Brief Definitive Report

T Cell Recognition of an HLA-A2-restricted Epitope Derived from a Cleaved Signal Sequence

By Maryse Guéguen, William E. Biddison,* and Eric O. Long

From the Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852; and the *Neuroimmunology Branch, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Summary

An alternative pathway for class I-restricted antigen presentation has been suggested on the basis of peptides bound to HLA-A2 molecules in cells lacking the transporter for antigen presentation (TAP). Most of these peptides were derived from signal sequences for translocation into the endoplasmic reticulum (ER). However, it is not known whether these peptides can be presented to T cells. The hydrophobic nature of an HLA-A2-restricted T cell epitope (M1 58-66) was exploited to test whether it could be presented to T cells when derived from a signal sequence. Replacing the signal sequence of the influenza virus hemagglutinin molecule H3 with an artificial sequence containing that HLA-A2-restricted T cell epitope resulted in efficient translocation of H3 molecules into the ER and transport to the cell surface. This signal sequence-derived epitope was presented to HLA-A2-restricted T cells. Involvement of cytosolic processing for this presentation is very unlikely, because (a) presentation occurred in cells lacking TAP; (b) expression of H3 molecules with the artificial signal sequence did not produce a detectable cytosolic form of H3; and (c) presentation of the same epitope expressed in cytosolic forms of antigen required TAP. Thus, a peptide derived from a signal sequence cleaved in the ER can provide an epitope for HLA-A2-restricted T cell recognition.

Cytosolic proteins constitute the major source of peptides for class I-restricted presentation to T cells. Peptides produced in the cytosol are transported to the early exocytic pathway where they bind to newly synthesized MHC class I molecules. Proper assembly of class I molecules and their transport to the cell surface require binding of peptide (1). Efficient delivery of cytosolic peptides into the endoplasmic reticulum (ER) requires an ATP-dependent transporter for antigen presentation (TAP). TAP-deficient mice express greatly reduced cell surface levels of MHC class I molecules and fail to establish a normal CD8+ T cell repertoire (2). An inefficient, ATP-independent peptide transport into the ER has been observed in the absence of TAP (3–5). Presentation of cytosolic peptides to class I-restricted T cells can be detected in the absence of TAP (6), but is improved by attachment of a signal sequence for ER translocation (7–9).

Cell surface expression of HLA-A2 is much less dependent on TAP than that of other class I alleles (10). Analysis of peptides eluted from HLA-A2 molecules in TAP-defective cells revealed that most were derived from signal sequences (11, 12). Similar peptides were also detected among peptides eluted from HLA-A2 in wild-type cells (12). However, the unusual length of these class I-bound peptides makes them poor candidates for T cell epitopes (13). Although T cells may recognize specific endogenous peptides bound to HLA-A2 molecules in TAP-deficient cells, it is not known whether such peptides are derived from signal sequences (14). On the other hand, signal sequence-derived peptides can provide T cell epitopes in normal cells (15, 16). Furthermore, several peptides with nine residues, derived from signal sequences, were eluted from HLA-B7 molecules in wild-type cells (17). It is possible that these signal sequence-derived peptides originated from processing in the cytosol, given the extreme efficiency of the class I processing pathway. Even a few precursor molecules that fail to translocate into the ER may provide sufficient cytosolic peptides derived from their signal sequence.

Thus, an important question remains: can signal sequence cleavage in the ER generate T cell epitopes from leader sequences? Evidence is presented here that such a mechanism can be employed for antigen presentation to HLA-A2-restricted T cells.

