An N-acetylated Natural Ligand of Human Histocompatibility Leukocyte Antigen (HLA)-B39: Classical Major Histocompatibility Complex Class I Proteins Bind Peptides with a Blocked NH$_2$ Terminus In Vivo

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Abstract

Sequence-independent interactions involving the free peptidic NH$_2$ terminus are thought to be an essential feature of peptide binding to classical major histocompatibility complex (MHC) class I proteins. Challenging this paradigm, a natural N$_\text{a}$-acetylated ligand of human histocompatibility leukocyte antigen (HLA)-B39 was identified in this study. It matched the NH$_2$-terminal sequence of two human helicases, was resistant to aminopeptidase M, and was produced with high yield from a synthetic 30-mer with the sequence of the putative parental protein by the 20S proteasome. This is the first reported natural ligand of classical MHC class I antigens that has a blocked NH$_2$ terminus.

Key words: human • antigen processing • biochemistry • molecular biology • tolerance

Introduction

Classical MHC class I molecules (HLA-A,B,C in humans) constitutively bind peptides from endogenously synthesized cell proteins and from intracellular pathogens, and present them at the cell surface for recognition by CD8$^+$ CTLs. Peptides bind the class I molecule in its peptide binding site. This is a groove formed by the α1 and α2 domains of the heavy chain, consisting of two long α-helical segments on top of an eight-strand antiparallel β-pleated sheet (1, 2). The fine structure of this groove can be described as a series of subpockets (pockets A–F) formed by side chains of residues from the MHC molecule (3, 4). Peptides bind to class I molecules in extended conformation, with the NH$_2$ and COOH termini interacting in pockets A and F, respectively, and various peptidic anchor residues interacting in the other pockets (5). MHC polymorphism occurs mainly in the peptide binding groove and, by affecting the size, shape, and polarity of the pockets, modulates the peptide binding specificity of class I molecules.

A major feature of the peptide binding groove of classical class I antigens, which distinguishes it from class II molecules, is that the two α-helical segments come close to each other at both ends. In addition, the ends of the groove become blocked by bulky side chains including Tyr84 (pocket F) and Trp167 (pocket A), which are highly conserved among class I proteins. This imposes strong restrictions on the size of class I-bound peptides, so that these have a size range of 8–12 residues, with a majority of nonamers (6). Size variations are usually accommodated by differential bulging or other conformational changes in the middle part of the peptide (7–9). The peptidic NH$_2$ and COOH termini bind the class I molecule through hydrogen bonds to conserved residues in pockets A and F, respectively, essentially in a peptide sequence-independent mode. These interactions have a significant contribution to the total binding energy of the peptide (10, 11). Nonetheless, class I ligands can also bind with the COOH-terminal residue positioned outside the peptide binding groove, and this noncanonical binding mode is stable at physiological temperature (12).

In contrast, it is currently assumed that there is a universal binding mode for peptidic NH$_2$ termini. All known natural ligands of classical class I antigens have a free NH$_2$ terminus. This interacts in the A pocket through a set of hydrogen bonds established directly with Tyr7 and Tyr171, and with Tyr59 through a water molecule (5, 13). Suppres-
amino acid changes in the protein molecule, including peptides (16–18). This is made possible through various
occurrence of a cocktail of protease inhibitors, and B from GIBCO BRL). Cells were lysed in 1% NP-40 in the pres-
first natural ligand with a blocked NH
occurrence of these hydrogen bonds by NH\textsubscript{2}-terminal methyla-
tion of a peptide ligand of HLA-A2 decreased the thermal stability of the peptide–MHC complex by 22°C, indicating
that the NH\textsubscript{2} terminus is an energetically important site for class I binding (10). N\textalpha-acetylation has been shown to sig-
ificantly decrease binding of peptide ligands to classical MHC class I proteins (14, 15). In contrast, the nonclassical murine antigen H2-M3 specifically binds N-formylated peptides (16–18). This is made possible through various amino acid changes in the protein molecule, including some that dramatically affect the structure of pocket A (19, 20).

