Identification of a Shared HLA-A*0201-restricted T-Cell Epitope from the Melanoma Antigen Tyrosinase-related Protein 2 (TRP2)

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ABSTRACT

Tyrosinase-related protein 2 (TRP2) is a melanosomal enzyme expressed in most mammalian melanocytes and melanomas. This protein has been identified as a melanoma antigen recognized by tumor-reactive CTLs derived from tumor infiltrating lymphocytes in the context of HLA-A31 and HLA-A33. The frequencies of these HLA-A alleles among melanoma patients in the United States is low (~6% for HLA-A31 and ~2% for HLA-A33) compared with that of HLA-A*0201 (~46%). Therefore, to extend significantly the use of TRP2-based immunotherapies for the treatment of patients with melanoma, we searched for new HLA-A*0201-restricted epitopes from this protein by screening TRP2-derived peptides for the induction of melanoma-reactive CTL. Fifty-one peptides were selected from TRP2 based on a permissive HLA-A*0201 binding motif, and the 21 peptides with the highest experimentally determined binding affinities were used to stimulate peripheral blood lymphocytes from HLA-A*0201+ melanoma patients in vitro. One peptide, TRP2(180–188) (SVYDFFVWL), induced CTLS from three of four patients that specifically recognized peptide-pulsed T2 cells, COS-7 cells expressing HLA-A*0201 and TRP2, and HLA-A2* TRP2+ melanomas. TRP2(180–188) is identical to a previously identified TRP2 epitope recognized by murine melanoma-reactive CTLS in the context of H-2Kb. These results suggest that TRP2 may be useful for the development of murine tumor immunotherapy models and for the treatment of melanoma patients who are diverse in HLA expression.

INTRODUCTION

Clinical evidence strongly suggests that T lymphocytes can mediate the regression of melanoma. In one study, the adoptive transfer of autologous TILs with IL-2 significantly reduced tumor burden in 30–40% of patients with metastatic disease (1, 2). Some populations of tumor-reactive CTLs derived from TILs or from lymphocytes stimulated in vitro with tumor cells recognize nonmutated proteins expressed on melanoma cells from multiple patients. At least 14 normal self proteins have been identified as melanoma antigens (3–5), and results from several clinical investigations suggest that class I MHC-restricted recognition of epitopes from such proteins by CTLs may be involved in tumor rejection. In one investigation, a significant correlation was demonstrated between T-cell recognition of HLA-A2-restricted epitopes from the melanoma antigen gp100 in vitro and tumor regression in patients receiving TIL therapy (6, 7). In another study, clinical responses were observed in some patients receiving peptides from the melanoma antigens MART-1, gp100, and tyrosinase (8). Furthermore, in a recent immunization trial with a gp100 peptide modified to enhance its binding affinity to HLA-A*0201, tumor regression was observed in 13 of 31 patients (42%) receiving the peptide with IL-2 (9).

The expression of nonmutated melanoma antigens is heterogeneous among tumors isolated from different patients and between individual cells from single lesions. Thus, the development of immunotherapies based on as many antigens as possible may be clinically beneficial. TRP2 is a melanosomal enzyme with dopachrome tautomerase activity involved in melanin synthesis and is widely expressed in mammalian melanomas (10, 11). It is a member of the tyrosinase-related gene family and shares 40% amino acid sequence identity with tyrosinase and TRP1. TRP2 has been identified as a melanoma antigen recognized by tumor-reactive CTLs in both the mouse (12) and human (13). In the murine model, several B16-reactive CTL lines generated from splenocytes of C57BL/6 mice immunized with irradiated B16 melanoma cells recognized TRP2(181–188) (VYDFFVWL) in the context of H-2Kb. In addition, a T-cell line raised by repeated in vitro stimulation of murine splenocytes with this peptide recognized B16 melanoma and eliminated 3-day-old established pulmonary micrometastases in vivo (12). In the human, TRP2(197–205) (LGGRPRYR) was identified as the HLA-A31-restricted epitope recognized by a CTL clone derived from a population of TIL (TIL586). The adoptive transfer of this TIL with IL-2 into the autologous patient resulted in an objective clinical response (13).

The same peptide was also recognized by an independent TIL (TIL1244) in the context of HLA-A33 (14). Based on these findings, we believe TRP2 is a good candidate tumor antigen for the immunotherapeutic treatment of human melanoma. However, in one study, among 412 patients with metastatic melanoma referred to the National Cancer Institute, the frequencies of HLA-A31 and HLA-A33 were 6% and 2%, respectively (15). Therefore, to increase the number of patients eligible for TRP2-based treatments, we began a search for new class I HLA-restricted epitopes from this protein.

