Direct identification of an endogenous peptide recognized by multiple HLA-A2.1-specific cytotoxic T cells

(class I major histocompatibility molecule/xenoreactivity/alloreactivity/mass spectrometry/antigen processing)

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ABSTRACT An endogenous peptide recognized by a murine T-cell clone specific for the human class I major histocompatibility complex-encoded molecule HLA-A2.1 was identified through the use of microcapillary high-performance liquid chromatography coupled with electrospray-ionization tandem mass spectrometry. The peptide was associated with HLA-A2.1 on both normal cells and the antigen-processing-mutant cell line T2. This observation demonstrates that a processing mechanism other than that involving the transporter associated with antigen processing (TAP) proteins 1 and 2 can produce peptides that can be recognized by T cells. The peptide was also recognized by four other independently derived murine HLA-A2.1-specific murine T-cell clones. This suggests that xenogeneic responses are directed at a restricted subset of major histocompatibility complex product-associated peptides. Finally, quantitation of this peptide in cell extracts using mass spectrometry showed it to be among the most dominant HLA-A2.1 associated species on human lymphoid cells. The potential relevance of this observation to models of alloreactivity will be discussed. The methodology described should be generally useful for the identification of peptide epitopes recognized by alloreactive, tumor-specific, and autoimmune T cells.

Cytotoxic T lymphocytes (CTLs) are a component of the immune system responsible for the recognition of host cells that express new antigens as a result of viral infection or transformation. CTLs do not recognize antigens directly, but only as small peptides bound to host class I major histocompatibility complex (MHC) molecules (1). CTLs also cause rejection of tissue transplanted from unrelated individuals of the same species (allograft) or other species (xenograft) through recognition of foreign MHC molecules. One hypothesis to explain the nature of the epitopes recognized by alloreactive and xenoreactive CTLs is that they are formed by the combination of the foreign class I molecule together with one of a large collection of endogenous peptides derived from the processing of intracellular proteins (2, 3). A second proposes that such T cells recognize the allelically polymorphic residues of the foreign class I molecule itself, independent of the presence or structure of associated peptides (4). A number of studies have suggested that the former hypothesis accounts for the epitopes recognized by the great majority of alloreactive and xenoreactive class I-specific T cells (refs. 5–9 and R.A.H., unpublished data). However, with a single exception (7), none of the peptides responsible has been characterized, and it is unclear whether they represent a restricted subset of MHC-associated peptides based on either structure or antigen density.

To date, the identification of class I-associated peptides that are recognized by T cells has relied on knowledge of the protein sequence from which the peptide was derived, coupled with mutagenesis (10–12) or class I MHC allele-specific peptide-binding “motifs” (13–15), and the use of synthetic peptides to reconstitute biological activity. The exact structures of naturally processed peptides have been inferred by comparison of elution times of various synthetic peptides with those of the naturally processed forms (13, 16, 17). However, this approach is not applicable to peptides recognized by alloreactive or xenoreactive T cells, because the source proteins are unknown and not easily identified. In principle, reverse-phase high-performance liquid chromatography (RP-HPLC) separation of the naturally processed peptides extracted from the class I molecule should allow the direct identification of the species responsible for the epitope regardless of its protein source. However, RP-HPLC cannot completely resolve these complex mixtures of up to 2000 peptides, and thus this approach appears to be of limited usefulness. Previously, we used the higher-resolution methodology of microcapillary HPLC coupled with electrospray-ionization tandem mass spectrometry to examine the complexity of self-peptides presented by class I MHC molecules and to sequence individual peptides at the subpicomolar level (18–20). Here we show that this technology can be used to directly identify and sequence peptide epitopes recognized by a murine CTL clone that recognizes the human class I MHC molecule HLA-A2.1. The characteristics of this peptide have several implications for understanding the production and recognition of such peptides by T cells.

MATERIALS AND METHODS

Cell Lines. The derivation and specificity of the murine CTL lines used have been described (21–23). Ltk-A2 is an HLA-A2.1-positive transfectant of the murine thymidine kinase-negative L-cell line Ltk[−] (21), and P815-A2 is a similar transfectant of the murine mastocytoma P815 (gift of J. Barbosa, Molecular Diagnostics).

