Brief Definitive Report

Mass Spectrometric Identification of a Naturally Processed Melanoma Peptide Recognized by CD8+ Cytotoxic T Lymphocytes

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Summary

We and others have previously reported that melanoma-specific, cytotoxic T lymphocytes (CTL) define a minimum of six class I-presented peptide epitopes common to most HLA-A2+ melanomas. Here we show that three of these peptide epitopes are coordinately recognized by a CTL clone obtained by limiting dilution from the peripheral blood of an HLA-A2+ melanoma patient. Tandem mass spectrometry was used to characterize and sequence one of these three naturally processed melanoma peptides. One of the potential forms of the deduced peptide sequence (XXTVXXGVX, X = I or L) matches positions 32-40 of the recently identified melanoma gene MART-1/Melan-A. This peptide (p939; ILTVILGVL) binds to HLA-A2 with an intermediate-to-low affinity and is capable of sensitizing the HLA-A2+ T2 cell line to lysis by CTL lines and clones derived from five different melanoma patients. A relative high frequency of anti-p939-specific effector cells appear to be present in situ in HLA-A2+ melanoma patients, since p939 is also recognized by freshly isolated tumor infiltrating lymphocytes. p939 represents a good candidate for the development of peptide-based immunotherapies for the treatment of patients with melanoma.

During the last several years, a number of studies have been conducted on the autologous CD8+ T cell–mediated response to human melanoma (1–17). The emerging picture indicates that melanomas express multiple T cell–defined epitopes, some of which are unique to a given tumor, while others are shared by allogeneic, HLA-matched melanomas (2–4, 6–9, 11–17). These epitopes appear to represent short 9–10 amino acid peptides derived from tumor-associated antigens that are presented by MHC class I antigens to CD8+ T cells (5–9, 17, 18). While many class I alleles have been reported to represent restriction elements for tumor-reactive CD8+ T cells (6, 7, 10), the HLA-A2.1 allele (expressed by 45% of melanoma patients) appears to play an immunodominant role in presenting melanoma epitopes (11). At least six different CD8+ T cell–defined epitopes appear to be expressed by multiple HLA-A2+ melanomas (12, 13, 19). The identification and sequencing of these individual epitopes should allow for the design and testing of peptide-based immunotherapies for the treatment of melanoma.

Materials and Methods

Cell Lines. Two melanoma cell lines have been used throughout this study. Mel 624 (HLA-A2, -A3; -B7, -B14; -Cw7: obtained from the Surgery Research Branch of the National Cancer Institute, Bethesda, MD) and Mel 9742 (HLA-A2, -A24; -B13, -B18; -Cw6, -Cw7: obtained from the Istituto Nazionale Tumori, Milan, Italy) were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all reagents from Gibco BRL, Gaithersburg, MD). The CTL clone A83 (autologous to Mel 9742) recognizes a common melanoma antigen and is restricted by HLA-A2 (16). The tumor infiltrating (TIL) 1235 line and TIL 501.A42 clone were kindly provided by Dr. Y. Kawakami (National Institutes of Health, Bethesda, MD) and have been previously reported to recognize p27-35 of the MART-1 antigen presented in the context of HLA-A2 (17). TILs 5403 and 6970 were isolated from metastatic melanoma lesions of HLA-A2+ patients as previously described (20), cultured for 7 d in the presence of autologous tumor, and used directly as effector cells in cytotoxicity experiments. All TIL lines and clones were cultured in AIM-V media (Gibco BRL) supplemented with 10% heat-inactivated human AB serum (Gibco), and 300 IU/ml rhIL-2 (Cetus Corp., Emeryville, CA).

Acid Elution of MHC Class I-presented Melanoma Peptides. Acid elution of, and reverse-phase high performance liquid chromatography (HPLC) resolution of, melanoma peptides was performed as previously described (12). Individual HPLC fractions were lyophilized and reconstituted in 200 μl of Hank's buffered saline (Gibco BRL) and stored at −20°C for use in cytolytic assays. Alterna-

tively, for mass spectrometric analyses, peptides were lyophilized and reconstituted in 50 μl of 50% acetonitrile/50% water and stored at −70°C until used.