Among melanoma patients in the United States, HLA-A2 is the most commonly expressed family of class I MHC molecules with an estimated frequency of 47% (15). HLA-A*0201 is the most common subtype of these alleles and is expressed in ~98% of HLA-A2+ Caucasians in North America (16). HLA-A*0201-restricted epitopes have been identified from six known melanoma antigens: MART-1 (17), gp100 (6, 7, 18-20), tyrosinase (21), MAGE-3 (22, 23), N-acetylglucosaminyl-transferase V (24), and the melanocyte-stimulating hormone receptor MC1R (4). Therefore, immunotherapies targeting these antigens are applicable to a large number of patients. The identification of new HLA-A*0201-restricted epitopes from additional melanoma antigens would lead to the extension of present immunotherapy protocols for a large fraction of the patient population.

The present investigation describes a new HLA-A*0201-restricted epitope from TRP2 identified by screening peptides for the induction of melanoma-reactive CTL. Fifty-one candidate peptides were selected from TRP2 based on an extended HLA-A*0201 binding motif. This set of criteria was adapted from previous studies that demonstrated a correlation between peptide binding affinity and the predomin-
iniance of certain amino acids at particular positions in peptide sequences (25–30). The 21 peptides with the highest experimentally determined binding affinities were used to stimulate PBLs from HLA-A*0201+ melanoma patients in vitro. One peptide [TRP2(180–188); SYVYDFFYWL] induced CTLs from three of four patients that specifically recognized peptide-pulsed T2 cells, COS-7 transfectants expressing HLA-A*0201 and TRP2, and HLA-A2+ TRP2+ melanoma cells. These results will enable the development of TRP2-based immunotherapies for the treatment of a large percentage of patients with melanoma.

MATERIALS AND METHODS

Cell Culture. The Skmel23 human melanoma cell line was kindly provided by Thierry Boon (Ludwig Institute for Cancer Research, Brussels, Belgium), and A375 was purchased from American Type Culture Collection (Rockville, MD). All other human melanoma cell lines were established in our laboratory (31). Melanoma cell lines and T2 cells (HLA-A*0201+ peptide transporter associated protein deficient T-B hybrid; Ref. 32) were routinely cultured in RPMI 1640 (Meditech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD) and 2 mM l-glutamine (Biofluids). The COS-7 monkey kidney cell line was kindly provided by W. Leonard (NIH, Bethesda, MD) and was maintained in DMEM (Meditech or Biofluids) containing 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, and 10 mM HEPES. Human lymphocytes were cultured in complete medium consisting of Iscove’s modified DMEM with 25 mM HEPES, 2 mM l-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin (Biofluids), and 10% heat-inactivated human AB serum (Sigma Chemical Co., St. Louis, MO; Valley Biomedical, Winchester, VA).

The presence of TRP2 mRNA in melanoma cell lines was assessed previously by Northern blot analysis (13). The expression of HLA-A2 was evaluated by FACS using an anti-HLA-A2 monoclonal antibody (One Lambda, Canoga Park, CA), and for some cell lines, DNA sequencing confirmed the presence of HLA-A*0201 (HLA laboratory, NIH). The expression of TRP2 and HLA-A2 in melanoma cell lines was as follows: 397mel (HLA-A2+, TRP2+); 888mel (HLA-A2+, TRP2+); A375 (HLA-A*0201+, TRP2+); 526mel (HLA-A2+, TRP2+); 624mel (HLA-A*0201+, TRP2+); 1300mel (HLA-A2+, TRP2+); Skmel23 (HLA-A*0201+, TRP2+); and 501mel (HLA-A*0201+, TRP2+). In addition, COS-7 cells expressing HLA-A*0201 and TRP2 or MART-1 were generated by cotransfection (Lipofectamine Plus; Life Technologies). MART-1 were generated by cotransfection (Lipofectamine Plus; Life Technologies) and were either used directly (peptide-loaded) or were washed twice prior to use (peptide-pulsed). Responder T cells (10⁵) were coincubated with 10⁴ stimulator cells (total, 250 ìl) ~20 h at 37°C and the concentration of human IFNγ in coculture supernatants was determined by gamma counting. The percentage of specific lysis was measured using a commercially available ELISA kit (Endogen, Cambridge, MA).

