Mining the Melanosome for Tumor Vaccine Targets: P.polypeptide Is a Novel Tumor-associated Antigen

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Abstract

To identify novel, tumor-specific target antigens for vaccine development, we studied immune responses to P.polypeptide, an Mf_110,000 integral melanosomal membrane protein associated with the Prader-Willi syndrome. Together with expressed sequence tag (EST) and serial analyses of gene expression (SAGE) library analyses, reverse transcription-PCR and Northern blotting verified that P.polypeptide expression was limited to melanoma and melanocytes. A single dominant epitope corresponding to positions 427–435 (IMLCLIAAV) was identified using allele-limited transgenic mice. This epitope was then used to generate de novo human P.polypeptide-specific CD8+ T cells capable of recognizing P.polypeptide expressing human tumor cell lines in an HL-A-A*0201-restricted fashion. Thus, P.polypeptide may be valuable in the creation of novel therapeutic anticaner vaccines.

Introduction

Molecular cloning of the antigens targeted by cellular immune responses to malignant melanoma has revealed that the targets of the cellular immune response are often a group of MDAs that are associated with melanosomes (1, 2). These antigens, MART-1, gp100, tyrosinase, TRP-1 and TRP-2, are often used in the design of recombinant and synthetic cancer vaccines that have been explored in animal models and in the clinic for vaccine development (3–8). It is not known why these antigens serve as the focus of the tumor-specific T-cell response in so many patients with melanoma. One hypothesis is that MDA glycoproteins are immunogenic because of the structural and functional similarities of melanosomes and compartments for peptide loading of MHC class II molecules. MDAs are transported to the low pH melanosomal organelle by a dileucine-based melanosomal transport signal, and mutations or deletions to the melanosomal transport signal abrogate antigen presentation in vitro and T-cell responses in vivo (9). Evidence for the therapeutic potential of an immune response specific for melanosomal MDA comes from studies showing that melanoma patients can be immunized with these self-antigens and that objective tumor regression can be observed when immunization with modified synthetic peptide immunogens is combined with IL-2 (4). In addition, the development of vitiligo, a patchy and usually permanent loss of melanocytes from the skin, is a good prognostic sign when it follows the administration of high-dose IL-2 to patients with metastatic melanoma and is associated with successful tumor treatment in a mouse melanoma model (10). This report represents the first demonstration that the melanosome-associated P.polypeptide can be processed and presented on the surfaces of melanoma cells for recognition by human T cells in the available T-cell repertoire. Thus, P.polypeptide may be useful in the development of a therapeutic vaccine for malignant melanoma.

Materials and Methods

Animals, Peptides, and Cell Lines. A2Kb Tg transgenic mice expressing a chimeric MHC class I molecule composed of the α1 and α2 domains from the HLA-A*0201 class I antigen and the α3, cytoplasmic, and transmembrane domains from the murine H-2Kb class I were kindly provided by Linda Sherman (The Scripps Research Institute, La Jolla, CA; Ref. 11). mice were maintained and bred in a barrier facility (Biocen, Inc., Rockville, MD) and were between 6 and 10 weeks old for all of the experiments. A2Kb transgene expression was confirmed using step-wise PCR amplification of genomic DNA with primers (forward: AGTTCGTTGCGGTTCAGG; reverse: GCAGCCTACATACCTCTGGGA) as follows: cycle 1, 96°C for 60 s; cycles 2–6, 96°C for 25 s, 70°C for 45 s, 72°C for 30 s; cycles 7–22, 96°C for 25 s and 65°C for 45 s, and 72°C for 30 s; cycles 23–33, 96°C for 25 s, 58°C for 50 s, and 72°C for 30 s. and cycles 34–40, 96°C for 25 s, 55°C for 50 s, and 72°C for 90 s. Mice were backcrossed onto a C57BL/6 strain background using a marker-assisted Speed Congenic protocol and matched C57BL/6 on 78 of 80 microsatellite markers (Research Genetics, Huntsville, AL) after six selected backcrosses.