The apparent inability of class I antigens to bind N\textalpha-acetylated ligands is in contrast with the relatively frequent occurrence of N\textalpha-acetylated proteins in eucariotic cells (21, 22). We report here that an N\textalpha-acetylated peptide is a natural ligand of HLA-A2-39. This is, to our knowledge, the first known N\textalpha-acetylated ligand of any class I molecule, and the first natural ligand with a blocked NH\textsubscript{2} terminus reported from a classical MHC class I protein. In addition, we show that this peptide is directly generated by the 20S proteasome as a major breakdown product of a precursor substrate with the sequence of the corresponding parental protein.

Materials and Methods

HLA-B39 Transfectant Cell Lines. HM2y2.C1R (C1R)\textsuperscript{1} is a human lymphoid cell line with low expression of its endogenous class I antigens. C1R transfectants expressing B*3901 or B*3909 have been described previously (23). The B*3905-C1R transfectant was obtained with the same electroporation and selection procedures as the previous transfectants, using a full-length cDNA clone with the coding B*3905 sequence (a gift from Dr. Peter Parham, Stanford University, Stanford, CA) subcloned into the RSVneo vector. HLA-B39-C1R transfectant cells were cultured in DMEM supplemented with 7.5% heat-inactivated FCS (both from GIBCO BR L). RMA-S is a transporter associated with antigen processing-deficient murine cell line (24, 25). The B*3909–RMA-S (23) and B*2705–RMA-S (26) cells used in this study, which also express human β2-microglobulin, have been described elsewhere. These cells were grown in RPMI 1640 medium containing 25 mM Hepes buffer and 5% heat-inactivated FCS (GIBCO BR L).

Isolation of HLA-B39–bound Peptides. This was carried out as described previously (27). In brief, ∼10\textsuperscript{10} cells were grown at 37°C in DMEM, pH 7.4 containing 7.5% heat-inactivated FCS, 100 μg/ml streptomycin sulfate, and 100 μM penicillin G (all from GIBCO BR L). Cells were lysed in 1% NP-40 in the presence of a cocktail of protease inhibitors, and B*3901, B*3905, or B*3909 were purified by affinity chromatography from the cell lysates using the W 6/32 mAb (IgG2a, specific for a monomorphic HLA-A,B,C determinant; reference 28). HLA-B39–bound peptide pools were obtained by acid extraction with 0.1% TFA, and were fractionated by HPLC.

\textsuperscript{1}Abbreviations used in this paper: C1R, HM2y2.C1R; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; NAc, N-acetyl.

Mass Spectrometry Analysis and Sequencing. The peptide composition of HPLC fractions or other samples was analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) using a calibrated Kompact Probe instrument (Kratos-Schimadzu) operating in the positive linear mode, as described previously (27). Peptide composition analysis, zoomscan, and sequencing by electrospray ion trap MS was carried out with an LCQ instrument (Finnigan Thermospray) and using the "nanospray" interface, as detailed elsewhere (23, 29). Zoomscan is a high-resolution method for determining accurate peptide mass and charge of ionic species, in which a precursor ion window is selected to incorporate several isotopomers. The charge states of individual product ions were determined at enhanced resolution by scanning across a limited m/z range. Synthetic Peptides. Peptides were synthesized using the standard solid-phase Fmoc chemistry, and were purified by HPLC. In the case of N-acetyl (NAc) peptides, acetylation was carried out manually as follows: some of the peptide resin was treated to remove the Fmoc group without removing the side chain protecting groups, then it was incubated with an excess of an acetylation cocktail containing 10% acetic anhydride in N-methyl-pyrroloidine-2 (SDS; Pepsin) and shaken for 10 min. The filtered peptide resin was then washed eight times with dichloromethane (SDS; Pepsin) before removing the protecting groups of the peptide side chains. The correct composition and molecular mass of purified peptides were confirmed by amino acid analysis using a 6300 amino acid analyzer (Beckman Coulter), which also allowed their quantification, and by electrospray ion trap MS, respectively.

