HLA-A2.1–Associated Peptides from a Mutant Cell Line: A Second Pathway of Antigen Presentation

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Peptides extracted from HLA-A2.1 class I major histocompatibility complex (MHC) molecules expressed on the antigen processing mutant CEMx721.174.T2 were characterized by electrospray ionization–tandem mass spectrometry. Only seven dominant peptides were found, in contrast to over 200 associated with HLA-A2.1 on normal cells. These peptides were derived from the signal peptide domains of normal cellular proteins, were usually larger than nine residues, and were also associated with HLA-A2.1 in normal cells. These results suggest that proteolysis of signal peptide domains in the endoplasmic reticulum is a second mechanism for processing and presentation of peptides for association with class I molecules.

CLASS I MOLECULES OF THE MHC bind to peptides derived from intracellular pathogens or normal cellular proteins, and present them to the immune system (*1–7*). The characterization of antigen processing mutants has suggested a model wherein proteins are degraded in the cytoplasm, translocated into the endoplasmic reticulum, and subsequently bound by newly synthesized class I molecules (*8–14*). Studies with these mutants have also shown that peptide binding is critical for the stable cell surface expression of most class I molecules (*11, 12, 14*). However, certain allelic forms of class I molecules continue to be expressed on the surface (*15*). It has been unclear whether these molecules are expressed in the absence of peptides or whether they are associated with peptides that arise through different mechanisms. We prepared acid extracts from the HLA-A2.1 molecules that were expressed on the surface of the antigen processing mutant CEMx721.174.T2 (T2). With the use of electrospray ionization–tandem mass spectrometry, we show that these molecules contain bound peptide, and we provide information on their complexity and sequence. The results suggest the existence of a second mechanism of peptide import into the endoplasmic reticulum for class I binding.

Extracts of HLA-A2.1 molecules purified from cells of the antigen processing mutant T2, as well as from the normal parental cell line T1, were analyzed for the presence and complexity of peptides by microcapillary high performance liquid chromatography (HPLC) and mass spectrometry (*7, 16*). The peptides isolated from T1 cells consisted of a complex mixture, representing at least 200 different species [Fig. 1A and (*7*)]. We have estimated that each of the 20 most prevalent peptides occupied between 0.3 and 1.2% of the HLA-A2.1 molecules (*7*), and similar numbers were observed for the peptides extracted from T1. The extract of HLA-A2.1 purified from T2 contained an amount of material similar to that found in the T1 extract (Fig. 1, B and D). This extract was dominated by seven major species that each represented between 1 and 21% of the total HLA-A2.1 from which the peptides were isolated (Fig. 1C and Table 1). These observations indicate that the normal mixture of peptides does not associate with the HLA-A2.1 molecules expressed on T2, consistent with the antigen processing defect in these cells. However, a small subset of peptides, represented in relatively high quantities, is found in association with these molecules.

Amino acid sequences of peptides identified in the T2 extracts were determined by mass spectrometry (*7, 16*). The peptide observed at mass-to-charge ratio (*m/z*) 898 has the sequence XXDVPTAAV (Table 1). Because of their identical mass Leu and Ile cannot be distinguished under the conditions used, and are therefore designated with an X. The peptides at *m/z* 1011 and 1211 also contained this sequence. These two peptides contained an additional NH_2-terminal Leu/Ile residue and the 1211 peptide contained the additional residues QA at the carboxyl terminus. These sequences were found in only a single protein, IP-30, in which all of the Xs corresponded to Leu residues (*17*). A fourth peptide at *m/z* 1056 had the sequence MXXSVPXXXG, and was represented in a single protein, calreticulin, in which all of the Xs were Leu residues (*18–20*). A reported sequence motif common to HLA-A2.1–associated peptides includes a 9-amino acid length, Leu at position 2, and an aliphatic hydrocarbon side chain (Val, Leu, Ile, or Ala) at position 9 (*5, 7*). This motif is present in the nine-residue 898 peptide (underlined residues in Table 1). Although the other three peptides are longer, each contains a Leu at position 2 and an aliphatic hydrocarbon side chain at position 9. The 1011 and 1211 peptides also contain a Leu at position 3 and an aliphatic hydrocarbon side chain at position 10, suggesting two possible modes of peptide binding. The location of the conserved hydrocarbon side chains in the 1211 and 1056 peptides also suggests that they need not be the COOH-terminal residue.