Peptides were synthesized using solid-phase N-(9-fluorenylmethoxycarbonyl) chemistry using a multiple peptide synthesizer (Model AMS 422; Gilson Co., Inc., Worthington, OH), and the molecular masses of peptides were verified by laser desorption mass spectrometry (Bio-Synthesis, Inc., Lewisville, TX). HLA serotypes and DNA genotypes of fresh human PBMC and tumor cell lines were determined by the NIH HLA Laboratory and were, for melanoma patient (TD), A*0201,68, B*07,44, C*0702,07, DRB1*0401,1101, DQ*0301,0302, DRB3*0202, DRB4*01; for tumor cell lines HLA-A* genotypes were T2 cells (0201), 397 Mel (01, 25), 526 Mel (0201, 03), 586 Mel (29, 31), 624 Mel (0201, 05), 697 Mel (0201, 11), 1088 Mel and EBV-B (01, 0201), 1102 Mel (0201, 24), 1300 Mel (0201, 24), and SK23 Mel (0201); and for fresh, unfultured, darkly pigmented tumors 1699 Fr Mel (0201), 1720 Fr Mel (0201, 2401), 1742 Fr Mel (0201, 2601), and 1752 Fr Mel (0201). The HLA-DRB1* genotypes of tumor cell lines used in the mAb blocking assay included 1088 EBV-B (0301, 0401) and 1102 Mel (0401, 1502). All of the mouse cell lines were maintained in culture medium as described (12). For human T-cell cultures, heat-inactivated AB serum (Biochemed, Winchester, VA) was substituted for fetal calf serum (FCS) and 50 Cetus units/ml of IL-2 was added to cultures of the human CD4+ T-cell line (Te-2; Ref. 12). Fresh human tumor samples for T-cell assays were prepared using adherent cells after overnight triple-enzyme digest of a surgically resected tumor mass.

Cloning of the Full-length P.polypeptide Gene. The full-length P.polypeptide gene was amplified from cdNA generated from 397 Mel cells by PCR using primers containing a forward-BamHI restriction site (CTGG-GATCTCCACATGCTTGAGGGCGAGAGA) and a reverse-Nor1 restriction site (CAAGCCCAGCGCTTCAAAACGTTGCGAGAG). PCR Amplification of the 2630-bp product (2515 bp of which is coding) using Platinum Pfx DNA Polymerase (Life Technologies, Inc., Rockville, MD) was performed as follows: 1 cycle, 94°C for 2 min; and 35 cycles, 94°C for 15 s, 55°C for 30 s, and 68°C for 3 min. The purified product was subcloned into a ZeroBlunt vector (Invitrogen, Carlsbad, CA) then transferred into an eukaryotic expression vector (VR1012) with a cytomegalovirus-based promoter (Vical Inc., San Francisco, CA) and introduced into 293 cells (Life Technologies, Inc., Rockville, MD) for transient expression. Following the selection of a stable cell line, the full-length P.polypeptide gene was characterized by DNA sequencing (13). The sequence included 50 s, and 72°C for 30 s; and cycles 34–40, 96°C for 25 s, 55°C for 50 s, and 72°C for 90 s. Mice were backcrossed onto a C57BL/6 strain background using a marker-assisted Speed Congenic protocol and matched C57BL/6 on 78 of 80 microsatellite markers (Research Genetics, Huntsville, AL) after six selected backcrosses.

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2 The abbreviations used are: MDA, melanocyte differentiation antigen; IL, interleukin; A2Kb, Tg, HLA-A*0201/Kb; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; EST, expressed sequence tag; SAGE, serial analysis of gene expression.
Depleted syngeneic splenocytes at a 10:1 ratio (5 × 10^5) Two A2K b transgenic mice were vaccinated three times at 2-week intervals. Total RNA was prepared with RNeasy (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instructions from multiple cell lines to evaluate P.polypeptide expression. RT-PCR amplification was performed using the Superscript One-Step RT-PCR System (Life Technologies, Inc.), with P.polypeptide-specific primers (forward: CAGGGAGGTGTCTGCTCTGT; reverse: CTGGGTCATTCGCAATACCA); and β-actin-specific primers (forward: ATCAAGATCTCCAGGACGC; reverse: TACTCTGCGCTCAGGAGGAGGAG). For Northern blot analysis, 10 μg of total RNA from 12 normal tissues, brain, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, stomach, thymus, uterus (Clontech, Palo Alto, CA), and neonatal melanocyte (line 9F1966; Clonetics, Walkersville, MD), were run overnight at 70V on a 1% agarose gel (containing 4-morpholinepropanesulfonic acid and formaldehyde), transferred to a positively charged nylon membrane with a downward-blotting apparatus, then cross-linked, incubated overnight at 65°C with salmon-sperm DNA and 32P-labeled full-length P.polypeptide and control β-actin probes for autoradiography.