Digestions with Aminopeptidase M. Approximately 70% of the HPLC fraction containing the NAc-SHVAVENAL peptide from the B*3905–B*3909–bound peptide pool was dried down, resuspended in 5 μl of methanol/water (1:1 vol/vol) containing 0.1% formic acid, and sonicated for 5 min. A 2-μl aliquot of this mixture (28% of the fraction) was used for MALDI-TOF MS analysis. Another 2-μl aliquot of the same mixture was digested as follows: 0.5 μl of a suspension containing 10\textsuperscript{–2} U of aminopeptidase M from pig kidney (Boehringer), 0.5 μl 100 mM Tris, and 2 μl H\textsubscript{2}O was added in an eppendorf tube and incubated for 10 min at 37°C. The digestion was stopped by adding 0.2 μl of TFA. This whole mixture was then subjected to MALDI-TOF MS analysis.

Epitope Stabilization Assay and Flow Microfluorometry Analysis. The epitope stabilization assay used to measure peptide binding was performed as described (23). In brief, B*3909–RMA-S or B*2705–RMA-S transfectants were incubated at 26°C for 24 h. They were then incubated for 1 h at 26°C with 10\textsuperscript{–4}–10\textsuperscript{–9} M peptide without FCS, transferred to 37°C, and collected for flow microfluorometry after 4 h. B*3909 or B*2705 expression was measured using 50 μl of hybridoma culture supernatant containing the mAb W 6/32 (28; for B*3909) or M E1 (30; for B*2705), as described previously (31). Binding was expressed as the C50, which is the molar concentration of any given peptide at 50% of the maximum fluorescence obtained at the concentration range used. Binding of acetylated peptides was assessed as the concentration of the acetylated peptide required to obtain the fluorescence value at C50 of the corresponding unblocked peptide. This was designated as EC50. Relative binding was calculated as the ratio between the C50 of the unblocked peptide and the EC50 of the corresponding N\textalpha-acetylated analogue.

Purification of 20S Proteasomes. Approximately 3 × 10\textsuperscript{10} B*2705–C1R cells were pooled lysed in 50 ml of 50 mM Tris/HCl and 25 mM KCl, pH 8. The homogenate was centrifuged at 1,500 g for 10 min. The supernatant was further centrifuged at 180,000 g for...
Results

MS analysis of HLA-B39-bound peptides reveals a putative N-\alpha-acetylated ligand. B*3901-, B*3909-, and B*3905-bound peptides were extracted from the corresponding immunopurified HLA molecules expressed on C1R transfectants and fractionated by HPLC. Full-scan analysis by nanoelectrospray ion trap MS of HPLC fraction number (N.) 155 from B*3901 (Fig. 1 A) revealed various ion peaks. Among these, those at m/z 848.4 and 870.4 were compatible with a peptide (M + H\(^+\)) and its Na\(^+\) adduct, respectively. MS/MS fragmentation analysis of the former ion allowed us to determine its amino acid sequence as THGPPVQL (23). A second peptide was revealed by ion peaks at m/z 981.4 (M + H\(^+\)), 502.4 (presumably M + H\(^+\) + Na\(^+\)), and 510.5 (presumably M + H\(^+\) + K\(^+\)). A peptide with the same mass was also detected in the corresponding HPLC fractions from B*3909 and B*3905 (Fig. 1, B and C).

Zoomscan analysis of the ion peak at m/z 981.4 from B*3901 and the corresponding ones from B*3909 and B*3905 yielded a monoisotopic peptide mass of 980.4-980.5 (Fig. 1 C). MS/MS fragmentation analysis of this ion peak from B*3901 yielded a daughter spectrum (Fig. 2 A) that was consistent with the sequence XHVAVENAL. Although residue X had a molecular mass corresponding to that of glutamic acid (E), no match with any peptide was
found when the MS/MS spectrum was used to search against the nr.fasta protein database. In addition, no peptide with the sequence EHVAVENAL was found upon scanning of multiple protein or nucleotide databases. However, a sequence SHVAVENAL was found, which was identical except for the NH2-terminal residue. It corresponded to the NH2-terminal sequence of the human protein RNA helicases of the DEAD box family DBX (32) and CAP-Rf (33). The mass difference between the E and S residues (42 Da) was consistent with an acetyl group. Because NH2-terminal acetylation is a common posttranslational modification, our results raised the possibility that XHVAVENAL was an Nα-acetylated ligand of B*3901. Identical results were obtained from the MS/MS analyses of the corresponding ion peaks from B*3909 and B*3905 (data not shown), indicating that the same peptide was present in the three HLA-B39 subtypes.

