (den Haan et al., 1995), as well as antigens expressed on murine tumors (Huang et al., 1996; Dubey et al., submitted) and cells infected with Listeria monocytogenes (Gulden et al., 1996). We also identified a peptide that defines a human H-Y epitope for an HLA-B*0702-restricted CTL (Wang et al., 1995). This 11 residue peptide is derived from SMCY, an evolutionarily conserved Y-chromosomal protein.

An important advantage of the use of this method for antigen identification is that the structure of the peptide responsible for the epitope is directly determined rather than inferred from the primary amino acid sequence and predictive motif information. The importance of this advantage was made clear when it was recently shown that a peptide epitope derived from the tyrosinase protein and recognized by melanoma-reactive CTL differed from the predicted sequence owing to a posttranslational modification of an asparagine residue to aspartic acid (Skipper et al., 1996). This modification did not significantly alter interaction with the MHC molecule, but it had a profound effect on T cell recognition.

In the present study, we report the identification of peptide epitopes that comprise H-Y antigens recognized by HLA-A*0201-restricted specific CTL clones and that also originate from the SMCY protein. The use of

# These authors contributed equally to this work.

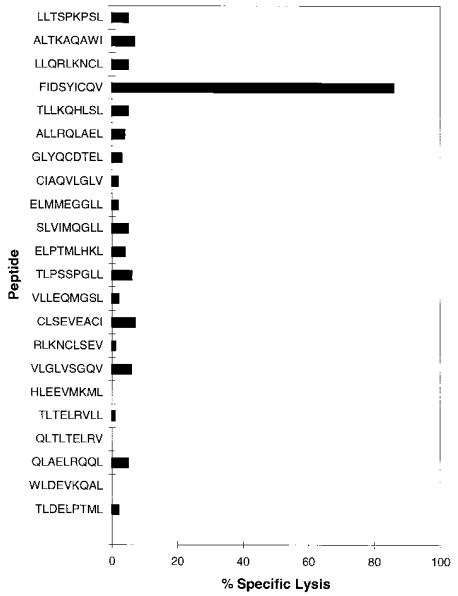


Figure 1. Reactivity of the H-Y-Specific, HLA-A*0201-Restricted CTL Clone 1R35 with Synthetic Peptides Derived from the Sequence of SMCY

Peptides (5 ng/ml) were preincubated with $^{51}$Cr-labeled T2 cells for 30 min at 37°C, followed by the addition of 1R35 cells at an E:T of 12.5:1.

direct antigen identification technology has demonstrated that these epitopes are posttranslationally modified. This newly described modification involves attachment of a second cysteine residue to a cysteine in the primary sequence via a disulfide bond. Data on the reaction pathway leading to this modification suggests that cysteinylated cysteine residues in naturally processed peptides are likely to be quite common.

## Results

### Definition of Epitopes for H-Y-Specific CTL Using Synthetic Peptides

Two previous reports identified the human and murine homologs of the Y-chromosomal protein SMCY as the source of peptide epitopes recognized by H-Y-specific CTL in the context of HLA-B*0702 and H-2K$^k$, respectively (Scott et al., 1995; Wang et al., 1995). This made SMCY the prime target in searching for H-Y epitopes restricted by other human class I molecules. To this end, a computer program (D'Amaro et al., 1995; Drijfhout et al., 1995) was used to predict and rank peptides from human SMCY that would bind to HLA-A*0201. From this list, twenty-two peptides whose sequences differed from the corresponding sequences encoded by the X-chromosomal homolog, SMCX, were synthesized. When these peptides were tested for their ability to reconstitute the epitope for the HLA-A*0201-restricted, H-Y-specific CD8$^+$ CTL clone, 1R35, only a single 9 residue peptide, FIDSYICQV, was active (Figure 1). This peptide was also recognized by the HLA-A*0201-restricted, H-Y-specific CD4$^+$ CTL clone R416 (46% lysis at 5 ng/ml peptide and an effector: target ratio [E:T] of 9:1).

### Definition of Epitopes for H-Y-Specific CTL in Naturally Processed Peptides

To verify that this was indeed the peptide naturally expressed on the cell surface and recognized by the T cell, HLA-A*0201 molecules were immunoaffinity purified from the H-Y$^+$ B lymphoblastoid cell line (B-LCL) Blk. The associated peptides were extracted in acid and fractionated by reverse-phase HPLC with heptafluorobutyric acid (HFBA) as the organic modifier. Aliquots of each fraction were assayed for their ability to reconstitute the epitope recognized by 1R35 and R416 after incubation with the HLA-A*0201$^+$, H-Y$^-$ target cell, T2. Interestingly, while both CTL clones recognized the same synthetic peptide, FIDSYICQV, they each recognized components in different HPLC fractions of the naturally processed material. For the R416 clone, a single peak of reconstituting activity was observed in fraction 34, while the 1R35 clone recognized two peaks, one at fractions 35–37 and one at fraction 39 (Figure 2A). The active fractions for each clone were pooled separately and rechromatographed with trifluoroacetic acid (TFA) as the organic modifier. A single peak of reconstituting activity was again observed for the R416 clone at fractions 43 and 44 (Figure 2B), while the 1R35 clone again recognized two separate peaks, one at fractions 26 and 27 and one at fractions 48 and 49 (Figure 2C).

