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31. We thank N. Lippmann, J. Fox, and K. Hewes for microbiologic assays, K. Bloch for serum immunoelectrophoresis, P. Della Pella for immunohistochemistry, M. Selig for assistance with the electron microscopy, H. Wu and R. Jaenisch for the 129Sv ES genomic library and β_2-microglobulin–deficient mice, and W. Hull for surfactant protein analysis. We acknowledge the technical assistance of K. Edelman, K. Brose, L. Kieckens, C. Crawford, and J. Salter. We are grateful to V. Tybulewicz, M. Goodell, T. Jacks, S. Connor, and C. Gee for their help and advice. Supported by a USPHS Physician-Scientist Award (G.D.) and NIH grant HL37569 (R.C.M.).

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Identification of a Peptide Recognized by Five Melanoma-Specific Human Cytotoxic T Cell Lines

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Of several thousand peptides presented by the major histocompatibility molecule HLA-A2.1, at least nine are recognized by melanoma-specific cytotoxic T lymphocytes (CTLs). Tandem mass spectrometry was used to identify and to sequence one of these peptide epitopes. Melanoma-specific CTLs had an exceptionally high affinity for this nine-residue peptide, which reconstituted an epitope for CTL lines from each of five different melanoma patients tested. Recognition by multiple CTL lines suggests that this may be a promising candidate for use in peptide-based melanoma vaccines.

Cytotoxic T lymphocytes that recognize tumor cells can often be isolated from cancer patients (*1*). Their existence suggests that there is an immune response to cancer in these patients and that its augmentation might be therapeutic. Immunotherapy as a treatment for cancer has focused on melanoma, both because melanoma spontaneously regresses more frequently than most human malignancies (*2*) and because melanoma-specific CTLs can be generated consistently in vitro (*3*). However, neither adoptive transfer of melanoma-specific CTLs nor specific active immunotherapy with whole melanoma cells or cell-derived preparations has led to the eradication of melanoma or long-term survival in more than a minority of patients (*4*). For immunotherapy to be improved, epitopes recognized by melanoma-specific CTLs must be identified. CTL epitopes are peptides derived from cellular proteins that are presented on the cell surface by major histocompatibility complex (MHC) class I molecules (*5, 6*). Peptides that are expressed by the tumors of many individuals may be useful for immunotherapy (*7–10*), but the most generally applicable would be those that also are recognized by lymphocytes obtained from a large number of different melanoma patients.

Melanoma-specific CTL lines restricted by HLA-A2.1 recognize epitopes present on most allogeneic A2.1$^+$ melanoma cells (*7, 8, 11*). Individual CTL lines have been shown to recognize at least six peptides common to multiple melanomas (*12, 13*), some of which may be recognized by CTL lines derived from different melanoma patients (*13*). The tyrosinase gene has been reported to encode two HLA-A2.1–restricted, melanoma-specific epitopes, each of which is recognized by a single CTL clone (*14*). Here, we report the direct identification, by tandem mass spectrometry, of a peptide epitope that is recognized by high-affinity CTLs from five different melanoma patients: VMM5, VMM6, LD1, DM169, and DM204.

Peptides associated with the HLA-A2.1 molecules expressed on a melanoma cell line, DM6, were isolated as described (*12*) and fractionated by high-performance liquid chromatography (HPLC). We identified HPLC fractions containing relevant peptides by testing their ability to reconstitute epitopes for melanoma-specific CTL lines from two different patients, VMM5 (*12*) and DM204 (*15*), after incubation with the HLA-A2.1–positive lymphoblastoid cell line T2 (*16*) (Fig. 1). Six peaks of activity were identified for cells from VMM5 and three for cells from DM204 (Fig. 1A). Fractions 2 to 3 and 14 to 15 contained peptides recognized by both CTL lines. Fractions 2 and 3 were pooled and rechromatographed (Fig. 1B). Two distinct peaks of activity were found for each CTL line, one of which contained peptides recognized by both (*17*). The number of peptides in the active fractions (greater than 50) exceeded the number that could be sequenced with available material, and third-dimension separations by standard HPLC methods caused loss of material without reducing the number of candidates.