To confirm the sequence assignment, synthetic EHVAVENAL and NAc-SHVAVENAL peptides were separately subjected to MS/MS fragmentation analysis. Whereas the daughter spectrum of NAc-SHVAVENAL (Fig. 2 B) was identical to that of XHVAVENAL, the MS/MS spectrum of EHVAVENAL yielded an additional set of ion peaks corresponding to the b1 series, generated from neutral loss of water from ions of the b series (Fig. 2 C). This difference between the two peptides was consistently found at various peptide concentrations, ionization conditions, or collision energies (data not shown). Taken together, these results strongly suggest that B*3901, B*3909, and B*3905 bind a natural Nα-acetylated ligand derived from the NH2-terminal region of human proteins.

The natural HLA-B39 Ligand Has the Same Retention Time as NAc-SHVAVENAL in HPLC. In a further analysis, we compared the HPLC retention times of the natural B*3901 ligand XHVAVENAL and the synthetic peptides NAc-SHVAVENAL and EHVAVENAL. The THGPPVQL peptide, which coeluted with XHVAVENAL in the HPLC fractionation of B*3901-bound peptides (shown in Fig. 1 A), was used as a marker for the retention time of XHVAVENAL. The three peptides were separately chromatographed under identical conditions in consecutive runs, and the chromatograms are superimposed.
fractionated in a single HPLC run (data not shown). This result confirms that the natural ligand with \( M + H + 981.4 \) coeluting with THGPPVQL in the HPLC fractionation of the B*3901-bound peptide pool is NAc-SHVAVENAL.

The natural HLA-B39 ligand is resistant to digestion by aminopeptidase M. The HPLC fraction from B*3905-bound peptides containing the putatively N\(^\alpha\)-acetylated ligand showed a complex composition when analyzed by MALDI-TOF MS (Fig. 3 A). Among the peptides present, those at m/z 848.3 and 981.4 were shown by MS/MS fragmentation analysis to be THGPPVQL and XHVAVENAL, respectively. An aliquot of this HPLC fraction identical to that used for MALDI-TOF analysis (~30% of the total) was digested with aminopeptidase M, an enzyme known to hydrolyze peptide bonds from small peptides with free, but not blocked, NH\(_2\) termini (34, 35). When the digestion mixture was analyzed by MALDI-TOF MS, most peak signals in the undigested aliquot were reduced to background levels, whereas the peaks corresponding to XHVAVENAL (m/z: 982.0) and its Na\(^+\) adduct (m/z: 1004.2) remained as the only significant signals above background. As a control for this digestion, an approximately equimolar mixture of the synthetic peptides THGPPVQL and NAc-SHVAVENAL was treated with aminopeptidase M under the same conditions, and the mixture was analyzed by MALDI-TOF MS before and after digestion (Fig. 3, C and D). Whereas the peak signal of the former peptide (m/z: 849.1) was reduced almost to background levels after digestion, the signals corresponding to NAc-SHVAVENAL (m/z: 982.2 and 1004.1) remained strongly significant. These results indicate that XHVAVENAL has a blocked NH\(_2\) terminus, further confirming the assigned NAc-SHVAVENAL sequence.

NAc-SHVAVENAL is generated with high yield by the 20S proteasome. An N\(^\alpha\)-acetylated synthetic 30 mer with the NH\(_2\) terminal sequence of the putative parental proteins of NAc-SHVAVENAL and the DBX and CAP-Rf helicases (32, 33) was digested in vitro with purified 20S proteasome for 4, 8, and 24 h, and the digestion mixtures were separately fractionated by HPLC. Approximately 26, 39, and 63% of the 30 mer, respectively, was digested at each of the three time points.