Materials and Methods

Cells and Viruses. The B lymphoblastoid cell line 721.45 and its derivative 721.174 (gift of R. DeMars, University of Wisconsin, Madison, WI) have been described (18). Mutant 721.174 has a homozygous deletion of most of the MHC class II region (19). Recombinant vaccinia viruses Vac-M1 (gift of B. Moss, National
Institutes of Health) and Vac-H3 (gift of G. Smith, Oxford University, Oxford, UK) have been described (20, 21). Vac-H3-D and Vac-H3-J were constructed as follows. The plasmid p3X31A (a gift from M. J. Getling, University of Texas Southwestern Medical Center, Dallas, TX) encoding the H3 hemagglutinin of influenza A virus was digested with either Sall (filled with dCTP and dTTP with Klenow polymerase) (H3-D) or BamHI (H3-J) and with EaeI and with EaeI. EaeI cuts the H3 coding region exactly at the signal sequence-mature H3 protein boundary, leaving a glycine residue at the NH2 terminus. BamHI cuts the H3 coding region exactly at the luminal-transmembrane region boundary. Sall cuts in the vector 3' downstream of the H3 coding region. The plasmid pEL1 (22) was digested with BglII (for H3-D and H3-J), filled with dATP and dCTP (only for H3-D), and with Sall. The oligonucleotides TCGATAATT-CTATTAATCATGCGACAGCTCATGGCATCCTAGGCTTCGTCTTCACGCTG-CTGCCGAGCAGCTGACGCT and GCCAGGCAGCTGCAGCTG were synthesized using standard automated procedures, purified on a 20% polyacrylamide gel, annealed, and ligated to the H3 fragment generated above and to the plasmid pEL1 in a triple ligation reaction. These oligonucleotides will provide 14 bp of the 5' untranslated region of H3 and a 17-amino acid peptide (MAEILGFVFTAAAG) that includes the HLA-A2-restricted epitope GILGFVFTL from the influenza A virus matrix protein M1 (23). The last glycine residue is part of the EaeI-compatible end. The algorithm of von Heijne (24) was used to predict the putative signal sequence cleavage site. The 5' end of the annealed oligonucleotides had a Sall-compatible end for ligation to the Sall site of pEL1. The 3' end of the annealed oligonucleotides had an EaeI-compatible end for ligation to the EaeI site of the H3 fragment. The 3' end of the H3 fragment was either BamHI (H3-J) or Sall (for H3-D) for ligation to the BglII site of pEL1. Sall and BglII ends were made compatible by partial filling as described above. In the construct H3-J, the stop codon is provided by the vector after a serine residue. pEL1-H3-D and pEL1-H3-J were used to generate recombinant vaccinia virus as described (25). Vac-H3-C was constructed as follows. The oligonucleotides TCGAAGGCATCTGATGATTAATAGAATTATC and CTCATATTACATGGCAGGCATCCTAGGCTTCGTCTTCACGCTGCTGCCGAGCAGCTGACGCT and GCCAGGCAGCTGCAGCTG were annealed and ligated to the unique XhoI site of the p3X31A plasmid. XhoI cuts the coding sequence of H3 at residue 411 of the mature H3 molecule (construct H3-D), as well as to a truncated form of H3 lacking the transmembrane region and cytoplasmic tail (construct H3-C) or Sall (for H3-D) for ligation to the BglII site of pEL1. These oligonucleotides provide 29 bp of the 5' untranslated region of H3 and the first two residues of the H3 coding region (MK) and an EaeI-compatible end that encodes a glycine residue. The resulting plasmid pEL1-H3-C was used to generate recombinant vaccinia virus as described (25). Recombinant vaccinia viruses were purified on a sucrose gradient as described (25). All these constructs are represented schematically (see Fig. 1).

**Immunoprecipitations.** 2 x 10⁶ cells were infected with 30 PFU/cell of vaccinia virus at 4°C for 1 h in 0.5 ml serum-free medium, and further incubated on a rotator for 1 h at 37°C. The infected cells were then starved in methionine-free medium for 1 h, and labeled with 50 μCi [³⁵S]methionine for 15 min. Cells were then washed twice in Tris-saline buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) and lysed in the same buffer containing 2% Triton X-100, 0.5 mM PMSF, 5 mM iodoacetamide, and 0.1 mM N-ethylmaleimide. The lysate was then centrifuged at 10,000 g for 5 min, at 4°C. The supernatant was first preclarified with a control mouse ascites and a polyclonal anti-mouse IgG antibody to protein A-Sepharose (all from Sigma Chemical Co., St. Louis, MO). The specific immunoprecipitation was performed with a pool of mAbs against H3 (gift of R. Webster, St. Jude Children's Research Hospital, Memphis, TN).