In some experiments, 4-h ⁵¹Cr release cytotoxicity assays were also performed to evaluate the recognition of TRP2 by bulk CTLs as described previously (36). Briefly, ⁵¹Cr-labeled T2 cells were incubated with 1 ìM peptide ~1 h at 37°C and washed twice. These cells and ⁵¹Cr-labeled melanoma cells were cocultured with effector cells (5000 targets/well; multiple E:T ratios; total, 150 ìl) 4 h at 37°C, and the radioactivity in coculture supernatants was determined by gamma counting. The percentage of specific lysis of target cells by CTLs was calculated, and spontaneous ⁵¹Cr release never exceeded 20% of the maximum.

RESULTS

Identification of HLA-A*0201 Binding Peptides from TRP2. Candidate peptides were selected from TRP2 that conformed to a permissive HLA-A*0201 binding motif. This set of criteria was derived from three previously published observations: (a) peptides that bind with high affinity to HLA-A*0201 are generally 9 or 10 amino acids in length and contain L or M at the second position from the NH₂ terminus (P2) and V or L at the COOH terminus (P9 or P10; Ref. 30); (b) amino acids at secondary anchor positions, most notably P1 and P3, can significantly affect peptide binding to HLA-A*0201 (28, 29); and (c) many previously identified HLA-A*0201-restricted epitopes from nonmutated melanoma antigens are nine amino acids in length and have a dominant residue at P2 or the COOH terminus, but not both primary anchor positions (37, 38). Based on these observations, peptides were selected manually from the amino acid sequence of the TRP2 protein. 9-mers were chosen that contained L/M at position 2 (P2) or V/L at the P9 or P10, and 10-mers were selected that contained L/M at both P2 and L/V at P10, and peptides with D, E, P, R, or H at P1 were eliminated. In total, 44 9-mers and 7 10-mers were synthesized (Table 1).

The binding affinities of TRP2 peptides to HLA-A*0201 were evaluated on the basis of the inhibition of binding of a standard radiolabeled peptide to purified class I MHC molecules (33).
concentration of test peptide necessary to inhibit the binding of the standard peptide by 50% (ID$_{50}$), relative binding affinity was defined as high (ID$_{50}$ < 50 nM), intermediate (50 nM < ID$_{50}$ < 500 nM), or weak (ID$_{50}$ > 500 nM).

### CTL Inductions with TRP2 Peptides

Results of previous investigations have demonstrated that immunogenic peptides generally bind to appropriate class 1 MHC molecules with high or intermediate affinity (39). Therefore, to identify new HLA-A$^{*}$0201-restricted epitopes from TRP2, the 21 peptides with ID$_{50}$ < 2000 nM (Table 1) were used to stimulate PBLs in vitro from four HLA-A$^{*}$0201$^+$ patients with metastatic melanoma. In this screening, PBLs were initially cultured with 5 ng/ml peptide and were restimulated weekly with peptide-pulsed autologous PBMCs beginning at day 11. IFN-γ secretion in response to peptide-loaded T2 cells and HLA-A$^{*}$0201$^+$ TRP2$^+$ melanomas was measured 7 days after the third and fourth restimulations (days 32 and 39). Lymphocytes from three of four patients proliferated during this study, and for a given patient, no significant or consistent differences were observed between lymphocyte expansions with different peptides. Peptide recognition by bulk T-cell cultures after four restimulations is presented in Table 2. In patients HU and IN, TRP2(431–439), TRP2(180–188), TRP2(217–225), and MART-1(27–35) induced peptide-reactive T-cell cultures, and in patient CA, peptide-specific CTLs were generated with TRP2(476–484) and TRP2(217–225). However, no TRP2 peptide-induced CTLs specifically recognized HLA-A$^{*}$2$^+$ TRP2$^+$ melanoma cells; only T cells from patients HU and IN stimulated with the positive control peptide MART-1(27–35) specifically secreted IFN-γ in response to HLA-A$^{*}$2$^+$ melanomas (data not shown).

One possible explanation for the lack of melanoma recognition by bulk T cells stimulated with TRP2 peptides was that these cultures contained only small numbers of high avidity T cells capable of recognizing low levels of TRP2 peptides expressed on the surfaces of melanoma cells. If this were the case, melanoma reactivity might be apparent in T-cell clones derived from peptide-specific cultures. Because these T cells were unavailable for further analysis, we stimulated PBLs from additional HLA-A$^{*}$0201$^+$ melanoma patients in vitro with the two TRP2 peptides that most efficiently induced peptide-reactive CTLs in the initial screening; PBLs from four HLA-A$^{*}$0201$^+$ melanoma patients (MU, KU, AN, and WE) were stimulated in vitro with TRP2(180–188), and PBLs from four separate patients (MC, WO, LE, and CA) were stimulated with TRP2(217–225). In this second set of experiments, PBLs were stimulated with peptides using a standard protocol that had previously been used successfully to generate melanoma-reactive CTLs from peptides from MART-1 and gp100 (40, 41). In particular, PBMCs were initially cultured with 1 μM peptide (instead of 5 μg/ml), and lymphocytes were restimulated weekly with peptide-pulsed autologous PBMCs beginning at day 7 (instead of day 11). IFN-γ secretion in response to peptide-loaded T2 cells, COS-7 cells expressing HLA-A$^{*}$0201$^+$ and TRP2, and HLA-A$^{*}$2$^+$ TRP2$^+$ melanoma cells were measured ~7 days after each stimulation beginning at 1 week.