All HPLC fractions from the 24-h digest eluting between 50 and 120 min, which encompasses virtually all eluted peptides (Fig. 4 A), were analyzed by MALDI-TOF MS. In addition, those HPLC fractions corresponding to peptide peaks were also analyzed by electrospray ion trap MS to detect possible additional peptides not seen by MALDI-TOF and to unambiguously characterize some of the digestion products through MS/MS sequencing. This analysis allowed us to determine the digestion pattern of the 30 mer, and revealed the presence of NAc-SHVAVENAL (1-9) as the main component of one of the major peptide peaks (Fig. 4 A).

As shown in Fig. 4 B, the NAc-SHVAVENAL peptide was directly generated by the 20S proteasome with high yield, accounting for ~16% of the total digest. This was due to a highly efficient cleavage at the L9-G10 peptide bond of the N\(^\alpha\)-acetylated precursor and to absence of any significant cleavage at peptide bonds between amino acids S1 to L9.

NAc-SHVAVENAL binds HLA-B39 in vitro with decreased efficiency. Because the peptidic NH\(_2\) terminus

![Figure 3](image-url)
plays a significant role in peptide anchoring to the class I molecule (10), we analyzed the effect of \( \text{Nac-} \)-acetylation on binding to \( B^*3909 \) in vitro using an epitope stabilization assay. The synthetic peptides SHVALENAL and N\( \text{Nac-} \)-SHVALENAL, as well as two unrelated natural ligands of \( B^*3909 \) (ARDETEFYL and YRPQTVAL; reference 23), were tested (Fig. 5 A). Although SHVALENAL bound to \( B^*3909 \) similarly as the two natural ligands with free \( \text{NH}_2 \) terminus, N\( \text{Nac-} \)-SHVALENAL bound \( \sim \)10-fold less efficiently than the nonacetylated analogue (relative binding 1:11). These results indicate that \( \text{Nac-} \)-acetylation of a Ser residue decreases but does not abrogate binding to \( B^*3909 \) in vitro.

**Limited Influence of P1 variability on binding of \( \text{Nac-} \)-acetylated peptides.** Molecular modeling suggested a binding mode for N\( \text{Ac-} \)-SHVALENAL in which the Ser1 side chain was switched towards the A pocket in order to locate the acetyl group towards the solvent (data not shown). In this model, hydrogen bonding through the \( \beta \)-hydroxyl group of Ser1 would partially compensate for loss of the canonic interactions involving the peptidic \( \text{NH}_2 \) terminus.
To test this model, we reasoned that variations in the size and/or polarity of the P1 side chain should significantly affect binding of the corresponding N-acetylated analogues. In contrast, if the binding mode of the N-acetylated ligand does not involve reorientation of the P1 side chain towards pocket A, the effect of N-acetylation should be less dependent on the P1 residue. Thus, synthetic THVAVENAL, AHVAVENAL, and their N-acetylated analogues were tested for binding to B*3909 in vitro (Fig. 5, B and C). The N-acetylated peptides bound B*3909 with similarly decreased efficiency relative to their nonacetylated counterparts (relative binding 1:6 in both cases) as NAc-SHVAVENAL. These results indicate that neither a moderately increased size (Thr) nor removal of the hydroxyl group (Ala) had a significant effect on binding of the corresponding N-acetylated peptides.

However, it could be argued that N-acetylation of small P1 residues might still be compatible with binding of the P1 side chain in the A pocket. Thus, we tested the effect of N-acetylation on binding of a peptide analogue with the bulky and apolar Phe1, whose size precludes its side chain from entering into the A pocket (Fig. 5 D). Binding of NAc-FHVAVENAL relative to its unblocked counterpart was somewhat lower than for other P1 residues (1:19). However, its binding efficiency (EC50: 38 μM) was similar to the other N-acetylated peptides tested (Fig. 5).

These results indicate that the size and polarity of the P1 residue have a limited influence on binding of N-acetylated peptides in our epitope stabilization assay, and suggest that their binding mode may not involve interaction of the N-acetylated P1 side chain in the A pocket.

Similar Effect of N-acetylation on Peptide Binding to HLA-B27. To test whether the effect of N-acetylation observed in HLA-B39 could be generalized to other class I molecules, a natural B*2705 ligand with Ser1, the influenza nucleoprotein epitope SYWAI RTR (36), and its N-acetylated analogue were tested in an epitope stabilization assay using B*2705 RMA-S cells. As shown in Fig. 6, the relative binding of the N-acetylated peptide (1:5) was similar as for the HLA-B39 ligand. This result indicates that the effect of N-acetylation on peptide binding is similar for at least two HLA-B allotypes.