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Consequently, second-dimension HPLC fractions 26 and 27 were pooled and analyzed with an apparatus that allows simultaneous immunological and mass spectrometric analyses of the effluent from a microcapillary HPLC column (Fig. 2). Both CTL lines showed a single coincident peak of activity. Mass spectra showed that these fractions contained at least 25 peptides (Fig. 2A), but the relative abundances of only three of these [with mass-to-charge ratios (m/z) of 1046, 946, and 864, herein referred to as peptides 1046, 946, and 864, respectively] matched the activity profile for CTLs from DM204 (Fig. 2B).

Collision-activated dissociation analyses performed on the $(M + H)^+$ ions for each of these three peptides defined their amino acid sequences as follows: m/z 864, SMAPGNTSV; m/z 946, YXEPGPVTA; and m/z 1046, AXYDATYET (*18*). The sequenced peptides were synthesized with an equimolar mixture of Leu and Ile in place of X. Peptide 946 reconstituted epitopes for CTLs from both VMM5 and DM204, with half-maximal reconstitution achieved between 1 and 10 pM (Fig. 3). Peptides 864 and 1046 had no epitope reconstituting activity at concentrations up to 10 nM (Fig. 3). Microcapillary HPLC fractionation experiments demonstrated that only the Leu-containing form coeluted with the peak of naturally processed material, showing that YLEPGPVTA is the naturally occurring species. The amount of peptide 946 present in well 41 (Fig. 2B) corresponded to a concentration of 8 pM, which indicates that the synthetic peptide sensitized at doses comparable to that of the naturally occurring species.

Peptide 946 reconstitutes T cell recognition at concentrations that are at least two orders of magnitude lower than those of several optimized HLA-A2.1–restricted epitopes of viral or cellular origin (*19*, *20*). This could be a result of high affinity of the peptide for the MHC or high affinity of the T cell receptor. With the use of a quantitative binding assay (*21*, *22*), the concentration of YLEPGPVTA giving 50% inhibition of binding of a standard peptide to purified HLA-A2.1 molecules was 1.06 μM (Fig. 4). This value lies well outside the 5 to 30 nM range observed for several other naturally processed peptides (Fig. 4) (*23*). Although this peptide contains the predicted motif residues at positions 2 and 9 that support peptide binding to HLA-A2.1 (*23*, *24*), its lower affinity may be due to the presence of a negatively charged residue at position 3 (*21*). The sequence of peptide 946 was obtained from 15 fmol of peptide