Specific peptide recognition by bulk CTLs stimulated with TRP2(217–225) was apparent as early as week 3 from one patient (WO), and by week 5, T-cell cultures from three of the four patients (MC, WO, and LE) specifically released IFN-γ in response to peptide-loaded T2 cells. Similar to the initial TRP2 peptide screening, none of the bulk cultures stimulated with TRP2(217–225) recognized COS-7 transfectants or HLA-A$^{*}$2$^+$ melanoma cells (data not shown). TRP2(180–188) induced peptide-reactive T cells from two of four patients (MU and KU) after only two restimulations (week 3). More significantly, at week 3, bulk CTLs from patient KU specifically recognized HLA-A$^{*}$0201$^+$ and TRP2, and HLA-A$^{*}$2$^+$ TRP2$^+$ melanoma cells (data not shown).

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A*0201+ TRP2 melanoma cell lines, all T-cell cultures specifically lysed T2 cells preloaded with the FluM I peptide. The IFN-γ release from T2 cells preincubated with TRP2(I180-I188) or HLA-A2* TRP2+ targets was 250 pg/ml and at least twice background with either media or T2 cells preloaded with the FluM I peptide.

**DISCUSSION**

This investigation describes the identification of TRP2(180–188) (SVYDFFVWL) as a new HLA-A*0201-restricted T-cell epitope capable of inducing melanoma-reactive CTLs. TRP2 is a melanosomal enzyme expressed in most mammalian melanocytic cells and may represent an ideal target antigen for the immunotherapeutic treatment of HLA-A2*1* patients. This protein has been identified previously as a melanoma antigen recognized by tumor-reactive T cells in the context of HLA-A*0201. Therefore, the identification of TRP2(180–188) as a new HLA-A*0201-restricted T-cell epitope will enable the treatment of a much larger group of melanoma patients with TRP2-based immunotherapies.

Most class I MHC-restricted epitopes from melanoma antigens have been identified previously by screening synthetic peptides for recognition by antigen-specific, tumor-reactive CTLs from TILs or from lymphocytes stimulated in vitro with melanoma cells. However, the frequencies of these alleles among melanoma patients in the United States are low compared with that of HLA-A*0201, which is the most commonly expressed class I HLA allele in the Caucasian population of North America (~6% for HLA-A31 and ~2% for HLA-A33 compared with ~46% for HLA-A*0201; Ref. 15). Therefore, the identification of TRP2(180–188) as a new HLA-A*0201-restricted T-cell epitope will enable the treatment of a much larger group of melanoma patients with TRP2-based immunotherapies.

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numbers of CTLs with high avidity for peptide-MHC complexes. If this were the case, some T-cell clones derived from peptide-reactive cultures might exhibit melanoma reactivity. In support of this idea, a recent investigation demonstrated that melanoma-reactive CTLs could be induced in vitro with three nonimmunodominant HLA-A2-restricted peptides from the melanoma antigen gp100, but only under conditions approaching those used in limiting dilution experiments (20). Specifically, for each peptide, multiple CD8+ T-cell microcultures (~5 x 10^4 cells) were initiated and restimulated individually. With a given peptide, only a small fraction of microcultures (5–17%) recognized both peptide and tumor, suggesting low frequencies of T-cell precursors in PBLs capable of recognizing these peptides in the context of HLA-A2 on the surfaces of melanoma cells. Because the TRP2 peptide-specific T-cell cultures were unavailable for further study, PBLs from additional HLA-A*0201+ melanoma patients were stimulated in vitro with the two TRP2 peptides that most efficiently induced peptide-reactive CTLs in the initial screening: TRP2(180–188) and TRP2(217–225). In the second set of experiments, TRP2(180–188) stimulated bulk T-cell cultures from three of four patients that recognized peptide-pulsed T2 cells as well as COS-7 cells expressing HLA-A*0201 and TRP2 and HLA-A2+ TRP2+ melanoma cells.