Discussion

This study provides the first demonstration that an N-acetylated peptide from an endogenous cell protein is a natural HLA class I ligand. This finding challenges the current paradigm that a free NH2 terminus is an essential feature for natural ligands of classical class I–bound peptides. Various posttranslational modifications have been reported among natural MHC class I ligands, including deamidation (37), cysteine modification (38), and glycosylation (39). However, these modifications do not affect the NH2 terminus of the peptide or otherwise alter sequence-independent MHC–peptide interactions. Other naturally occurring modifications also not affecting the peptide NH2 terminus, such as phosphorylation, are compatible with class I binding (40), but to our knowledge, no natural phosphorylated class I ligands have been reported. The possibility that N-acetylation might have occurred during peptide purification is ruled out because it requires different and more drastic chemical treatments than those used for isolation of class I ligands. Moreover, multiple peptides with unblocked NH2-terminal Ser or other residues have been sequenced in our laboratory after using the same isolation procedure (23, 41).

Three issues are raised by our results: (a) the mode of interaction of N-acetylated ligands with the class I molecule; (b) the mechanism by which such ligands might be processed in vivo; and (c) the biological significance of class I–mediated presentation of N-acetylated peptides.

The first issue can be definitively addressed only by x-ray crystallography. However, an attempt was made here to distinguish between two alternative possibilities. In the first one, predicted by molecular modeling (data not shown), the P1 side chain would be accommodated in the A pocket with the acetyl group directed outwards. This binding mode would be expected to be strongly influenced by the size and polarity of the P1 side chain. A second alternative would be that the acetyl moiety is located inside the A pocket whereas the P1 side chain points towards solvent. Presumably, this binding mode should be less sensitive to the nature of the P1 side chain. Our results showing that even a significant increase in the size and hydrophobicity of the P1 residue had only a limited influence on binding of the N-acetylated analogue do not support the first alternative and are more compatible with the second one. However, other possibilities cannot be ruled out. For instance, if the acetylated P1 residue is Ser or another small one, the side chain might bind in the A pocket, and alternative binding modes might only be adopted if the side chain is too big. Finally, neither the P1 side chain nor the acetyl moiety might bind in the A pocket. The cavity created in pocket A might then be filled by a water molecule hydrogen bonded to Tyr7 and Tyr171, as seen in the x-ray struc-
ture of HLA-A*0201 in complex with a peptide for which the free amino group was replaced by a methyl moiety (11).

In spite of its presence in vivo, NAc-SHVAVENAL bound in vitro ~10-fold less efficiently than other natural ligands. This might be due to the fact that the epitope stabilization assay used in this study is significantly influenced by the association rate of the peptide, whereas binding in vivo depends on the stability of the MHC–peptide complex. Thus, although the N α-acetylated ligand is sufficiently stable for binding in vivo, its association rate at the cell surface might be lower than for its counterpart with free NH₂ terminus. In vitro assays, including our epitope stabilization assay, do not reflect peptide binding as it occurs in vivo. In particular, the kinetics of MHC–class I peptide association may be strongly influenced in vivo by the mechanisms of assisted loading involving transporter associated with antigen processing–tapasin–MHC protein complexes.

The interaction of N α-acetylated peptides with a classical class I protein differs significantly from the nonclassical class I molecule H-2-M3 associated to an N-formyl peptide (19). H-2-M3 presents N-formylated peptides from prokaryotic and mitochondrial proteins to CD8⁺ T cells (18, 20). It can also bind nonformylated peptides, but with a restricted specificity for Gly at P1 (42). This unusual binding motif is explained by several amino acid changes (Trp167Leu, Tyr171Phe) that dramatically reduce the size of pocket A and shift the P1 side chain into pocket B (19).