Reconstitution of T Cell Epitopes. In peptide-pulsing assays, 10 μl of reconstituted peptides was added to microwells containing 10^4 ^{51}Cr-labeled T2 cells, 0.2 μg human β_2-microglobulin (β_2-m; Sigma Chemical Co., St. Louis, MO), and 0.2 μg MA2.1 (anti-HLA-A2.1; American Type Culture Collection, Rockville, MD, reference 21) monoclonal antibody in a total of 125 μl. Peptide loading was facilitated by the presence of the β_2-m and MA2.1 reagents (22–24), and was allowed to proceed for 2 h at room temperature. Effector T cells were then added at a 10:1 effector-to-target cell ratio (unless otherwise stated) and cytolytic assays performed as previously described (12).

HLA-A2 Stabilization Assay. Various concentrations of synthetic peptides (as noted in text) were incubated for 18 h at room temperature with 10^6 T2 cells (25), 1 μg β_2-m, and 2 μg MA2.1 monoclonal antibody. Cells were then washed twice with buffered saline and stained with FITC-conjugated $F(ab')_2$ goat anti-mouse Ig (Organon Teknika, Durham, NC) for 30 min at 4°C. After two additional washes with buffered saline, cells were fixed with 4% formalin (Fisher Scientific Co., Pittsburgh, PA) and flow cytometry performed on a FACScan® (Becton Dickinson, Mountain View, CA) as previously noted (22). Results are reported as percentage of increase (over control) of MA2.1 mean fluorescence channel number reactivity. Controls represent T2 cells cultured with β_2-m and MA2.1, in the absence of synthetic peptide.

Mass Spectrometric Analysis. Mel 9742 HPLC fractions 47 and 48 were pooled, lyophilized, then reconstituted in 50 μl of 50% acetonitrile, 50% double distilled H_2O, and stored at −70°C. A 10 μl aliquot of this material was introduced into an API III tandem mass spectrometer (PE-Sciex, Ontario, Canada) via the articulated ion-spray interface. The sprayer needle was held at 4,500 V with a coaxial sheath of nebulizing gas (compressed air) flow. Profile mass spectrum was obtained for peptide samples by scanning the first quadrupole from mass-to-charge (m/z) 500 to 1,600 in 3.37 s. The final spectrum was averaged from 10 scans. A mass spectra/mass spectra (MS/MS) product ion spectrum was obtained for the p941 by scanning the fragment ions resulting from collision with Ar gas. The sequence was assigned as XXGVXXTVX, where X = isoleucine or leucine, each with residue mass of 113.

Synthetic Peptides. Peptides were synthesized using FMOC chemistry by the Peptide Synthesis Facility (Shared Resource) of the Pittsburgh Cancer Institute. Each peptide was purified to >95% homogeneity by reverse-phase (RP) HPLC and the identity of each peptide confirmed by MS/MS. The following peptides were synthesized: p939/MART-1 32-40:ILTVILGVL; gp100 280-288: YLEPGPVTA (9); HIV-nef 73-82:QVPLRPMTYK (26); influenza A matrix, Flu M1 58-66:GILGFVFTL (24); p53 186-196:DGLAPPQHLIR (22); and p53 264-272:LLGRNSFEV (22).

Results

Specific Recognition of HLA-A2–presented Melanoma Peptides by CTL Clone A83. Melanoma 9742 (Mel 9742) and melanoma 624 (Mel 624) were acid treated (12) and peptides obtained from ∼5 × 10^9 cell equivalents. These peptides were then resolved by RP-HPLC (see Materials and Methods for details). Aliquots of individual fractions were pulsed onto the HLA-A2$^+$ T2 cell line, in the presence of human β_2-m and the anti-HLA-A2 monoclonal antibody MA2.1, and tested for recognition by antimelanoma CTL clone A83 in 4-h cytotoxicity assays. Three bioactive peaks were identified for autologous Mel 9742 peptides, resolving in HPLC fractions 42-43, 45, and 47-48 (Fig. 1). A similar pattern of bioactive peaks was identified for allogeneic HLA-A2$^+$ Mel 624 fractionated peptides (Fig. 1). Peak 1 (HPLC fraction 42-43) and peak 3 (HPLC fractions 47-48) exhibited comparable efficacy in sensitizing the T2 target cell to lysis by CTL clone A83, with peak 2 (HPLC fraction 45) displaying a somewhat lesser capacity to do so. HLA-A2 restricted, melanoma-specific TIL 1235 and CTL clone A42 also recognized these same A83-identified bioactive peaks as detected by cytolysis of T2 peptide-pulsed target cells, with peak 2 reactivity approximating that of peaks 1 and 3 (Fig. 1).