IP-30 has been found in both extracellular and lysosomal locations (*17*), whereas calreticulin is located in the lumen of the endoplasmic reticulum. The sequences corresponding to these four HLA-A2.1–associated peptides are found in the signal peptides that mediate translocation of these proteins into the endoplasmic reticulum (Fig. 2). These results suggest the existence of a second mechanism capable of generating peptides that can associate with class I MHC molecules that is distinct from the described cytoplasmic pathway. We suggest that the ability of this subset of peptides to

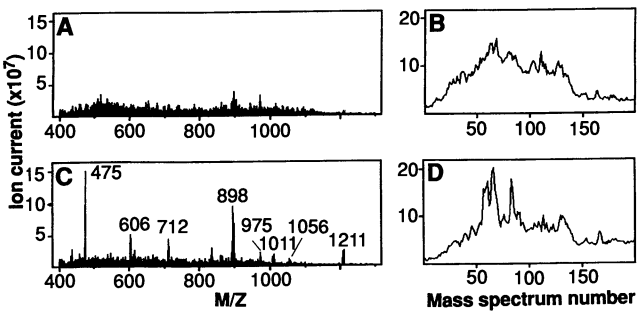

Fig. 1. Complexity of HLA-A2.1–associated peptides isolated from T1 and T2 cells. HLA-A2.1 molecules were purified, acid extracts were prepared, and material equivalent to the extract from 10^8 cells was analyzed on a Finnigan-MAT (San Jose, California) TSQ-70, triple quadrupole mass spectrometer equipped with an electrospray ion source, all as described (*7*). The data in (**A**) and (**C**) represent ion chromatograms, whereas the data in (**B**) and (**D**) represent total ion signal, obtained by scanning *m/z* values between 300 and 1500 every 2 s and summing each of the spectra. (A) and (B) are material extracted from T1 and (C) and (D) are material extracted from T2.

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*Contributions of these authors were equivalent and their order should be considered arbitrary.

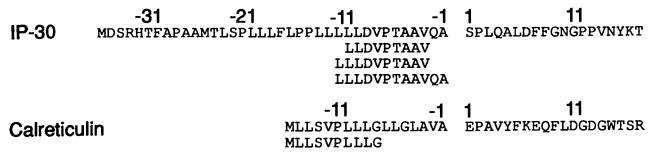

Fig. 2. Location of peptide sequences in signal peptide domains of candidate source proteins. Source proteins and regions of homology were determined using release 70 of Genbank (December 1991).

circumvent the antigen processing defect in T2 cells is due to their entry into the endoplasmic reticulum as intact signal sequences during cotranslational protein translocation. These sequences are then cleaved by the signal peptidase and subsequently become associated with newly synthesized class I molecules. The COOH-terminus of the 1211 peptide corresponds precisely to the COOH-terminus of the signal peptide, suggesting that this end of the peptide is generated directly by the signal peptidase. The involvement of other proteases in the endoplasmic reticulum cannot be ruled out. However, the predominance of peptide sequences from signal peptide domains, as opposed to internal sequences from proteins that are present in the lumen of the endoplasmic reticulum, suggests that such proteases are at best inefficient participants in this pathway.

We have also identified peptides in extracts of normal cells that have *m/z* values and HPLC elution characteristics appropriate for five of the seven major peptides associated with HLA-A2.1 in T2 cells (Table 1). Collision-activated disassociation (CAD) spectra have established that the sequences of the 1011 and 1210 peptides in normal cells are identical to the peptides isolated from T2 cells (*21*). The peptides processed by this second pathway are associated with a larger fraction of the HLA-A2.1 molecules on T2 cells, presumably because there is no competition from peptides generated by the cytoplasmic pathway (Table 1). However, the relative amounts of the IP-30–derived 9-, 10-, and 12-residue peptides were similar in T2 and in normal cells. Thus, this second pathway for the generation of class I MHC-associated peptides appears to operate in normal cells as well.

All naturally processed peptides previously isolated from class I molecules have been, with a single exception (*22*), nine residues long (*7, 23–25*). This may reflect the binding of the class I molecule to a larger peptide, which is then trimmed by a protease to a size that is protected from further digestion by the binding site (*26*). However, our data indicate that longer peptides bind to MHC molecules and are not necessarily trimmed further. On the other hand, modeling studies suggest that free NH_2- and COOH-termini of nine-residue peptides can form additional hydrogen bonds with side chains in the class I binding site (*6*) and might therefore bind with particularly high affinity. For at least some sequences, nonamers bind to class I molecules with higher affinity than longer related species (*27–29*). Consequently, the relative binding affinity of the IP-30–derived peptides was assessed in a competitive binding assay. Cells of the HLA-A2.1 expressing line JY were incubated with the $M1_{57-68}$ peptide of influenza virus in the absence or presence of various concentrations of synthetic peptides corresponding to the 898, 1011, and 1211 sequences and then assayed for their ability to be lysed. All three peptides inhibited equivalently at all concentrations tested (Fig. 3). These peptides had been rigorously purified by HPLC and assessed for the presence of lower molecular weight contaminants prior to analysis. Taken together with the observation that all three species are found in association with HLA-A2.1 in vivo, these results suggest that peptides longer than nine residues can bind to class I MHC molecules with affinities similar to those of nonameric species.