Preparation of HLA-A2.1-Associated Peptides. HLA-A2.1 was purified from detergent-solubilized extracts of 10¹⁰ JY cells as described (18, 19), except that the supernatant was passed over a protein A-Sepharose column prebound with the HLA-A2.1-specific antibody BB7.2. The column was washed and eluted with 0.2 M acetic acid. The eluate was acidified to pH 2.1 with glacial acetic acid, boiled for 5 min, centrifuged

Abbreviations: CTL, cytotoxic T lymphocyte; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; HFA, hexafluoroacetone; CAD, collision-activated dissociation; MHC, major histocompatibility complex.

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through an Ultrafree-CI unit (5-kDa cutoff; Millipore), and concentrated by vacuum centrifugation to 100 μ l. Peptides were separated by RP-HPLC (see Fig. 2 legend).

Reconstitution of T-Cell Epitopes. A 10- μ l aliquot of each HPLC fraction was concentrated by vacuum centrifugation to approximately half of its original volume, diluted with 75 μ l of assay medium containing human β_2 -microglobulin (Calbiochem) at 6 μ g/ml, and added to individual wells of a V-bottom microtiter plate. 51 Cr-labeled Ltk-A2 or P815-A2 cells (2×10^3) were added in 75 μ l and incubated for 2 hr at 37°C. CTLs (2×10^4) were added in 50 μ l and a standard 4-hr 51 Cr-release assay was performed.

Mass Spectrometry. All data were acquired on a Finnigan-MAT (San Jose, CA) TSQ-70 triple-quadrupole mass spectrometer interfaced to a polyimide-coated fused-silica microcapillary HPLC column (i.d., 100 μ m; o.d., 360 μ m; length, 65 cm; Polymicro Technologies, Phoenix), the last 12 cm of which were packed with C_{18} -derivatized 10- μ m silica beads (YMC, Kyoto) (18).

Synthetic Peptides. Peptides were synthesized by the solid-phase method with standard tert-butoxycarbonyl chemistry on an Applied Biosystems 430A peptide synthesizer and were purified to >98% homogeneity by RP-HPLC on a Vydac C_4 column with 0.05–0.085% trifluoroacetic acid (TFA) in water and an acetonitrile gradient. Purity was established on an analytical RP column and the identity of each peptide was confirmed by mass spectrometry.

RESULTS

Mice immunized with human lymphoid cells produce CTLs directed against human class I and class II MHC products (24, 25). The fine specificity of these xenoreactive T cells is similar to that of human alloreactive CTLs. They recognize allele-specific rather than species-specific epitopes and are able to discriminate among closely related structural variants of class I molecules (23, 25–27). More recently, indirect evidence has suggested that as with alloreactive T cells, the epitopes recognized by xenoreactive T cells are dependent upon the association of the target MHC molecule with a specific bound peptide (22, 28). In particular, many of these CTLs fail to recognize the target MHC molecules when they are expressed on murine, rather than human, target cells. The murine T-cell clone AHIII12.2 recognized HLA-A2.1 expressed on the surface of human cells but not HLA-A2.1 transfected into murine cells (Fig. 1A). AHIII12.2 also recognized the antigen-processing mutants T2 and 721.134 (29, 30), which present only a small subset of the endogenous peptides that are associated with HLA-A2.1 in normal cells (19, 31) (Fig. 1B). This pattern of specificity and other evidence (22, 28) suggest that CTL clone AHIII12.2 recognizes a peptide that is presented by HLA-A2.1 in both normal human cells and the antigen-processing mutants but not by murine HLA-A2.1 transfectants.

To identify the peptide recognized, HLA-A2.1 molecules were immunoaffinity purified from the human lymphoblastoid cell line JY, and the associated peptides were acid extracted and separated by microbore RP-HPLC. Fractions were incubated with the murine HLA-A2.1 transfectant Ltk-A2 and tested for their ability to reconstitute the epitope recognized by AHIII12.2. In three separate extractions, the epitope was reconstituted with RP-HPLC fractions 28 and 29 (Fig. 2A). A mass spectrum of this material showed that six peptides accounted for 25% of the total ion current, while the remaining 75% of the material was distributed among several hundred lower-abundance peptides (Fig. 3A). Fractions 28 and 29 were pooled and subjected to RP-HPLC using HFA in place of TFA to achieve a different separation of these peptides. Fractions 23 and 24 from the HFA separation reconstituted recognition (Fig. 2B), and mass spectrometric analysis of fraction 23 showed that only one of the six major