Mass Spectrometric Analysis. Mel 9742 HPLC fractions 47 and 48 (peak 3) were pooled and analyzed using tandem mass spectroscopy. 10 μl of fractionated material, corresponding to 20% of the pooled fractions and representing 10^9 cell equivalents, were injected into the electrospray ionizing source and the MS of the peptides recorded on a triple quadrupole mass spectrometer (PE-Sciex). The summation of mass spectra for peptides with m/z = 500–1,600 is reported in Fig. 2 *A*. At least 39 different peptides were detected, with one peptide (m/z = 941) representing the predominant species. Collision-induced dissociation was performed on this ion yielding the daughter ion spectrum shown in Fig. 2 *B*. The spectra was interpreted as defining a nine–amino acid peptide of M_r = 939 with the sequence XXXTVXXGVX, where X = isoleucine or leucine (single letter amino acid designations). Since isoleucine and leucine exhibit identical masses (M_r = 113), 32 potential variants of this peptide sequence are possible. A search of the GenBank database yielded a complete homology for one of these sequences, ILTVILGVL, with a recently cloned melanoma-associated gene Melan-A (accession number HSU06654, 15) also known as MART-1 (14).

p939 Binds to HLA-A2 with Intermediate-to-Low Affinity. The p939 sequence ILTVILGVL was synthesized and shown to elute in HPLC fractions 48/49, approximating the naturally processed bioactive form eluted from Mal 9742. This peptide was then analyzed for its ability to bind to HLA-A2 using an HLA-A2–specific stabilization assay implementing the T2 cell line (22, 27). The T2 cell line exhibits severely depressed cell surface class I expression resulting from a genetic lesion affecting peptide transport into the endoplasmic reticulum (25). T2 expression of class I (in particular HLA-A2) at the cell surface can be enhanced by incubation at reduced temperature in the presence of exogenous β_2-m and peptides capable of binding to the HLA-A2 allele (22–24). We incubated a panel of synthetic peptides, including p939, with the T2 cell line at room temperature (23°C) for 18 h and evaluated their capacity to stabilize HLA-A2 expression on the cell surface of T2 cells. Those peptides binding to HLA-A2 yielded an elevated reactivity with the MA2.1 (anti-HLA-A2) monoclonal antibody compared with non–peptide-treated or irrelevant peptide-treated T2 cells. By performing a peptide dose titration analysis, a qualitative assessment of the relative binding capacity of each peptide for HLA-A2 was obtained (22). Analysis of a series of peptides revealed that

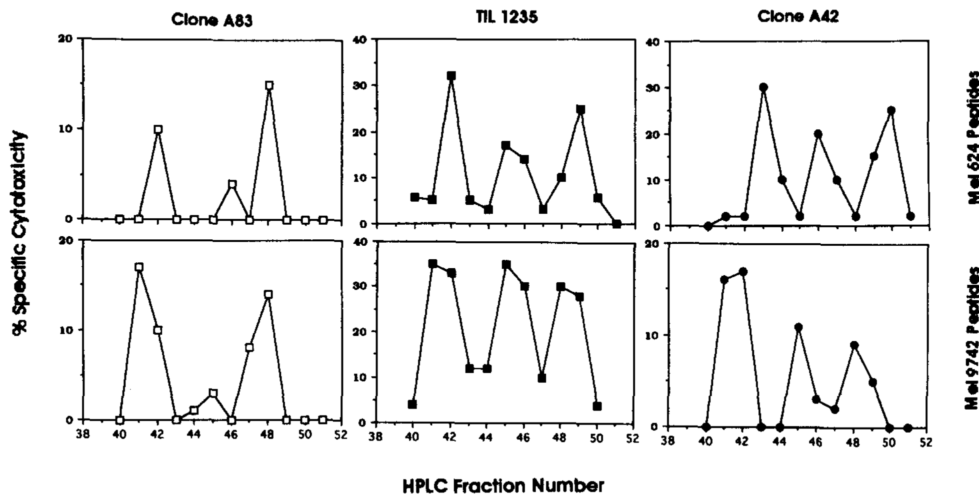

Figure 1. Melanoma TIL lines and clones recognize three predominant epitopes resolved by RP-HPLC. MHC class I–presented peptides derived from Mel 9742 or Mel 624 were extracted and fractionated by RP-HPLC. After lyophilizing, each fraction was reconstituted in 200 μl of buffered saline and 10 μl used to pulse onto ^{51}Cr-labeled T2 target cells in the presence of human β_2-m and the anti-HLA-A2 monoclonal antibody MA2.1 (as described in Materials and Methods). CTL were then added at an effector-to-target cell ratio of 2:1 (*Clone A83*) or 10:1 (*TIL 1235* and *Clone A42*) in a standard 4-h cytotoxicity assay. HPLC fractions 1–38 pulsed onto the T2 cell line were not recognized by CTL.