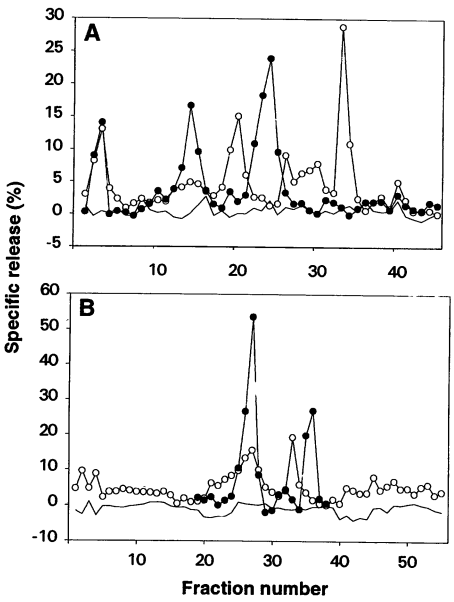

Fig. 1. Reconstitution of epitopes for melanoma-specific CTLs with HPLC fractions derived from naturally processed peptides extracted from HLA-A2.1 molecules. (**A**) Peptides bound to A2.1 molecules were extracted and fractionated by reversed-phase HPLC as described (*12*), with the use of a gradient of acetonitrile and 0.085% trifluoroacetic acid (TFA) in 0.1% TFA, with the concentration of acetonitrile increasing from 0 to 9% (0 to 5 min), 9 to 36% (5 to 55 min), and 36 to 60% (55 to 62 min) (v/v) and with fractions collected every minute. (**B**) Fractions 2 and 3 from the separation shown in (A) were pooled and rechromatographed with a 55-min gradient of 0 to 30% acetonitrile and 0.1% heptafluorobutyric acid (HFBA) in 0.1% HFBA. For both panels, peptide fractions were preincubated for 2 to 3 hours with 2×10^3 ^{51}Cr-labeled T2 cells before incubation with CTLs, at an effector:target ratio of 10:1. Open circles, VMM5 CTLs; closed circles, DM204 CTLs; solid line, media only. Lysis of T2 cells without peptide by VMM5 CTLs was 0.3% in (A) and 0.8% in (B) and by DM204 CTLs was 0.7% in (A) and −0.2% in (B); positive control lysis of HLA-A2$^+$ melanoma cells by VMM5 CTLs was 41% in (A) and 67% in (B) and by DM204 CTLs was 28% in (A) and 78% in (B).

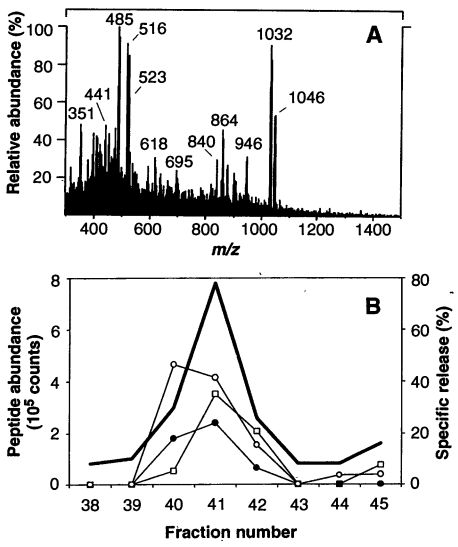

Fig. 2. Identification of candidate peptides by mass spectrometry. Second-dimension HPLC fractions 26 and 27 (from Fig. 1B) were separated with an on-line microcapillary column effluent splitter. A PRP-1 (Hamilton) microcapillary HPLC column (100 μm by 22 cm) was butt-connected with a zero dead volume union (Valco) to two capillaries of different lengths and interior diameters (25 μm and 40 μm; Polymicro Technologies) and eluted with a 34-min gradient of 0 to 60% acetonitrile. The 25-μm capillary deposited one-sixth of the material into 50 μl of culture media in microtiter plate wells for analysis with CTLs as in Fig. 1. The 40-μm capillary directed the remaining five-sixths of the material into the electrospray ionization source, and mass spectra of the peptides deposited in each well were recorded on a Finnigan-MAT (San Jose, California) triple-quadrupole mass spectrometer as described (*21*). Chromium release assays were performed as described (Fig. 1). (**A**) Summation of mass spectra recorded on peptides deposited in well 41. Many of these were eliminated as candidates because their relative abundance failed to correlate with the observed lysis. (**B**) Percent of specific release (solid line). Peptide ion abundance in wells 38 to 45 for peptide 1046 is shown by open circles; for peptide 946, by solid circles; and for peptide 864, by squares.

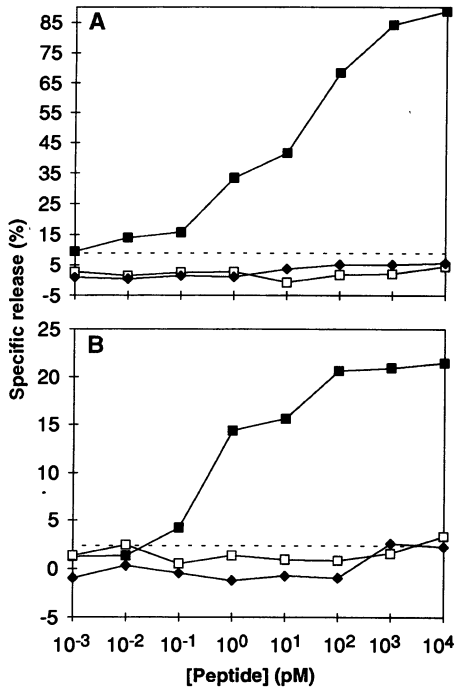

Fig. 3. Epitope reconstitution with synthetic peptides. Melanoma-specific CTLs from patient VMM5 (**A**) or patient DM204 (**B**) were assayed at an effector:target ratio of 10:1 on T2 cells that had been incubated with the indicated concentration of peptides 946 (■), 864 (□), and 1046 (◆). Lysis of T2 cells without peptide is shown by a dotted line.