FIG. 1. HLA-A2.1 specificity of murine CTL clone AHIII12.2. Target cells (2×10^3) were incubated with CTL clone AHIII12.2 at the effector/target ratios indicated in a standard 4-hr 51 Cr-release assay. (A) The target cells and their MHC types were as follows: ■, HSB (human, HLA-A1, 2.1; B12, 17); ●, JY (human, HLA-A2.1, 2.1; B7, 7; -DR4, 6); ○, Ltk-A2 (murine, H-2^k, HLA-A2.1); □, P815-A2 (murine, H-2^d, HLA-A2.1). (B) The target cells were HLA-A2.1⁺ antigen-processing mutants: ■, T2; ●, 721.134.

peptides observed in fraction 28 from the TFA separation, m/z 1049, was still present (Fig. 3B). The possibility that this peptide was recognized by CTL clone AHIII12.2 was supported by the observation of a peptide with the same m/z in the mass spectrum of naturally processed HLA-A2.1-associated peptides isolated from the T2 cell line (Fig. 3C).

Collision-activated dissociation (CAD) analysis (32, 33) performed on $(M+H)^+$ ions defined the amino acid sequence of peptide 1049 as AXWGFFPVX (X designates either leucine or isoleucine, which are indistinguishable by mass) (Fig. 4). CAD analysis also established that the identical peptide

FIG. 2. Reconstitution of the epitope recognized by T-cell clone AHIII12.2. (A) Naturally processed peptides extracted from HLA-A2.1 molecules were injected onto a Brownlee C_{18} Aquapore column (column dimensions, 2.1 mm $\times$ 3 cm; pore size, 300 Å; particle size, 7 μ m) and eluted with a 40-min gradient of 0–60% (vol/vol) acetonitrile/0.085% TFA in 0.1% TFA at a flow rate of 200 μ l/min. Fractions were collected at 1-min intervals. (B) Second-dimension separations were performed with the same type of column and elution conditions, but the TFA organic modifier was replaced with 0.1% hexafluoroacetone (HFA) that had been adjusted to pH 8.1 with 14.8 M ammonium hydroxide. ■, Peptide-containing fractions; □, medium alone.

FIG. 3. Mass spectra of HLA-A2.1-associated peptides that reconstitute the epitope recognized by AHIII12.2. Spectra were recorded on 5% of the material in RP-HPLC fraction 28 from the first-dimension separation with TFA/acetonitrile (A), 95% of the material in fraction 23 of the second-dimension separation with HFA (pH 8.1)/acetonitrile (B), and 2% of the unfractionated peptide mixture extracted from HLA-A2.1 molecules expressed on the surface of T2 cells (C). Spectra were acquired every 1.6 sec over the mass range m/z 300–1400 and then added together. In B, signals within the envelope from m/z 433–900 result largely from contaminants introduced during the isolation procedure. Full scale on the ion-current axis is 5.13×10^6 .

was present in extracts from T2 cells (A.L.C., unpublished data). Although coelution experiments have been used successfully to distinguish isoleucine from leucine in other peptides obtained from class I mixtures (18), peptides based on the above sequence with either leucine or isoleucine at position 9 could not be chromatographically resolved. Accordingly, an equimolar mixture of peptides containing leucine at position 2 and either leucine or isoleucine at position 9 was synthesized. When incubated with murine L cells expressing HLA-A2.1, this peptide reconstituted the epitope recognized by AHIII12.2, whereas another HLA-A2.1-binding peptide with a similar sequence did not (Fig. 5A). Eight other peptides known to bind to HLA-A2.1 (18) had no reconstituting activity (Fig. 5A and R.A.H., unpublished data). These results establish that this xenogeneic murine T-cell clone specifically recognizes peptide 1049 in association with the HLA-A2.1 molecule.

We next determined whether peptide 1049 constituted an epitope for other HLA-A2.1-specific CTLs. Split-well analyses of limiting-dilution cultures had previously established that murine CTLs that fail to recognize HLA-A2.1 on murine targets represent 15–40% of the total HLA-A2.1-specific xenogeneic response (ref. 34 and V.H.E., unpublished data). Between 1982 and 1988, 21 different HLA-A2.1-specific murine T-cell clones derived from nine independent experiments were isolated and characterized (21–23). Nine of these clones, including AHIII12.2, are similar in their inability to recognize HLA-A2.1 on murine cells, and of these, six use an identical T-cell receptor β chain based on the $V_{\beta 8.1}$ variable region (R.A.H., unpublished data). Peptide 1049 reconstituted the epitope recognized by all five tested $V_{\beta 8.1}$ -positive clones, including AHIII12.2, but not that of a sixth $V_{\beta 8}$ -negative clone (Fig. 5B). Again, the epitopes recognized by these different clones were not reconstituted by any of eight other peptides known to bind to HLA-A2.1 (R.A.H., unpublished data). The five $V_{\beta 8.1}$ -positive clones originated from three different mice and experiments. This establishes that m/z 1049 is the naturally processed peptide specifically recognized by a significant subset of murine HLA-A2.1-specific T-cell clones.