Tandem mass spectrometry was employed to search for the $(M+2H)^{2+}$ ion of FIDSYICQV at *m/z* $544^{2+}$ in each of the biologically active second dimension fractions. Although collision-activated dissociation (CAD) spectra were recorded on several different ions of *m/z* $544^{2+}$ in these fractions, none corresponded to FIDSYICQV. These data suggested either that each CTL clone recognized a different modified form of this peptide or that the true epitope for either or both CTLs was a naturally processed peptide with a primary sequence different from FIDSYICQV. The most likely site for modification of FIDSYICQV is the sulfhydryl group of the cysteine residue at position 7. If this modification contains a disulfide bond, it should be susceptible to reduction. Indeed, when aliquots of each of the biologically active fractions were treated with dithiothreitol (DTT), a new ion at *m/z* $544^{2+}$ appeared in the mass chromatogram (compare top panels in Figures 3A and 3B) and was confirmed by CAD to be the SMCY sequence FIDSYICQV (Figure 4A).

### The Peptide Epitope Recognized by CTL Clone R416 Contains a Cysteinylated Cysteine Residue

In order to identify the parent peptide that gave rise to FIDSYICQV upon reduction, mass spectra were searched for signals that disappeared on treatment with DTT. In second dimension fractions 43 and 44 of Figure 2B, we found a peptide at *m/z* $604^{2+}$ that disappeared upon treatment of the fraction with DTT (compare middle panels of Figures 3A and 3B). The CAD spectrum of this peptide (Figure 4B) confirmed the sequence as FIDSYICQV with a cysteinylated cysteine residue in position 7. The HPLC fractions 43 and 44, in which this peptide was found in the extract of Blk cells, corresponded to those recognized by CTL clone R416. Adjacent fractions 42 and 45 neither contained this peptide nor showed

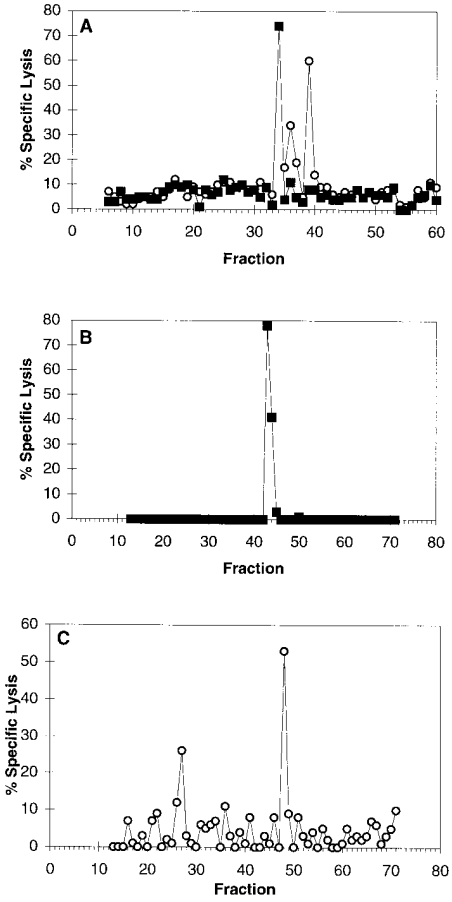


Figure 2. Reconstitution of the H-Y Epitope with HPLC-Fractionated Peptides Extracted from HLA-A*0201 Molecules

Peptides were prepared from HLA-A*0201$^+$ H-Y$^+$ Blk cells and fractionated by reverse-phase HPLC as described in Experimental Procedures. Aliquots of each fraction were preincubated with $^{51}$Cr-labeled T2 cells at 37°C for 30 min, followed by addition of the CTL clone.

(A) First dimension separation of peptides with HFBA as the organic modifier. The reconstitution assay was performed using 3% of each fraction. The R416 clone (closed square) was used at an effector-to-target ratio (E:T) of 14:1 and 1R35 (open circle) at an E:T of 16:1.

(B) Fraction 34 from the separation shown in (A), which contained reconstituting activity for clone R416, was rechromatographed with TFA instead of HFBA as the organic modifier and the gradient described in Experimental Procedures. The R416 clone was used at an E:T of 14:1 to assay the reconstituting activity of 3% of each fraction.

(C) Active fractions 36 and 39 from the separation shown in (A), which contained reconstituting activity for clone 1R35, were separately pooled and rechromatographed with TFA instead of HFBA as the organic modifier and the gradient described in Experimental Procedures. The 1R35 clone was used at an E:T of 13:1 to assay the reconstituting activity of 8% of each fraction.