Another major difference of H-2-M3 with classical class I molecules is the presence of a positively charged His at position 9 that interacts with the formyl oxygen atom. H-2-M3 does not bind N α-acetylated peptides (16). Consequently, binding of N-formylated peptides by H-2-M3 is very strongly favored over unblocked peptides. In contrast, for the N α-acetylated peptide bound to a classical class I protein described in this study, pocket A is composed of consensus amino acids, and binding is less efficient than for a counterpart with free NH₂ terminus. The similar effect of N α-acetylation on binding of peptide analogues with Thr1 or Ala1 suggests that acetylation of some P1 residues other than Ser might be tolerated among natural class I ligands. In addition, the fact that the effect of N-α-acetylation of Ser1 on binding to B*2705 was similar in HLA-B39 suggests that other MHC class I molecules besides HLA-B39 might also bind N α-acetylated peptides.

Processing of the NAc-SHVAVENAL ligand was approached in this study by analyzing its generation from a precursor substrate by the 20S proteasome. The high yield of the natural ligand in vitro demonstrates that in the sequence context of its parental protein, a blocked NH₂ terminus does not impair cleavage at the precise COOH-terminal residue of the natural ligand by the 20S proteasome. This experiment does not necessarily reproduce the mechanism by which this ligand is processed in vivo, as much protein breakdown in the cytosol occurs through the action of the 26S proteasome on ubiquitinated proteins (43). N α-acetylated proteins are degraded via the ubiquitin system without removal of the blocked NH₂ terminus (44-47). Therefore, it is likely that NAc-SHVAVENAL is generated in vivo by an ubiquitin-dependent mechanism. However, the cleavage pattern and high yield of the natural ligand observed with the 20S proteasome in vitro strongly suggest that it is directly generated by the proteasome also in vivo.

What is the possible biological significance of class I–mediated presentation of N α-acetylated peptides? N α-acetylation is a common modification of eucariotic proteins, and also occurs in viral and bacterial proteins. Although many N H₂-terminal residues can be acetylated in vivo, Ser is the most frequent one, occurring in ~40% of the N α-acetylated proteins (22). As shown in our study, an N α-acetylated peptide with appropriate size for MHC class I binding can be generated by proteasomal cleavage with high yield. Thus, N α-acetylated peptides with the size of class I ligands (8–11 amino acids) might be common breakdown products of eucariotic cell proteins.

It is generally assumed that a major mechanism of immunological tolerance against self-proteins is achieved through negative selection of autoreactive T cell precursors. Because MHC polymorphism modulates peptide specificity, a given MHC allelic product is able to bind a limited number of peptide ligands among those produced during the metabolic breakdown of a given endogenous protein. It is likely that not all of these peptides are equally immunogenic or tolerogenic. This is clear, for example, in self-restricted T cell responses against viral antigens. For a given restriction element, these responses are usually directed against one of very few immunodominant peptides from given viral protein (48). Because of the likely limitation in the number of immunogenic and/or tolerogenic self-peptides from an endogenous protein that can be presented by a particular class I allotype, the capacity of class I molecules to bind N α-acetylated peptides might contribute to enlarge the spectrum of self-antigens for negative selection of autoreactive T cell repertoires.

On the basis of known MHC class I ligands, it may be argued that N α-acetylated peptides are rare in class I–bound peptide pools. However, it should be noted that these ligands escape detection by classical Edman sequencing, and only a very small fraction of the natural class I–bound peptide repertoires have yet been sequenced by M.S. For this reason, the presence of putative N α-acetylated natural ligands of MHC class I proteins might have been underestimated.

We thank Anabel Marra, Samuel Oqueta, Fernando Barahona, Fernando Roncal (Centro Nacional de Biotecnología, Madrid), José Gavilanes (Universidad Complutense, Madrid), and Juana Busín for technical assistance in M.S., peptide synthesis, amino acid analysis, and tissue culture. We also thank José G. Castaño (Instituto de Investigaciones Biomédicas, Madrid) for help in proteasome purification, and Peter Parham for the B*3905 DNA.

This work was supported by grant SAF99/0055 from the Plan Nacional de I+D and grant 083/0022/1998 from the Comunidad Autónoma de Madrid (to J.A. López de Castro), and by grant 31-45504-95 from the Swiss National Science Foundation (to D.
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