To directly quantitate the amount of peptide 1049 expressed on cells, known quantities of the synthetic peptide were added to unfractionated acid extracts of HLA-A2.1 to establish a correspondence between ion current and peptide quantity. On this basis, the typical yields of peptide 1049 from 10^9 JY and T2 cells were 2.6 and 0.67 pmol, respectively. Assuming a 90% overall peptide yield, this corresponds to approximately 1600 and 400 peptide-MHC molecule complexes per cell, respectively. The yield of this peptide places it among the most dominant 10% of peptides associated with HLA-A2.1 on JY cells (18). A dose-response experiment showed that the concentration of synthetic peptide necessary for half-maximal lysis of a murine HLA-A2.1 transfectant by AHIII12.2 was ≈ 27.5 nM (Fig. 6). By using a RP-HPLC fraction derived from the separation of HLA-A2.1-associated peptides and containing 48 nM peptide 1049, it was also established that both the naturally processed and synthetic forms of peptide 1049 reconstitute this epitope at similar concentrations.

DISCUSSION

In the present study, we combined microcapillary HPLC—electrospray-ionization tandem mass spectrometry with epitope reconstitution to identify a peptide recognized by a T cell in the absence of prior knowledge of its source protein. Class I molecules have been shown to associate with >2000 different species of endogenous peptides (18, 20). While RP-HPLC can partially resolve this mixture, individual fractions usually still contain 50–100 different peptides. In the present case, peptide 1049 was not the dominant species in the biologically active fractions, and even after additional HPLC purification, the remaining material was still too complex and insufficient in quantity (<1 pmol) for Edman sequencing. Nine hundred femtomoles was used in the CAD analyses to obtain the 1049 sequence, and sequences of other peptides in these mixtures have been obtained with as little as 500 fmol (A.L.C., unpublished). Udaoka *et al.* (7) used repeated HPLC fractionation and Edman sequencing to identify a peptide recognized by an alloreactive H-2L^d-specific CTL clone. However, 3–5 pmol of material was needed to derive the sequence of the first six amino acids. A molecular mass measurement on an additional 2 pmol of material was used to predict the rest of the sequence. Unless the majority of T-cell epitopes are highly abundant and easily resolved from other species, we believe that tandem mass spectrometry will be more generally useful than HPLC and Edman degradation for

FIG. 4. CAD mass spectrum of peptide $(M + H)^+$ ions at m/z 1049 from a 600-fmol sample derived from 2×10^8 JY cells. Predicted masses for fragment ions of types b and y (32, 33) are shown above and below the deduced sequence, respectively. Ions of type a that differ from type b ions by the loss of carbon monoxide are also labeled. Subtraction of m/z values for any two fragments that differ by a single amino acid generates a value that specifies the mass and thus the identity of the extra residue in the larger fragment. Residues 3–7 and 8–9 were deduced from ions of types y and b , respectively. Confirmation of the above assignments was obtained from a CAD spectrum of an additional 100 fmol of sample that had been converted to the corresponding methyl ester derivative. Residues 1 and 2, with a combined mass of 185 Da, remained undefined. Only three pairs of amino acids, Pro-Ser, Ala-Leu, and Ala-Ile, add to give 185 kDa. Manual Edman degradation performed on an additional 200 fmol of sample was employed to distinguish these possibilities. A mass measurement performed on the resulting truncated peptide identified the released amino acid as alanine and facilitated assignment of residue 2 as either leucine or isoleucine. Since these two residues are of identical mass, they cannot be distinguished on the triple quadrupole instrument and are specified as Lxx. Approximately 1.5 pmol of peptide material was used to derive this sequence.

identification and complete sequencing of individual peptides within complex RP-HPLC fractions.