R416 sensitizing activity. In addition, in extracts of two other HLA-A*0201$^+$, H-Y$^+$ cells, Rp and DM, the same cysteinylated peptide was found only in HPLC fractions that showed R416 sensitizing activity (data not shown). We conclude that the epitope for the H-Y-specific, HLA-A*0201$^+$ CTL clone R416 is FIDSYICQV, where the Cys has been modified by the attachment of another Cys via a disulfide linkage.

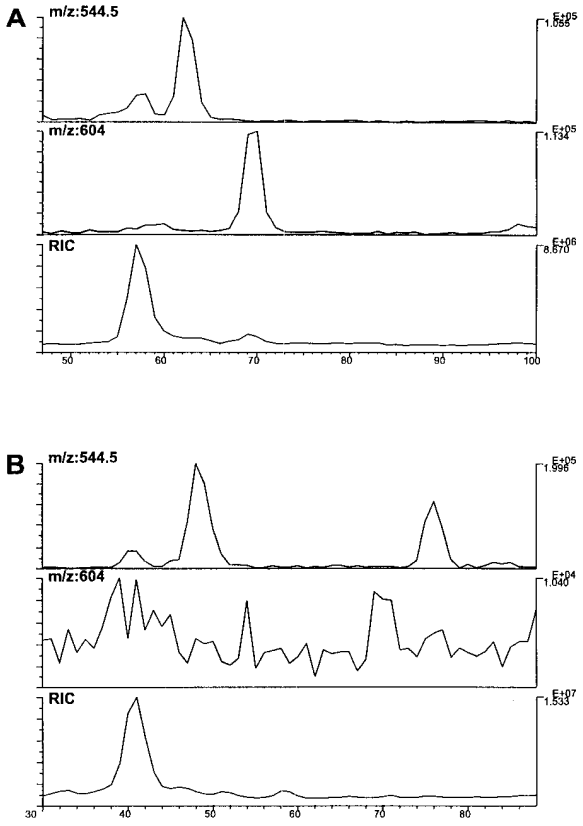


Figure 3. Ion Chromatograms for Recorded *m/z* 544.5 and 604

Plots of ion abundance versus chromatographic retention time for peptides in fraction 43 (Figure 2B) before (A) and after (B) treatment with DTT. Sample preparation and mass spectrometric analysis are described in Experimental Procedures. After mass spectra were acquired, a computer search was conducted to determine the abundance of ions in a two mass unit window centered on *m/z* 544.5 or 604. Twice the amount of sample was loaded for the ion chromatogram in (B).

### Effect of Cysteinylation on CTL Recognition and Binding to HLA-A*0201

One interesting aspect of these results is that the fractions that contained epitope reconstituting activity for R416 did not contain activity for the 1R35 CTL clone, despite the fact that both clones recognized the synthetic FIDSYICQV peptide. In addition, RPMI-1640 contains 0.21 mM cystine, the oxidized form of cysteine, and this could participate in a disulfide exchange reaction leading to cysteinylation of cysteine containing peptides incubated in this medium. Consequently, the ability of RPMI-1640 to mediate peptide cysteinylation and the impact of this modification on epitope recognition by 1R35 and R416 were examined. Target cells were pulsed with varying doses of unmodified and cysteinylated FIDSYICQV peptide in regular RPMI-1640 medium and then incubated with CTL in the same medium (Figure 5A). Under these conditions, both forms of the peptide were recognized similarly by the R416 clone. However, the unmodified peptide was recognized about 30-fold better than the cysteinylated form by the 1R35 clone. In contrast, when target cell incubation with peptide and the CTL assay were done in cystine-free medium for the