present in a second-dimension HPLC fraction derived from 4×10^{10} cells. As a result, we calculate that as few as two 946–HLA-A2.1 complexes may be present per melanoma cell (*25*). However, the low affinity of peptide 946 for HLA-A2.1 likely results in disproportionate losses during affinity purification. Thus, there may be as many as several hundred 946–HLA-A2.1 complexes per cell.

The 946 peptide sensitizes for CTL-mediated lysis at concentrations that are 10^4 to 10^5 lower than the concentration giving 50% inhibition of binding (IC_{50}). The T2 cell line used for the sensitization experiments does not express an unusually large number of the cell adhesion molecules LFA-1 or LFA-3 (*26*). In the absence of an alternate explanation for the efficacy of the 946 peptide, the affinity of the specific T cell receptor appears to be the critical component. The CTL lines from both patients, VMM5 and DM204, respond similarly to peptide 946; therefore, it appears that these CTL lines have a very high affinity for this epitope.

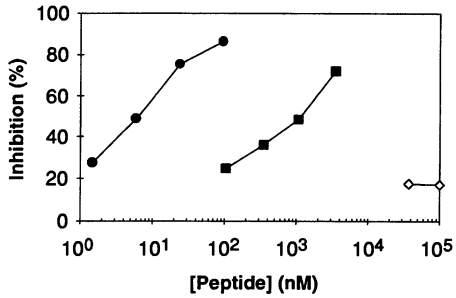

Fig. 4. Binding of synthetic 946 peptide to HLA-A2.1. The ability of the test peptides to compete with the radiolabeled standard peptide FLPSDYFPSV for binding to purified HLA-A2.1 molecules was measured with an equilibrium binding assay as described (*22, 23*). Solid squares, YLEPGPVTA; solid circles, ALWGFFPVL, another endogenous peptide isolated from HLA-A2.1 (*21*); open diamonds, APRTVALTA, an endogenous peptide isolated from HLA-B7 (*31*).

To determine whether peptide 946 was a generally relevant epitope for HLA-A2.1–restricted, melanoma-specific CTLs, we tested lines from three additional patients. The 946 peptide reconstituted recognition in each case, whereas neither peptide 864 nor peptide 1046 had any detectable activity (Fig. 5). Dose-response curves obtained for two of these lines again showed half-maximal lysis in the range of 1 to 10 pM. Thus, five of five patients examined had high-affinity T cells that recognized the HLA-A2.1–associated peptide 946. This peptide is likely to have been expressed on the tumor cells used to establish and maintain the T cell lines. An additional peptide, YMNGTMSQV, which originates from tyrosinase and is recognized by an HLA-A2.1–restricted, melanoma-specific T cell clone (*14, 27*), was also evaluated. None of the four CTL lines tested recognized cells incubated with this peptide (Fig. 5).

The Leu-containing version of peptide 946 was identified as part of Pmel 17 (*28*), a protein specific to melanocytes and melanoma (*28*). Of unknown function, Pmel 17 has been postulated to be a component of the melanin biosynthetic pathway (*28*). Its expression in melanocytes as well as melanoma is consistent with observations that some melanoma-specific CTL clones recognize melanocytes (9) and that spontaneous remissions of human melanoma have been observed in conjunction with the simultaneous development of vitiligo (*2*). These observations suggest that an autoimmune response to melanocytes may naturally accompany the development of immunity to melanoma. Because high-affinity T cells to autoantigens are probably deleted in the thymus, it is unclear how the high-affinity, 946-specific T cells normally exist in the periphery in the absence of discernible autoimmune disease. One possibility is that 946-A2.1 complexes expressed on melanocytes can be recognized by activated CTLs but that they are incapable of initiating a primary response in vivo. Alternatively, 946-specific T cells may escape negative selection and become anergized in the periphery. This might also explain their failure to eradicate melanoma despite their presence at tumor sites. However, it is also possible that peptide 946 may not be presented by HLA-A2.1 on the surface of these normal cells, in which case it would be a tumor-specific, rather than a tissue-specific, antigen.