The sequence of peptide 1049 conforms closely to the motif described for other peptides associated with HLA-A2.1 (18, 35), as it was nine amino acids long, with leucine at position 2 and leucine or isoleucine at position 9. This peptide was associated with HLA-A2.1 on the cell surface of both normal cells and the antigen-processing mutant T2. Its presence in the latter cell indicates that it does not require the transporter associated with antigen processing (TAP) protein 1- and 2-dependent pathway for peptide transport into the endoplasmic reticulum (36–38) in order to associate with HLA-A2.1. An alternative pathway involving the processing and presen-

tation of signal sequence peptides in association with HLA-A2.1 and HLA-B7 has been shown to operate in both normal cells and T2 (19, 20, 31), and it is tempting to conclude that peptide 1049 may be generated by such a mechanism. Unfortunately, this hypothesis cannot be confirmed because no sequence match was found in protein or DNA data bases.

FIG. 5. Reconstitution of T-cell epitopes using synthetic peptide ALWGFFPVX. (A) CTL clone AHIII12.2 was assayed on Ltk-A2 cells that had been incubated for 2 hr with synthetic peptide (40 μ g/ml): ■, ALWGFFPVX; □, AICTWFPVI; ●, no peptide. (B) The following CTL clones were assayed on Ltk-A2 cells that had been incubated for 2 hr with synthetic peptide ALWGFFPVX (40 μ g/ml): ◆, AH10-4; ■, AH17-8.6; ▲, AH17-12.30; ●, AH17-22.19; ○, AH9-2.

FIG. 6. Dose-response curve for peptide 1049 and AHIII12.2. P815-A2 cells (2×10^5) were added to serial dilutions of peptide 1049 and incubated for 2 hr at 37°C. CTLs were added to give an effector/target ratio of 10 and a standard 4-hr 51 Cr release was then performed. Each point is the mean $\pm$ SD of quadruplicates. The horizontal line is the level of lysis in the absence of any peptide.

Nevertheless, our present results clearly show that an alternative pathway of peptide processing and presentation by class I MHC molecules can generate epitopes that can be recognized by T cells. Previous observations in two systems indicated that $\approx 15\%$ of alloreactive T cells could recognize the target molecule when expressed on T2 cells (8, 9). The present data suggest that some, if not all, of these T cells are specific for peptides that are also generated by an alternative pathway.

A number of alloreactive CTLs have now been shown to depend upon endogenous peptides for their recognition (5–9), and the results reported here and our unpublished data indicate that this is an important mode of recognition for xenoreactive T cells as well. However, allogeneic responses are among the strongest based on the fraction of responding T cells, whereas xenogeneic responses are among the weakest (34). Thus, it is particularly striking that a significant number of the murine HLA-A2.1-specific T-cell clones that have been isolated in independent experiments recognize this molecule together with peptide 1049. In addition, quantification of peptide 1049 in unfractionated extracts indicated that it was present at 1600 copies per JY cell; thus it is among the most dominant 10% of peptides associated with HLA-A2.1 (18). These observations suggest that xenoreactive cells are preferentially elicited by a small subset of class I-associated peptides that are present at high density on the cell surface.

The recognition of a small number of highly represented peptides may be a consequence of other factors that limit recognition of xenogeneic class I molecules. However, an extrapolation of these results suggests an interesting hypothesis to explain alloreactivity. Most studies to date have been interpreted to support the hypothesis that the extraordinary strength of the allogeneic response is due to the recognition by different T cells of one of the array of >2000 peptides associated with allogeneic MHC molecules. The alternative hypothesis to explain alloreactivity is that a high density of foreign epitopes expressed on the MHC molecules themselves results in the recruitment of relatively low-affinity T cells. A hybrid of these two models is that alloreactive T cells are stimulated by the subset of class I-associated peptides that are expressed at the highest relative densities on the cell surface. Even if alloreactive T cells were able to recognize only 10% of the species associated with a given class I molecule, >200 different peptide epitopes would be represented. Thus, this model is not inconsistent with the diversity of alloreactive responses. This model is also attractive because it accounts for the seeming paradox that alloreactive T cells have been selected for preferential recognition of peptides presented by host MHC molecules and thus would be expected to show low reactivity toward peptides presented in the context of non-host MHC molecules. This low reactivity may be overcome in cases where the peptide-MHC molecule complexes are expressed at a relatively high density. Validation of this hypothesis will require the identification and quantitation of such peptides. We think that mass spectrometry offers the most efficient means to accomplish this objective.

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