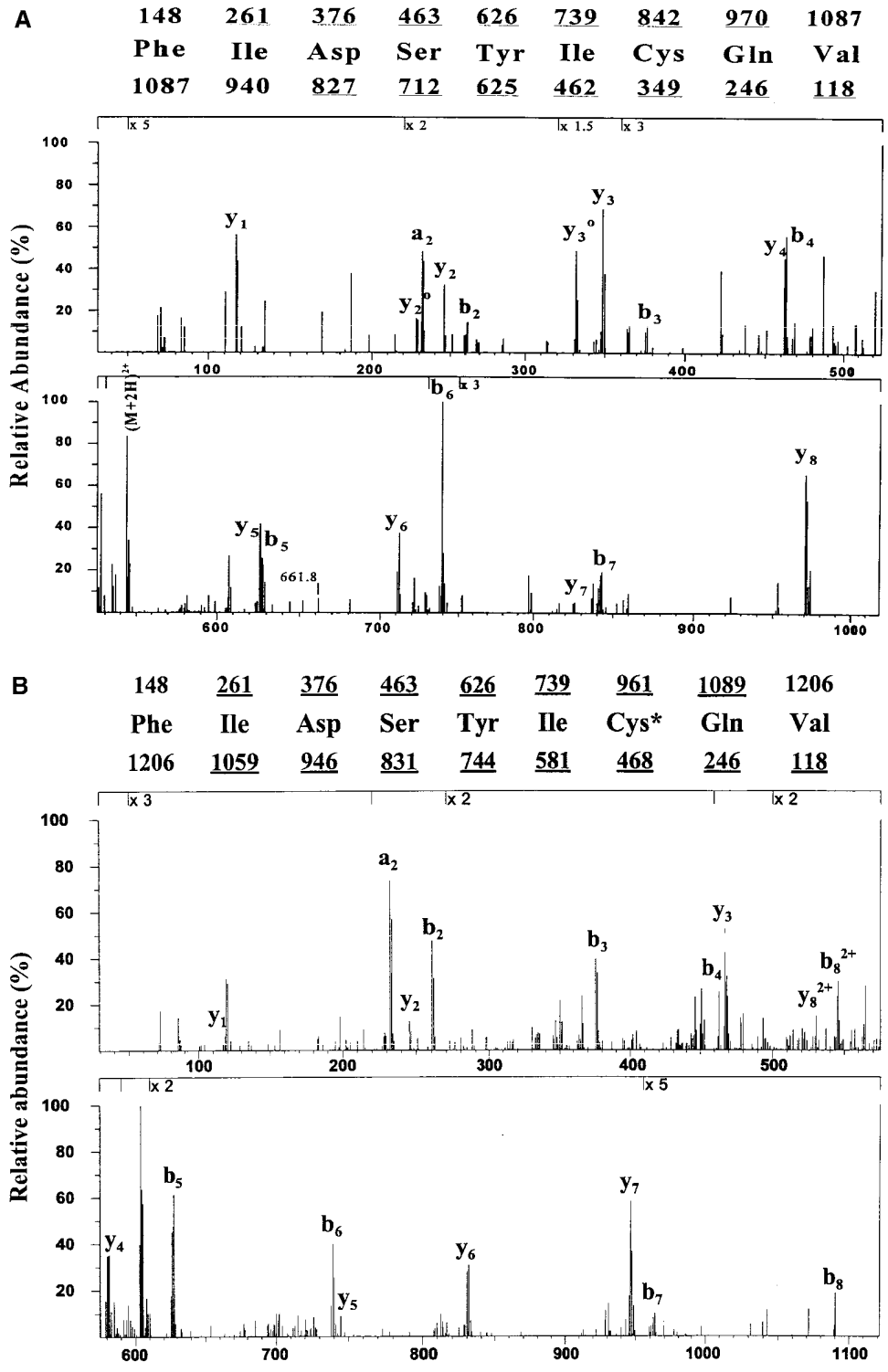


Figure 4. Mass Spectra

CAD mass spectra of peptide 1087 (A) recorded on $(M+2H)^{2+}$ ion at *m/z* 544 and the peptide 1207 (B) recorded on the $(M+2H)^{2+}$ ion at *m/z* 604. The (y) and (b) ions (Hunt et al., 1986) have been labeled. In (B), Cys* represents a cysteinylated cysteine residue of 222 mass units.

unmodified peptide and in cystine containing medium for the cysteinylated peptide, very large differences in the sensitizing activity of the two peptides became apparent for both CTL clones (Figure 5B). Half-maximal target cell lysis by the R416 CTL clone was achieved by using a 100 pM concentration of the cysteinylated peptide, which is typical of the concentrations required for reconstitution of several other HLA-A*0201-associated peptide epitopes (Cox et al., 1994; den Haan et al., 1995; Wang et al., 1995). However, a concentration of

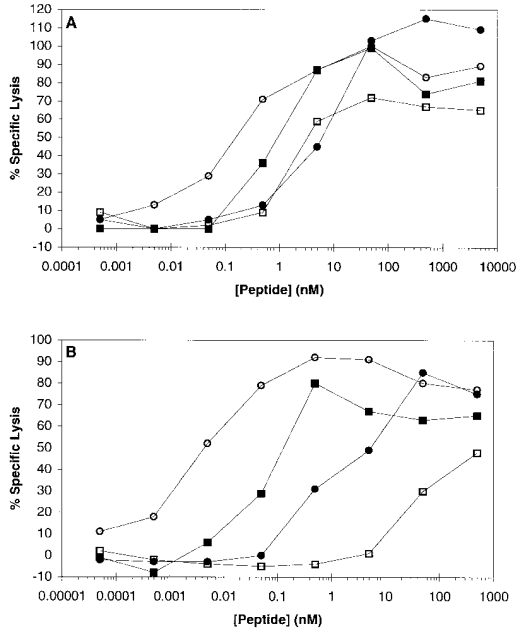


Figure 5. H-Y Epitope Reconstitution with Synthetic Peptides

In (A), both the unmodified (open symbols) and cystine-treated (closed symbols) FIDSYICQV peptides were tested in RPMI-1640 medium containing 15% HS. In (B), the unmodified peptide was initially reduced by incubation of the stock peptide in 2mM DTT prior to addition to target cells, and the assay was done in cystine-free RPMI-1640 (Gibco) containing 1% BSA. The cystine-treated peptide was tested in RPMI-1640 containing 1% BSA. After preincubation of the peptides with $^{51}$Cr-labeled T2 cells for 30 min at 37°C, either R416 (squares) or 1R35 (circles) was added, and a 4 hr $^{51}$Cr release assay was conducted.