The lack of specific melanoma recognition by bulk T-cell cultures stimulated with TRP2(180–188) in the initial peptide screening may have been due to a technical difference between that experiment and the latter. In the second set of experiments, PBLs were stimulated with peptides in vitro using a protocol that had been used successfully to generate melanoma-reactive CTLs with MART-1 and gp100 peptides (40, 41). This protocol differed from that used in the initial peptide screening in that GM-CSF and IL-4 were absent in the initial culture period, and the first restimulation was performed on day 7 as opposed to day 11. In addition, in the second set of CTL inductions, a lower concentration of peptide was used (1 μM versus 5 μg/ml). This may account for the induction of melanoma-reactive CTLs in the latter experiment but not the former because in a previous study, high avidity CTLs capable of clearing a viral infection in mice could only be generated in vitro using comparatively low concentrations of peptide (47). Another potential explanation was that a lower responder:stimulator ratio was generally used for restimulations in the preliminary TRP2 peptide screening than in the later CTL inductions (average, 1:7 versus 1:10) because of the large numbers of autologous PBMCs needed to restimulate cultures with 22 different peptides. In the second set of experiments, specific recognition of TRP2(180–188) preceded that of tumor by 1–3 weeks. Therefore, tumor reactivity may have become apparent in the initial screening with TRP2(180–188) if additional restimulations had been performed.

The approach of screening peptides for CTL induction has been used previously to identify epitopes from other potential tumor antigens such as MAGE3 (22, 23, 48), gp100 (20), p53 (49), HER2/neu (50), and MCI R (4), as well as from viral proteins like those derived from hepatitis C (51). Recently, many tumor or tissue-specific proteins have been identified using techniques including serological analysis of recombinant cDNA expression libraries (SEREX; Refs. 52 and 53), serial analysis of gene expression (SAGE; Ref. 54), differential display (55), and the use of cDNA subtraction libraries (56–58). Screening peptides for induction of tumor-reactive CTLs may be one of the best means for identifying epitopes from such candidate tumor antigens. In addition, if a known tumor antigen contains a few immunodominant epitopes like MART-1 [MART-K27-35] and gp100 [gp100(209–217), gp100(280–288), and gp100(154–162)], this approach may be the only means of identifying subdominant epitopes that may have therapeutic value. Furthermore, for breast, prostate, colon, and other prevalent cancers, the isolation and maintenance of tumor-reactive CTLs from TILs or from lymphocytes stimulated in vitro with tumor has been considerably more difficult than for melanoma. Consequently, screening synthetic peptides for recognition by
specific T-cell lines has been largely unsuccessful for identifying T-cell epitopes for these cancers. Screening peptides derived from tumor-specific proteins for CTL induction may enable the development of new immunotherapies for the treatment of patients with a variety of cancers other than melanoma.

TRP2(180–188) is unusual in that it is identical to a defined peptide previously from TRP2 recognized by murine melanoma-reactive CTLs. In the murine model published previously, several B16-reactive CTL lines recognized TRP2(181–188) (VYDFFVWL) in the context of H-2Kb, and a CTL line generated by in vitro stimulation of splenocytes with this peptide effectively treated established B16 lung micrometastases (12). However, in later studies, the 9-amino acid peptide generated by extending this 8-mer in the NH2 terminal direction [TRP2(180–188): SVYDFFVWL] was recognized more efficiently by B16-reactive T cells than the 8-amino acid peptide. Murine models for the prevention and treatment of B16 melanoma may greatly aid in the development and optimization of human melanoma vaccine strategies. Because TRP2(180–188) is recognized by melanoma-reactive CTLs in both the mouse and human and its amino acid sequence is identical between human and murine TRP2, this peptide may represent an ideal target in murine melanoma treatment models that may lead to more effective immunotherapies for human melanoma.

Peptides that are immunogenic in the context of multiple class I MHC molecules may be valuable for the development of vaccines for the treatment of patients diverse in HLA expression. In addition to TRP2(180–188), several peptides from viral and tumor antigens have been identified previously that act as T-cell epitopes across several class I MHC boundaries including P18100 (a peptide from the HIV-1 gp160 envelope protein; Refs. 59–62), several HBV peptides (63), TRP2(197–205) (Refs. 13 and 14), and a peptide from the MUC1 breast cancer antigen (64–66). One major factor influencing the presentation of a particular peptide in the context of a specific MHC molecule on the surface of a cell is the MHC binding affinity of the peptide. In the work presented here, TRP2(180–188) bound to HLA-A*0201 with high affinity, and in previous studies, this peptide was predicted to be within the top 25 presenting peptides on human melanoma cells. Cancer Res., 57: 4348–4355, 1997.


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