**Flow Cytometry.** 10⁶ cells were infected as described above. At the end of the incubation at 37°C in serum-free medium, FCS was added to a final concentration of 5% and the cells were incubated for 3 h at 37°C. Cells were then washed twice with PBS supplemented with 2% FCS and incubated with the anti-H3 mAbs in the same buffer. The cells were then washed twice and incubated with a FITC-conjugated goat anti-mouse Ig antibody (Tago Inc., Burlingame, CA) in the same buffer. The cells were then washed twice, fixed in 1% paraformaldehyde in PBS, and analyzed on a FACScan cytometer (Becton Dickinson & Co., Mountain View, CA).

**Cytotoxicity Assays.** 5 x 10⁵ 721.45 and 721.174 cells were infected as described above but incubated for 2 h at 37°C in the 5% FCS medium. Cells were labeled with 50 μCi sodium [³¹Cr]chelate for 1 h in 0.2 ml, washed, counted, and plated at 5 x 10⁴ cells/well in V-bottom 96-well plates containing previously aliquoted effector cells.

**Results and Discussion**

The HLA-A2-restricted M1 Epitope Can Serve as a Signal Sequence. Signal sequences do not contain specific amino acid residues other than a hydrophobic stretch of about eight residues (24). Cleavage usually occurs after a small nonpolar residue located five to six amino acids downstream of the hydrophobic segment. Thus, it appears that a hydrophobic peptide corresponding to a T cell epitope may fulfill the requirement to serve as a signal sequence. To test this possibility, the signal sequence of the influenza virus hemagglutinin protein H3 was replaced by a sequence containing the HLA-A2-restricted epitope of the influenza virus matrix protein M1 (residues 58-66). The nine amino acid-long epitope (GILGFVFTL) was preceded by the initiator methionine and an alanine residue and was followed by a spacer of five alanines (Fig. 1). According to the predictive algorithm of von Heijne (24), signal peptide would cleave after the last alanine residue of the spacer or after the adjacent glycine residue, yielding a peptide of 16 or 17 residues, respectively. Synthetic DNA corresponding to this 17 amino acid-long extension was added to the coding region of the mature H3 molecule (construct H3-D), as well as to a truncated form of H3 lacking the transmembrane region and cytoplasmic tail (construct H3-J). The M1 epitope was also inserted into a cytosolic form of the H3 molecule (H3-C in Fig. 1). Constructs encoding these chimeric molecules were inserted into recombinant vaccinia viruses.

To determine whether the 17 amino acid-long extension could serve as a signal sequence, cells infected with recombinant vaccinia viruses were metabolically labeled with [³⁵S]methionine and H3 molecules analyzed by SDS-PAGE after immunoprecipitation (Fig. 2). A nonglycosylated cytosolic form
of H3 has an apparent molecular weight of ~62,000 (22). Cells infected with Vac-H3-C produced a molecule of the expected size (Fig. 2 b). Due to a high number of glycosylation sites, the H3 molecule translated into the ER appears as a precursor with a molecular weight of ~83,000. Cells infected with Vac-H3 produced a molecule of the expected size (22, and Fig. 2 d). It is interesting to note that the product of Vac-H3-D was indistinguishable from that of Vac-H3 in both size and amount, suggesting that the H3-D molecule was efficiently translocated into the ER (Fig. 2 c). Furthermore, the product of Vac-H3-J migrated slightly faster (Fig. 2 e) as expected for a glycosylated molecule lacking 34 amino acids of the transmembrane region and cytoplasmic tail. It is worth noting that cells infected with Vac-H3-D did not produce detectable cytosolic H3-D molecules. Cytosolic H3-D molecules, which would migrate at the level of H3-C, were not seen even after long exposures of the gels. Unless the half-life of a cytosolic H3-D is much shorter than that of cytosolic H3-C, this result suggests that translocation into the ER mediated by the artificial leader sequence is very efficient. In pulse-chase studies (data not shown), the cytosolic H3-C molecule displayed a half-life of ~1 h, whereas the H3 and H3-D molecules were much more stable. The truncated molecule H3-J appeared in the cell supernatant during the chase, showing that it was secreted after translocation into the ER.