180 nM of the unmodified peptide was required to achieve the same result. Conversely, half-maximal target cell lysis by the 1R35 CTL clone was achieved by using a 3 pM concentration of the unmodified peptide, while similar sensitization with the cysteinylated form required 20 nM. These results demonstrate that the 1R35 and R416 clones preferentially recognize the unmodified and cysteinylated forms of FIDSYICQV, respectively. In addition, the less disparate recognition of both forms in normal RPMI-1640 medium containing 0.21 mM cystine demonstrates that peptide cysteinylation by disulfide exchange with cystine can occur rapidly under these conditions.

We also examined whether cysteinylation affected the ability of the peptide to bind to HLA-A*0201 (Figure 6). The unmodified FIDSYICQV showed an IC50 value in an in vitro binding assay with purified HLA-A*0201 of 16 nM, which is indicative of a high affinity interaction. Interestingly, the cysteinylated form of this peptide showed an IC50 of 75 nM, demonstrating that this modification inhibited binding by about 5-fold. Nonetheless, this IC50 value is still within the range of values observed for other peptides that are HLA-A*0201-restricted epitopes (Figure 6). It is important to note that this difference in peptide binding is still substantially less than the 3–4 orders of magnitude differences in CTL recognition of the two peptides in cystine-free medium. Thus, the major impact of cysteinylation is on the structure of the epitope recognized by each CTL clone.

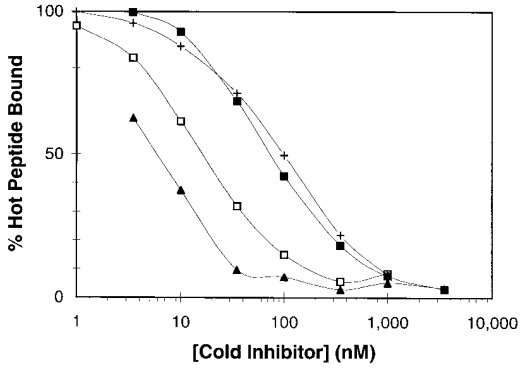


Figure 6. Peptide Binding to HLA-A*0201

HPLC-purified peptides were assayed for their ability to inhibit the binding of the iodinated peptide FLPSDYFPSV to affinity-purified HLA-A*0201 molecules in a cell-free peptide-binding assay. Open squares indicate unmodified FIDSYICQV; closed squares, cysteinylated FIDSYICQV; closed diamonds, IP-30 signal sequence peptide LLDVPTAAV; and (+), MHN24 alloantigen epitope YLDPAQQNL.

## Cysteinylation Is Not an In Vitro Culture Artifact

Despite the fact that the R416 clone showed preferential recognition of the cysteinylated form of the peptide, the demonstration that cysteinylation of peptides can be mediated by RMPI-1640 medium led us to consider whether it was an artifact of in vitro culture of the cells from which the MHC molecules were isolated. In order to address this possibility, HLA-A*0201-associated peptides were extracted from normal noncultured human spleen cells. The sensitizing activity for the R416 clone in the spleen cell peptides eluted at the same position on reverse-phase HPLC as did the activity in extracts of cultured B-LCL (Figure 7). CAD spectra recorded on the ions of *m/z* $604^{2+}$ in the sensitizing fractions of the spleen cell extract confirmed the presence of the cysteinylated form of FIDSYICQV (data not shown). This demonstrates that cysteinylated MHC-associated peptides

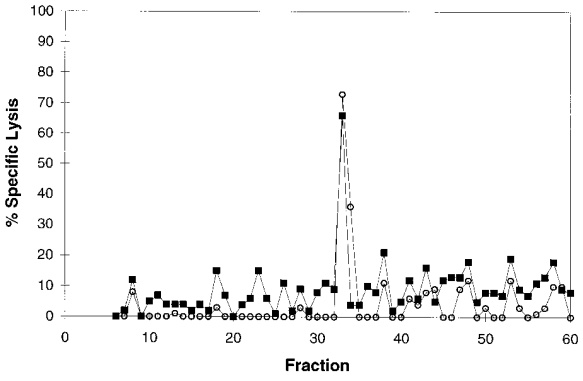


Figure 7. Reconstitution Activity of HPLC-Fractionated Peptides from HLA-A*0201$^{+}$ Human Spleen Cells

HLA-A*0201-associated peptides were extracted from affinity-purified molecules isolated from either noncultured human spleen (closed squares) or the B-LCL line Rp (open circles), and fractionated by reverse-phase HPLC with HFBA as organic modifier. Both preparations were run sequentially on the same column, with the spleen-derived peptides run first. Aliquots of each fraction corresponding to 2.5 × $10^9$ cell equivalents were incubated with $^{51}$Cr-labeled T2 cells and then CTL clone R416.

are produced in vivo and are not an artifact of in vitro culture.