Here, we used tandem mass spectrometry together with a different chromatography separation method to identify a peptide epitope of very low abundance in a mixture estimated to contain at least 10,000 species (*29*). Direct analysis of peptides eluted from MHC class I molecules also allowed the selection of those recognized by more than one CTL line in advance of epitope identification. The subsequent demonstration that this epitope was recognized by CTLs from several additional individuals illustrates the usefulness of this approach. Knowledge and techniques learned from the melanoma system can be applied to other solid tumors (*30*). Identification of peptide epitopes for CTLs that are specific for these tumors should permit a wider understanding of how best to stimulate cellular immune responses against solid tumors. This provides a foundation on which to develop immunological treatments to augment the surveillance, recognition, and destruction of malignant cells in humans.

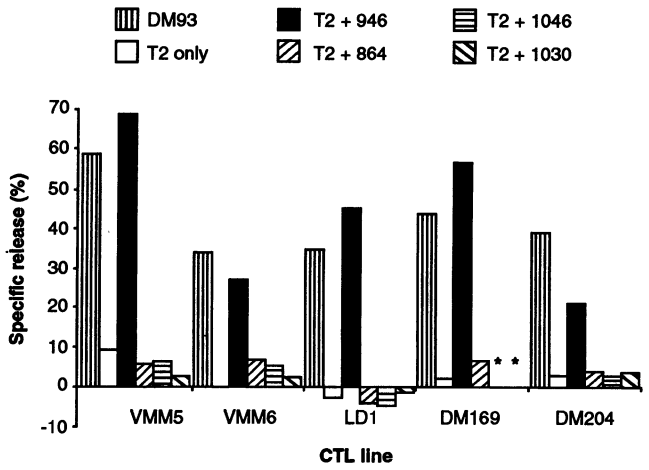

Fig. 5. Peptide 946 reconstitutes an epitope for five melanoma-specific CTL lines. The CTL lines were derived from five different patients, each with melanoma metastatic to regional or in-transit nodes. CTL lines from patients VMM5 and VMM6 were generated by repeated stimulation with fresh autologous tumor cells. VMM5 CTLs were maintained on allogeneic A2.1$^+$ melanoma line DM6 after specificity was obtained (*12*). DM169 and DM204 CTLs were generated by stimulation with allogeneic A2.1$^+$ melanoma line DM13 (*15*), then maintained with DM6. LD1 CTLs were stimulated on a rotating schedule with DM6, DM13, and DM93. Each of these lines reacts with several melanoma peptide epitopes. T2 cells were preincubated with the peptides whose $(M + H)^+$ values are indicated. Peptide 946 was used at a concentration of 0.1 nM, except for DM169 (1 nM). All other peptides were used at a concentration of 10 nM, except for DM169 (250 nM) and for LD1 (0.1 nM). DM93 is an HLA-A2.1 melanoma used as a positive control. Asterisks indicate peptides not evaluated with DM169 CTLs. The sequences of the peptides 864, 946, and 1046 are described in the text. Peptide 1030 has the sequence YMNGTMSQV (*19*).