The relatively high affinity of the 1011 and 1211 peptides could reflect an unusual interaction with the HLA-A2.1 binding site, and it remains possible that a nine-residue length is preferred for the majority of class I–associated peptides because of favorable binding interactions. Alternatively, nonameric peptides may be preferentially generated by cytoplasmic proteases or preferentially transported into the endoplasmic reticulum. Nonetheless, peptides of different lengths that bind efficiently to class I molecules are clearly generated by this second processing pathway and may also be generated by the cytoplasmic pathway. The relative proportion of such peptides is not yet clear, but peptides of longer or shorter length will almost certainly be found to comprise particular T cell epitopes.

The observation that HLA-A2.1 is exceptional among human class I molecules in its ability to be expressed on the surface of T2 cells (*15*) may reflect the importance of hydrophobic signal sequence residues in the motif that supports the binding of peptides to HLA-A2.1 (*7, 30*). The relatively low expression of other class I molecules on the surface of T2 may indicate that they use peptide binding motifs that are not well represented in signal peptides. Because most cells express many secreted and membrane-bound proteins with signal peptides, the small set of peptides that predominate in association with HLA-A2.1 may reflect the level of protein expression or the rate at which appropriate signal peptide fragments are generated. Alternatively, as suggested

Table 1. Characteristics of HLA-A2.1–associated peptides expressed on T2 cells. Aliquots of extracted peptides corresponding to 20 pmol of HLA-A2.1 (representing 2.5×10^8 T1 and 4×10^8 T2 cells) were analyzed by microcapillary high-performance liquid chromatography (HPLC) and electrospray ionization–mass spectrometry. Yields are based on comparisons with synthetic peptide standards. Sequences were deduced from collision-activated dissociation mass spectra with the use of a triple quadrupole mass spectrometer (*7, 16*). Binding motif–related residues are underlined. Single letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Peptide	$(M+H)^+$ (*m/z*)	Peptide yield (fmol)		Sequence*
		T2	T1	
1	898	1780	540	XXDVPTAAV
2	1011	510	90	XXXDVPTAAV
3	1211†	1740	320	XXXDVPTAAVQA
4	1056	230	—	MXXSVPXXXG
5	974	460	—	
6	1426‡	4100	190	
7	1784†	370	510	

*Leu and Ile cannot be differentiated on the triple quadrupole instrument and are therefore designated with an X. †Also observed as a doubly charged $(M+H)^{2+}$ ion at 1/2 this *m/z* value. ‡Observed as a triply charged $(M+H)^{3+}$ ion at an *m/z* of 475, and as a doubly charged $(M+H)^{2+}$ ion at an *m/z* of 712.

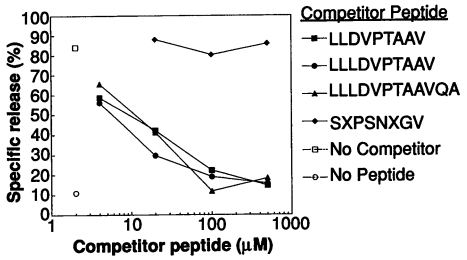

Fig. 3. Binding of peptides to HLA-A2.1 molecules. JY cells were incubated with the $M1_{57-68}$ peptide of influenza A (6 μg/ml) in the presence or absence of the indicated concentration of competitor peptide in RPMI 1640 supplemented with 5% newborn calf serum (Gibco) and ^{51}Cr (50 μCi) for 2 hours. Cells were washed and assayed for their sensitivity to lysis by the M1 peptide–specific, HLA-A2.1–restricted cytotoxic T lymphocyte clone 5.2-16 (*32*) at an effector:target of 10. Maximal release was determined in the presence of 2 N HCl. Peptides whose sequences contain the letter X were synthesized with an equimolar mixture of Leu and Ile at that position.

above for the IP-30 peptides, this set of peptides may all share common sequence elements that confer an unusually high affinity for HLA-A2.1. Despite the fact that the use of this second pathway is dominated by a few peptides in the case of HLA-A2.1, the number of peptide-MHC complexes necessary for T cell recognition is quite small (*25, 29, 31*). Consequently, even signal peptides that are processed relatively inefficiently may be capable of generating peptide antigens that are expressed in association with HLA-A2.1 or other class I molecules and recognized during normal immune responses.

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"Take two of these and call me a century from now."