### Cysteinylation Occurs in Multiple MHC-Associated Peptides

Work related to the identification of naturally processed epitopes had led to the discovery of two additional HLA-A*0101-associated peptides that are derivatized by cysteinylation. These were initially identified because they contained an unusual residue mass of 222. In each case, treatment of the sample with DTT converted the 222 Da residue mass to that of cysteine, a net change of 119 Da. This suggested that the extra mass in the untreated samples resulted from cysteinylation. To confirm this hypothesis, synthetic peptides were prepared and shown to coelute with the naturally occurring material and also to have identical CAD spectra. The two peptide sequences are FXDTDTXC*Y and FTESCEXC*Y, where (X) refers to Leu or Ile, two residues of identical mass that cannot be distinguished on the triple quadrupole instrument, and (C*) refers to a cysteinylated cysteine residue. In the case of the latter peptide, while the cysteine at P5 was not cysteinylated, it was amidocarboxylmethylated. This modification occurred as a result of the inclusion of iodoacetamide as a protease inhibitor in the buffer used to lyse the B-LCL. This reagent was not present in the culture medium or in any of the last wash or elution buffers used in affinity purification of the MHC molecules or the elution of the associated peptides, indicating that this cysteine residue is accessible to this modification while it is bound to the MHC molecule. Collectively, these results suggest that cysteinylation is likely to be a widespread modification of MHC-associated peptides, but that both modified and unmodified cysteine residues may be present in individual peptides.

## Discussion

In this paper, we have identified a peptide epitope for the H-Y-specific, HLA-A*0201-restricted CTL clone R416 and provided partial identification information for a second epitope for the H-Y-specific, HLA-A*0201-restricted CTL clone 1R35. The source of both epitopes appears to be SMCY, which was previously identified as the source for two other H-Y epitopes restricted by other murine and human MHC molecules (Scott et al., 1995; Wang et al., 1995). SMCY is expressed in multiple tissues and maps to the same Yq deletion interval as the human H-Y antigen controlling locus HY (Cantrell et al., 1992; O'Reilly et al., 1992). Interestingly, genetic mapping of the mouse Y chromosome has suggested that at least two and up to five distinct loci, including SMCY, encode H-Y antigens (King et al., 1994). With a single exception (Greenfield et al., 1996), all of the epitopes identified to date from both mouse and human have been derived from the SMCY protein. SMCX, a homolog of the SMCY protein located on the X chromosome, is 85% identical to SMCY at the amino acid sequence level. SMCX is ubiquitously expressed by both the active and the inactive X chromosomes in both mice and humans (Agulnik et al., 1994a; Agulnik et al., 1994b; Wu et al., 1994a, 1994b). Since self-tolerance to SMCX will limit the number of SMCY peptides that could give rise to H-Y epitopes in association with different MHC molecules, it may seem surprising that there is sufficient polymorphism in this sequence to allow the generation of different peptides that are both male-specific and capable of binding to different MHC molecules. This is likely to be due to the fact that SMCY is a very large protein ($>$1500 residues) that differs from the female homolog by over 200 residues that are relatively uniformly distributed throughout its length. These create the potential to generate a large number of distinct SMCY-specific peptides as H-Y epitopes. It will be interesting to determine whether the proteins that encode other mHag also share these characteristics.

An important result of this study is that the peptide epitope recognized by R416 CTL clone in extracts from HLA-A*0201 molecules has undergone a posttranslational modification in which a second cysteine residue has been covalently linked to a cysteine in the peptide sequence via a disulfide bond. This modification augments recognition by R416 by over 1000-fold, despite the fact that it also reduces the binding affinity of the peptide for HLA-A*0201 by about 5-fold. This same modified peptide was also identified as the only active species in HPLC fractions recognized by R416 from 3 different H-Y$^+$, HLA-A*0201$^+$ cell lines, as well as in human spleen cells. Taken together, these data clearly define the epitope recognized by the CD4$^+$ clone, R416, as the cysteinylated form of FIDSYICQV.

The results of epitope reconstitution experiments suggest that one possible mechanism for cysteine modification of peptides extracted from cultured cells is disulfide exchange with the 0.21 mM cystine present in the slightly basic RPMI-1640 medium. Although this might suggest that its occurrence is an artifact of in vitro culture, the presence of the same cysteinylated species in peptides extracted from freshly isolated human spleen cells argues against this possibility. Serum also contains up to 0.1 mM cystine and has a pH that would support cysteinylation of any free cysteine residues accessible on the cell surface, so it is possible that the same mechanism of modification could occur in vivo. However, our experiments do not rule out the possibility that cysteinylation might occur before or after peptide binding to MHC molecules in the endoplasmic reticulum (ER), since this compartment contains the components that mediate disulfide bond formation and exchange during protein folding. Regardless of the exact mechanism, these results suggest that this modified form of the peptide is the epitope that stimulated CTL clone R416 in vivo.