the Flu M1 58-66 and p53 274-282 peptides were quite effective in stabilizing HLA-A2 expression on the T2 cell line (half-maximal activity at ∼5 nM), while the p939 peptide was less effective (half-maximal activity at ∼200 nM) (Fig. 3). The efficacy of the p939 peptide was comparable to that observed for the gp100 280-288 peptide, recently identified by Cox et al. (9) as a melanoma-associated, HLA-A2–presented T cell epitope. The gp100 280-288 peptide was similarly reported to bind HLA-A2 with intermediate-to-low affinity (9). As controls, the p53 186-196 stabilized HLA-A2 very poorly and the HLA-A3 binding HIV-nef 73-82 peptide did not stabilize HLA-A2, as previously reported (22).

Reconstitution of a T Cell Epitope by p939. Epitope reconstitution experiments using p939 were performed and sensitization of T2 cells to lysis by antimelanoma CTL was evaluated over a wide range of peptide concentrations (250 nM–2.5 pM) (Fig. 4). Four different HLA-A2 restricted, antimelanoma CTL were evaluated in these studies: the bulk cultured TIL 1235 line, two CTL clones (A83 and A42), and the fresh TIL 6970 line. Despite the apparent low affinity of p939 for HLA-A2, each of these CTL recognized peptide-pulsed T2 targets. Interestingly, while half-maximal sensitization for lysis mediated by TIL 1235, clone A83, and TIL 6970 were reached at ∼10 pM of peptide, the A42 clone required ∼3,000 pM of peptide for half-maximal sensitization. TIL 1235 and CTL clone A42 recognition of p939-pulsed T2 targets also

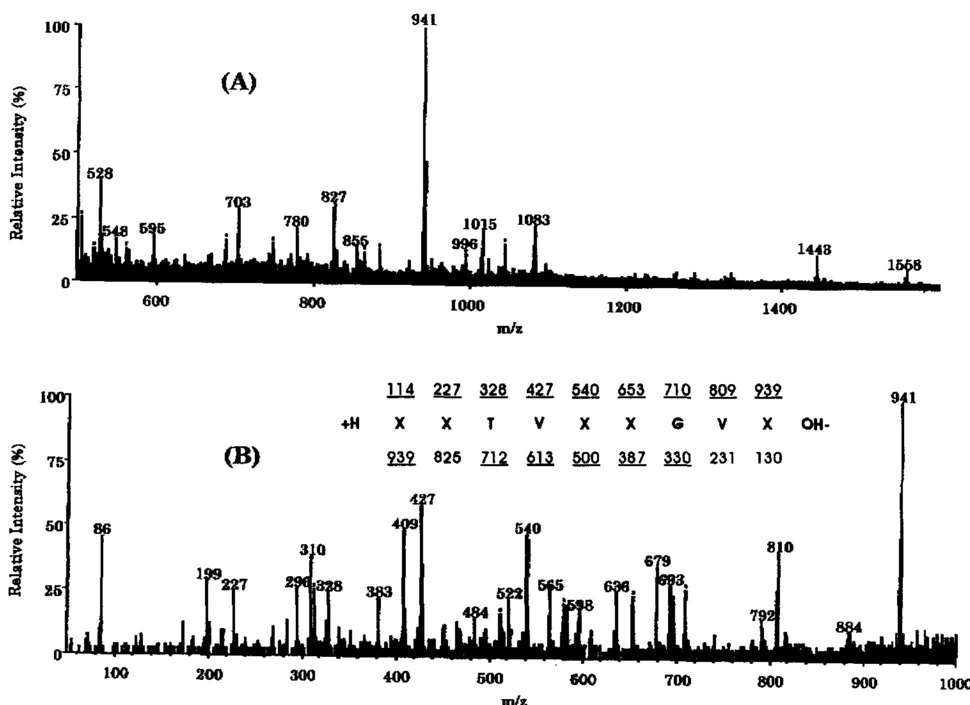

Figure 2. Mass spectrometric analysis of Mel 9742 fraction 47/48 peptides. HPLC fractions 47 and 48 derived from 5 × 10^9 melanoma 9742 cells were combined and 10 μl corresponding to one-fifth of the original volume was injected into the electrospray ionization source of a triple quadrupole mass spectrometer (PE-Sciex). Summation of the mass spectra obtained in the 500–1,600 m/z range is depicted in *A*. The $(M+H)^+$ ion at m/z = 941 was selected for fragmentation to generate sequence data and used to generate a similar CID spectrum (shown in *B*). Predicted (and actual, underlined) masses for the fragments of the type b (carboxyl terminal cleavage) are shown above and type y (amino terminal cleavage) below the deduced sequence XXTVXXGVX (M_r = 939), where X = isoleucine or leucine.