Proper folding of H3 molecules is a requirement for their transport out of the ER and through the Golgi apparatus to the cell surface (26). To determine whether the H3-D molecule fulfilled this requirement, cell surface levels of H3 molecules were analyzed by flow cytometry on cells infected for 4 h with Vac-H3 and Vac-H3-D (Fig. 3). Surface levels of H3-D (Fig. 3 b) were similar to those of H3 molecules (Fig. 3 d). In contrast, H3-C molecules were undetectable at the cell surface (Fig. 3 e). These data demonstrate that H3 molecules translocated into the ER via the artificial signal sequence containing the M1 epitope are transported to the cell surface in a form recognizable by mAbs and in amounts comparable to H3 molecules expressed with their native signal sequence.

Surface expression of H3-J molecules was also detected, although at reduced levels (Fig. 3 c). Such cell-bound truncated H3 molecules may represent secreted molecules that bind to cells via interaction of the H3 globular domain with sialic acid residues.

**HLA-A2-restricted T Cells Recognize a Peptide Derived from a Signal Sequence.** To test whether a cleaved signal sequence may be a source of peptide for presentation to class I-restricted T cells, it is necessary to use cells deficient in the classical
class I presentation pathway. Processing of cytosolic antigen for class I-restricted presentation is extremely efficient, such that even minute amounts of cytosolic signal sequences could lead to detectable recognition by T cells. To avoid this possibility, the mutant B cell line 721.174 was used. This B-LCL carries a deletion of the entire class II region of the MHC that includes the TAP1 and TAP2 genes (27). 721.174 cells pulsed with synthetic peptide M1 58-66 were lysed by the HLA-A2-restricted T cell line Q157 (Fig. 4 a). No lysis occurred with cells infected with a control vaccinia virus Vac-H3 (Fig. 4 b). Cells infected with Vac-M1 and with Vac-H3-C were lysed very inefficiently by Q157 (Fig. 4, c and d). In contrast, presentation of the M1 epitope within the signal sequence of H3-D and H3-J molecules was very efficient (Fig. 4, e and f). The lack of presentation of M1 and H3-C molecules is not due to poor expression in infected cells, because the Vac-M1 and Vac-H3-C viruses led to good protein expression (Fig. 2 and data not shown), and because 721.45 cells (with normal expression of TAP and large multifunctional protease [LMP] genes) infected with Vac-M1 and Vac-H3-C were recognized by the T cell line Q157 (Fig. 5).

The data presented here demonstrate that insertion of the M1 epitope at the NH2 terminus of the H3 molecule leads to the presentation of this signal sequence-derived epitope to HLA-A2-restricted T cells. There are essentially two pathways of processing that could lead to presentation of this peptide. First, precursor molecules which fail to be translocated into the ER may be processed in the cytosol and follow the classical class I presentation pathway. Although formally possible, it is unlikely that cytosolic processing accounts for the results presented here because 721.174 cells expressing an exclusively cytosolic form of antigen (M1 or H3-C) did not present the epitope, whereas cells expressing ER-translocated forms (H3-D and H3-J), with undetectable levels of cytosolic antigen, did. The second possibility is that the signal peptidase-mediated cleavage of the leader sequence in the ER provides a substrate for binding to HLA-A2. Trimming of such peptides to an optimal size for binding to HLA-A2 is not efficient, as peptides eluted from HLA-A2 molecules in 721.174 cells were heterogeneous in length, from 9 to 13 amino acids (11, 12). HLA-A2 molecules can bind some of these longer peptides with high affinity (28). Presentation of peptides derived from signal sequences is based either on the ability of T cells to recognize longer peptides bound to HLA-A2 or, more likely, to trimming of these peptides to the usual length. A biochemical characterization of peptides eluted from HLA-A2 in cells expressing H3-D molecules would not resolve this question, because it would only reveal the predominant forms of peptides, whereas T cells may selectively recognize a subset of appropriately-sized peptides.

In summary, the data presented here show that signal sequence-derived peptides can serve as a source of epitopes for T cells. This mechanism may be useful for immune surveillance, particularly of cells that fail to utilize the classical
class I pathway, such as tumor cells (29) or cells infected by certain viruses (30). The high prevalence of HLA-A2 (~40% among Caucasians) may be due to the propensity of this class I allele to bind hydrophobic peptides, including signal sequence-derived peptides that, as shown here, can be presented to T cells.

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Address correspondence to Dr. Eric O. Long, LIG-NIAID-NIH Twinbrook II, 12441 Parklawn Drive, Rockville, MD 20852-1773.

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