Our results also established that two other MHC-associated peptides have undergone cysteinylation of cysteine residues in their sequences, but that not every cysteine residue is modified in this way. If modification occurs after peptide binding to the MHC molecule, either in the ER or at the cell surface, then it would depend upon accessibility of the cysteine side chain to aqueous solution. The ability of an uncysteinylated residue to be derivatized by iodoacetamide while it is bound to MHC molecules suggests that there may be additional constraints on cysteinylation, even among accessible residues. Alternatively, if modification occurs prior to peptide binding to the MHC molecule in the ER, then

cysteinylation may exert a pronounced influence on the ability of peptides to bind and, in some cases, might preclude binding altogether. In sum, we conclude that cysteinylation is not rare, but is a highly probable modification of any free cysteine residues in peptides that point out of the MHC-binding pocket.

Our results also showed that a second H-Y-specific, HLA-A*0201-restricted $CD8^+$ CTL clone, 1R35, preferentially recognizes the unmodified, as opposed to cysteinylated, form of the same SMCY-derived peptide. However, this unmodified form of the peptide was not present in cell extracts in any measurable quantity. Instead, epitope-reconstituting activity for this CTL clone was present in two fractions, neither of which have retention times that correspond to that of the unmodified synthetic peptide. DTT treatment of these two active fractions resulted in the appearance of the unmodified peptide. However, we have as yet been unable to identify the modified parent species. Nonetheless, these results suggest that the 1R35 CTL clone is specific for a second distinct modification of the FIDSYICQV peptide. In this regard, a recent report described a modification involving the addition of an unidentified mass of 102 Da to a cysteine residue in an HLA-A*0201-associated peptide derived from vinculin (di Marzo Veronese et al., 1996). Further work will be necessary to establish the source, mechanism, and generality of other modifications of cysteine residues in class I–associated peptides.

The cysteinylation demonstrated here represents the second example of a naturally occurring posttranslational modification of class I MHC-associated peptides. We previously identified a peptide originating from tyrosinase that was modified by the conversion of an asparagine residue to aspartic acid, apparently owing to N-linked glycosylation and deglycosylation reactions (Skipper et al., 1996). Both of these modifications were detected only through the use of mass spectrometry for peptide sequencing and were not immediately predictable by techniques of antigen identification based on cDNA cloning and peptide-binding motifs (Wolfel et al., 1994). In addition, both of these modifications have a profound impact on T cell recognition, while they have a modest effect of peptide binding to the MHC molecule. Class II MHC–associated peptides have also been shown to be posttranslationally modified by the attachment of carbohydrate side chains (Chicz et al., 1993; Michaelsson et al., 1994), the extent of which affects T cell recognition of the peptides (Michaelsson et al., 1994), and we think it very likely that class II–associated peptides will undergo both asparagine residue–to–aspartic acid conversion and cysteinylation. Collectively, all of these results indicate that the occurrence of several types of posttranslational modifications of MHC-associated peptides is commonplace and that the influence of these modifications must be taken into account in attempts to predict immunologically active peptides from known protein sequences and in the design of peptide-based immunotherapeutics.

## Experimental Procedures

### Cell Culture

The $CD8^+$ cytotoxic T cell clone 1R35 and the $CD4^+$ cytotoxic and proliferative T cell clone R416 were derived by limiting dilution culture from the PBMC of a female aplastic anemia patient who had undergone multiple transfusions and who had rejected HLA-identical male bone marrow (Goulmy et al., 1977; de Bueger et al., 1992a). Both clones recognize H-Y in the context of HLA-A*0201. The clones were cultured by weekly stimulation with irradiated allogeneic PBMC and EBV-transformed B-LCL in RPMI-1640 medium containing 15% human serum, 3 mM L-glutamine, 1% leucoagglutinin, and 20 U/ml recombinant IL-2. Frozen aliquots were thawed and cultured for 1–3 days in RPMI-1640 containing 5–15% human or fetal bovine serum and 20 U/ml recombinant IL-2 before use in cytotoxicity assays. The HLA-$A*0201^+$ male B-LCL Blk, DM, and Rp and the HLA-$A*0201^+$ antigen-processing mutant cell line T2 were maintained in IMDM containing 5% FCS or in RPMI-1640 containing 3 mM L-glutamine and 10% FCS.