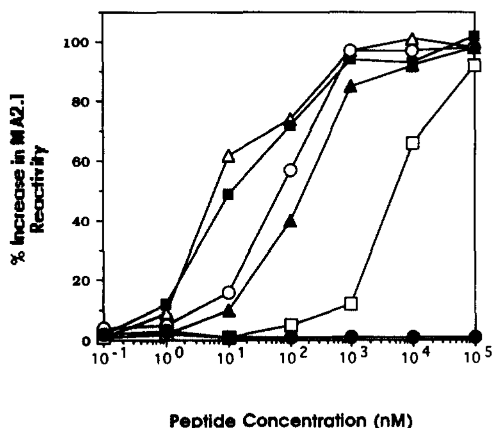

Figure 3. p939 binds to HLA-A2 with moderate-to-low affinity. The stabilization of HLA-A2 on the T2 cell line in the presence of exogenous peptide was evaluated as outlined in Materials and Methods using the indicated synthetic peptides. HLA-A2 expression was evaluated using the MA2.1 (anti-HLA-A2 monoclonal antibody) in indirect immunofluorescence assays monitored by flow cytometry. Data are reported as percent increase in MA2.1 reactivity relative to T2 incubated in the absence of peptide. △, p53-1; ■, Flu M1; ○, gp100; ▲, p939; □, p53-2; ●, HIV-nef.

resulted in peptide-specific release of interferon-γ at p939 doses as low as 1 pM (data not shown). An additional HLA-A2–restricted fresh melanoma TIL 5403 was also able to recognize and lyse T2 target cells pulsed with p939 peptide (data not shown). CTL recognition of T2 presented p939 could be inhibited by addition of the anti-HLA-A2 monoclonal antibody CR11-351 (data not shown).

Discussion

We have identified a naturally processed, melanoma-associated epitope recognized by five distinct HLA-A2–restricted, tumor-specific CTL lines and clones. This peptide was isolated from melanoma cell surface HLA-A2 class I complexes by mild acid elution. The sequence of this peptide (p939: MART-1/Melan-A 32-40; ILTVILGVL) was deduced by mass spectrometry and a GenBank database search for homology, representing the second successful use of this technology to identify a tumor-associated epitope (9).

Interestingly, the p939 epitope represents one of three epitopes coordinately recognized by two distinct antimelanoma CTL clones (A83 and A42) derived from two different HLA-A2^{+} patients (14, 16). The other two epitopes may be represented by MART-1-derived nonameric peptides MART-1 27-35;AAGIGILTV and MART-1 29-37;GIGILTVIL, since the CTL clone A42 and TIL 1235 used in this study have been recently reported to react with these sequences (17). These latter two sequences have not been proven to be naturally processed and presented by melanoma. If they represent naturally processed and HLA-A2–presented epitopes, this would support the recognition of three distinct and overlapping peptide sequences (all share the MART-1 32-35;ILTV sequence) derived from a single protein precursor (MART-1) by two independent CTL clones.

The high avidity of clone A83 for p939 is somewhat surprising since clone A83 is an autoreactive CTL; cross-reacting with melanoma and with normal melanocytes (16). If antitumor reactivity represents autoreactivity in human melanoma, the role of positive and negative selection in maintaining such clonotypic T cells in vivo is of significant interest. The "differential avidity" model (28) could account for the positive selection of clones such as A83. In this model, the critical parameter is the number of TCR engaged with peptide–MHC complexes on APC. When this number is moderate, positive selection may occur. The combination of a high affinity TCR with a specific peptide epitope displaying a low