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17. Considering the first- and second-dimension HPLC fractions assayed with these two CTL lines (Fig. 1), a total of nine distinct CTL epitopes were identified.
18. Single-letter abbreviations for the amino acids are A, Ala; D, Asp; E, Glu; G, Gly; I, Ile; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; S, Ser; T, Thr; V, Val; and Y, Tyr. X refers to either Leu or Ile, which cannot be distinguished by triple-quadrupole mass spectrometry.
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25. Fifteen femtomoles from 4×10^{10} cells corresponds to 0.23 copies per cell-equivalent after two rounds of HPLC separation. A 12% yield was estimated on the basis of the following values, which we have determined experimentally to be valid for a broad range of MHC-associated peptides: 70% recovery of peptide during class I MHC purification and extraction; 25% recovery in the first two rounds of HPLC; and 70% recovery that is a result of discarding off-peak fractions in two rounds of HPLC. Thus, as few as two copies of peptide 946 may be present per cell.
26. V. H. Engelhard, unpublished data.
27. We have identified a nonamer peptide YMDGTMSQV, extracted from HLA-A2.1 molecules of the melanoma cell line DM93, which is presented at high copy number. A protein data bank search identified its homologous sequence in the tyrosinase molecule as YMNGTMSQV.
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32. Supported by U.S. Public Health Service grants CA57653 (C.L.S.), GM37537 and AI33993 (D.F.H.), and AI20963 (V.H.E.), by American Cancer Society grant IN149H (C.L.S.), by the Diabetes Center (NIH P30DK38942) and the Cancer Center (NIH P30CA44579) at the University of Virginia, and by a Cancer Research Institute postdoctoral fellowship (J.S.). We thank D. Pappin for suggesting the support used in the chromatographic separation; H. F. Seigler for providing cellular reagents; M. C. Wilhelm for providing surgical tissue from patients VMM5 and VMM6; and C. Murphy, D. Graham, D. Taylor, T. Natale, and M. Fontanilla for large-scale culture of human melanoma cells.

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Mutagenesis and Mapping of a Mouse Gene, *Clock*, Essential for Circadian Behavior

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In a search for genes that regulate circadian rhythms in mammals, the progeny of mice treated with *N*-ethyl-*N*-nitrosourea (ENU) were screened for circadian clock mutations. A semidominant mutation, *Clock*, that lengthens circadian period and abolishes persistence of rhythmicity was identified. *Clock* segregated as a single gene that mapped to the midportion of mouse chromosome 5, a region syntenic to human chromosome 4. The power of ENU mutagenesis combined with the ability to clone murine genes by map position provides a generally applicable approach to study complex behavior in mammals.

Progress has been made at the physiological and cellular levels in our understanding of circadian systems (*1*), yet the molecular mechanism of circadian clocks has not been fully elucidated (*2*). The isolation of "clock mutants" and the widespread requirement for protein synthesis in circadian clock systems imply that gene expression is an integral component of the oscillator (*2*). Recent molecular work with the *Drosophila period* (*per*) and *Neurospora frequency* (*frq*) genes suggests that a circadian cycle of *per* and *frq* transcription, respectively, may lie at the heart of the oscillator mechanism in these species (*3*). However, no information exists concerning the molecular elements of the clock system in mammals. In the absence of specific mechanistic information, genetics has been a powerful approach to uncover unknown elements. We report here the isolation of a mutation in the mouse that changes two central properties of circadian rhythms: the intrinsic period length and the persistence of rhythmicity. Taken together, our results define a gene, named *Clock* (for circadian locomotor output cycles kaput), that is essential for normal circadian behavior.

Because the majority of clock mutants isolated in other organisms have been semidominant (*4*), we screened heterozygotes directly in the mouse. With the mutagen ENU, average forward mutation frequencies of 0.0015 per locus per gamete (1 in 700) can be achieved in the mouse (*5*). Male mice of the inbred strain C57BL/6J (B6) were treated with a single injection of ENU and after recovery of fertility were mated with untreated B6 females (*6*). First-generation (G_1) offspring would be heterozygous for any induced mutations but otherwise possess an isogenic B6 background (Fig. 1A). Normal B6 mice exhibit a robust circadian rhythm of wheel-running activity; we used this behavioral assay to screen for circadian mutants (Fig. 1B). Activity rhythms were monitored during exposure to a light-dark cycle (LD) to assess synchronization or entrainment behavior and in constant darkness (DD) to determine the circadian period of the locomotor activity rhythm (*7*). Laboratory mice typically have circadian periods of less than 24 hours, with B6 mice having periods that average between 23.3 and 23.8 hours (*8*).

We tested a total of 304 G_1 offspring of ENU-treated males (*9*). The distribution of period lengths of the activity rhythms from the G_1 mice was normal, with a mean of 23.7 hours and a standard deviation of 0.17 hours (Fig. 1C). One animal (G_1-25) expressed a circadian period that gradually lengthened over the first 30 days of DD,

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