### Extraction and HPLC Fractionation of Immunoaffinity-Purified Peptides

HLA-A*0201 molecules were immunoaffinity-purified from $10 \times 10^{10}$ $H\text{-}Y^+$ HLA-$A*0201^+$ Blk cells, and the bound peptides were separated as described (Hunt et al., 1992; de Bueger et al., 1993; den Haan et al., 1995), except that 1% CHAPS was substituted for NP-40 in all preparative steps. Peptides were eluted from the HLA-A*0201 molecule with 10% acetic acid, 1% TFA and separated from the HLA-A*0201 heavy chain and β2-microglobulin with a 10 kDa Centricon filter (Amicon). Peptides extracts were fractionated by HPLC on a reverse-phase $C_2/C_{18}$ column (3 μm particles, 120 Å pore size, 2.1 mm inner diameter, 10 cm length), using the Pharmacia Smart System. For the first dimension, separation buffer A was 0.1% HFBA in water, and buffer B was 0.1% HFBA in acetonitrile. The gradient was 100% buffer A (0–20 min), 0%–12% buffer B (20–25 min), and 12%–50% buffer B (25–80 min) at a flow rate of 100 μl/min, and 100 μl fractions were collected. For second dimension separations, TFA was substituted for HFBA. For separation of the first dimension fraction that contained epitope activity for the 1R35 clone, the gradient was 100% buffer A (0–29 min), 0%–22% buffer B (29–34 min), 22% buffer B (34–39 min), and 22%–27.9% buffer B (39–98 min) at a flow rate of 100 μl/min. For separation of the first dimension fraction that contained epitope activity for the R416 clone, the gradient was 100% buffer A (0–29 min), 0%–18% buffer B (29–34 min), 18% buffer B (34–39 min), and 18%–23.9% buffer B (39–98 min) at a flow rate of 100 μl/min. In both cases, 100 μl fractions were collected.

### Epitope Reconstitution Assay

To test for the epitope reconstituting activity of HPLC fractions, aliquots were diluted into 25 μl of Hank's BSS containing 50 mM HEPES and incubated with 2500 $^{51}Cr$-labeled T2 cells in 25 μl of RPMI-1640 containing 3 mM L-glutamine and 15% human serum for 30 min at 37°C. CTL were then added to give a final volume of 150 μl. After 4 hr at 37°C, $^{51}Cr$ release in the supernatant was measured by standard methodology. The same methodology was employed for the analysis of the activity of synthetic peptides. However, to avoid cysteinylation of peptides during incubation with target cells and CTL, assays were also performed in cystine-free RPMI-1640 containing 3 mM L-glutamine and 1% BSA, using peptides that had been dissolved in Hank's BSS containing 50 mM Hepes and 2 mM DTT.

### Mass Spectrometry

Fractions from second dimension HPLC separations that contained epitope reconstitution activity were analyzed by microcapillary HPLC-electrospray ionization mass spectrometry (Hunt et al., 1992). Peptides were loaded onto a C18 microcapillary column (75 μm i.d. × 10 cm) and eluted directly into the mass spectrometer with a gradient of 0%–80% acetonitrile in 0.1% acetic acid over 12 min at 0.5 μl/min. In some experiments, samples were reduced by loading them onto the microcapillary column and washing them with 5 μl of a 10 mM solution of DTT in ammonium acetate (pH 8.5). Mass spectra and CAD mass spectra were recorded on a Finnigan-MAT TSQ-7000 (San Jose, California) triple quadrupole mass spectrometer equipped with an electrospray ion source. Electrospray ionization was accomplished with a voltage differential of 4.6 keV on the needle and a sheath liquid consisting of a 70:30 mixture of methanol/ 0.1%

acetic acid flowing at 1–2 μl/min. Mass spectra were acquired every 1.5 s over the range *m/z* 300–1400.

**Synthetic Peptides**

Peptides were synthesized by solid-phase FMOC chemistry and Wang resins on an AMS 422 multiple peptide synthesizer (Gilson Medical Electronics, Middletown, WI). Cysteinylation was accomplished by incubating synthetic peptides in a solution of aqueous ammonium hydroxide containing excess cystine for 5 min at room temperature. The reaction was terminated by acidifying the solution with glacial acetic acid. Peptides were purified to greater than 90% homogeneity by reverse-phase HPLC and characterized by mass spectrometry.

**Class I Peptide–Binding Assay**

This was carried out as described (Ruppert et al., 1993; Chen et al., 1994; Sette et al., 1994). Briefly, affinity-purified HLA-A*0201 molecules were incubated at room temperature with the iodinated indicator peptide, FLPSDYFPSV, and graded doses of test peptides in PBS (pH 7.0) containing 0.05% NP-40, 1 μM human β2 microglobulin (CalBiochem, La Jolla, CA), 1 mM PMSF, 1.3 mM 1,10-phenanthroline, 73 μM pepstatin A, 8 mM EDTA, and 200 μM TLCK. After 48 hr, class I peptide complexes were separated from free peptides by gel filtration, and the dose of individual test peptides that reduced the binding of indicator peptide by 50% ($IC_{50}$) was calculated.

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