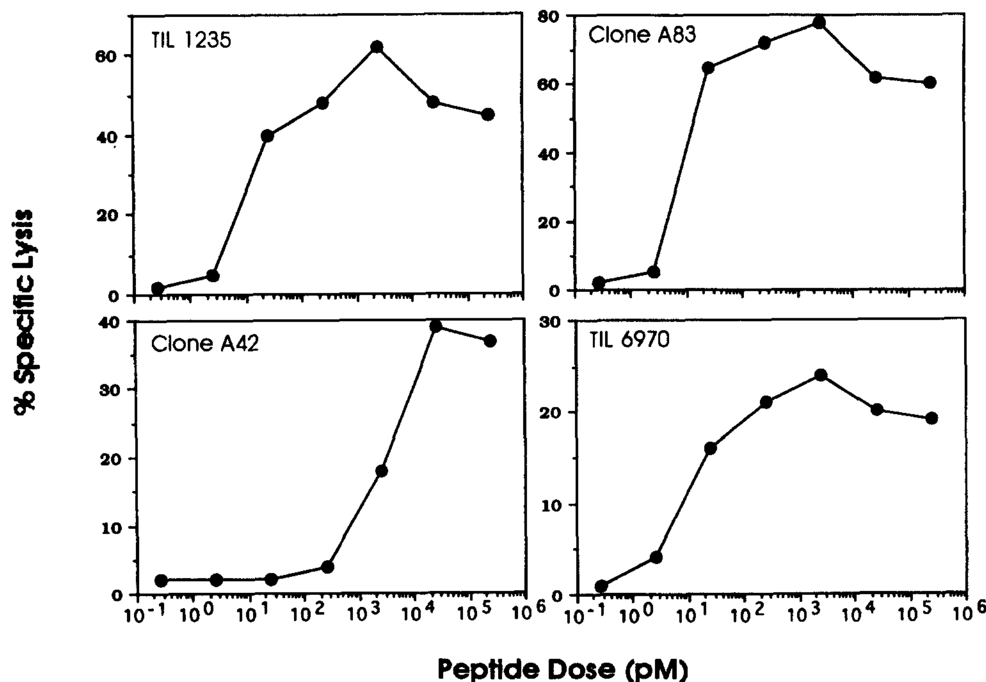

Figure 4. Epitope reconstitution with p939. Melanoma-specific CTL (cultured TIL 1235, clone A83, clone A42, and fresh TIL 6970) were assayed in 4-h cytotoxicity assays at effector-to-target ratios of 10:1 against ^{51}Cr-labeled T2 cells pulsed with p939 at the indicated concentrations. Lysis of T2 cells not pulsed with p939 was <5%.

binding affinity for the MHC restriction element may accommodate such moderate TCR engagement. Such a model makes it harder to justify positive selection of clones such as A42, displaying very low avidity for p939, unless we assume that clone A42 could have been positively selected by a different peptide (such as MART-1 27-35; AAGIGILTV or MART-1; GIGILTVLI) for which it may have a far higher avidity. We are currently evaluating this possibility by comparative epitope titrational analyses using the T2 presenting cell line.

Of some concern, Kawakami et al. (17) have recently screened a series of overlapping synthetic 9-mer peptides (including the p939 sequence) derived from the MART-1 gene for CTL reactivity. In particular, they failed to demonstrate CTL recognition of the p939;ILTVILGVL sequence) presented by the T2 cell line using two of the same cellular reagents (TIL 1235 and clone A42) used in this study. This discrepancy appears to be due to the peptide-loading conditions used in their study. Unlike Kawakami et al. (17), we allow epitope reconstitution to occur in the presence of exogenous human β_2-m and MA2.1 (anti-HLA-A2 monoclonal antibody). This facilitates peptide loading into HLA-A2 complexes by 100–1,000-fold (8, 22–24). Without these additions, we have similarly observed only marginal CTL reactivity (5–10% over background) against p939-loaded T2 target cells. This requirement for β_2-m and MA2.1 appears to reflect the apparent low affinity of p939 for HLA-A2 in the assay system we used for assessing CTL reactivity and may represent an important consideration in the development of p939-based vaccine strategies. Despite this low affinity, enough p939 peptide was presented in HLA-A2 complexes on the cell surface of Mel 9742 cells to allow for CTL reactivity as well as p939 identification and sequencing. This suggests that p939 is efficiently processed and loaded into HLA-A2 molecules via the endogenous pathway in melanoma cells.

Since anti-p939 reactivity could be demonstrated for five distinct CTL populations derived from unrelated HLA-A2$^+$ melanoma patients, p939 may serve as an important component in an effective peptide-based vaccine for the treatment of melanoma. Given the apparent subtleties in the p939-HLA-A2 loading requirements, such therapies may best take the form of ex vivo pulsed autologous antigen presenting cells (i.e., dendritic cells) used as a vaccine (29) or moderate-to-high dose intradermal immunization of p939. Alternatively, p939 may be used to expand antimelanoma CTL in vitro for subsequent adoptive immunotherapy.

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