Generation and Assessment of Mouse and Human CD8+ T-Cell Lines. Two A2K transgenic mice were vaccinated three times at 2-week intervals using the Accell gene gun (Powderject, Middleton, WI) with plasmid DNA encoding the full-length P.polypeptide gene. Plasmid DNA was precipitated onto 1.6-μm gold beads (2 μg of DNA/mg of gold) using CaCl2 in the presence of spermine. Mice received 5 μg of DNA divided between five nonoverlapping shots to the shaved abdomen at a hemolysis pressure of 400 psi (13). Two weeks after the final immunization, erythrocytes were ACK-lysed from pooled splenocytes populations and stimulated with 10 μm of candidate HLA-A*0201-restricted 9-residue peptides (6 × 10^6 cells/well in 24-well plates). Seven days later, individual cultures were tested in duplicate against peptide pulsed T2 cells, and cultures demonstrating specificity were restimulated (as above), and 10 days later each subline was screened for peptide and tumor specificity by coculturing in duplicate 10^5 T cells with 10^5 HLA-A*0201-restricted 9-residue peptides (6 × 10^6 cells/well in 24-well plates). Seven days later, individual cultures were tested in duplicate against peptide pulsed T2 cells, and cultures demonstrating specificity were restimulated (as above), and 10 days later each subline was screened for peptide and tumor specificity by coculturing in duplicate 10^5 T cells with 10^5 HLA-A*0201-restricted 9-residue peptides (6 × 10^6 cells/well in 24-well plates). Seven days later, individual cultures were tested in duplicate against peptide pulsed T2 cells, and cultures demonstrating specificity were restimulated (as above), and 10 days later each subline was screened for peptide and tumor specificity by coculturing in duplicate 10^5 T cells with 10^5 HLA-A*0201-restricted 9-residue peptides (6 × 10^6 cells/well in 24-well plates). Seven days later, individual cultures were tested in duplicate against peptide pulsed T2 cells, and cultures demonstrating specificity were restimulated (as above), and 10 days later each subline was screened for peptide and tumor specificity by coculturing in duplicate 10^5 T cells with 10^5 HLA-A*0201-restricted 9-residue peptides (6 × 10^6 cells/well in 24-well plates). Seven days later, individual cultures were tested in duplicate against peptide pulsed T2 cells, and cultures demonstrating specificity were restimulated (as above), and 10 days later each subline was screened for peptide and tumor specificity by coculturing in duplicate 10^5 T cells with 10^5 HLA-A*0201-restricted 9-residue peptides (6 × 10^6 cells/well in 24-well plates). Seven days later, individual cultures were tested in duplicate against peptide pulsed T2 cells, and cultures demonstrating specificity were restimulated (as above), and 10 days later each subline was screened for peptide and tumor specificity by coculturing in duplicate 10^5 T cells with 10^5 HLA-A*0201-restricted 9-residue peptides (6 × 10^6 cells/well in 24-well plates). Seven days later, individual cultures were tested in duplicate against peptide pulsed T2 cells, and cultures demonstrating specificity were restimulated (as above), and 10 days later each subline was screened for peptide and tumor specificity by coculturing in duplicate 10^5 T cells with 10^5 HLA-A*0201-restricted 9-residue peptides (6 × 10^6 cells/well in 24-well plates). Seven days later, individual cultures were tested in duplicate against peptide pulsed T2 cells, and cultures demonstrating specificity were restimulated (as above), and 10 days later each subline was screened for peptide and tumor specificity by coculturing in duplicate 10^5 T cells with 10^5 HLA-A*0201-restricted 9-residue peptides (6 × 10^6 cells/well in 24-well plates).

To generate human CD8+ T cells, fresh PBMC from patient TD were cultured at 2 × 10^6 cells/well in 48 wells in a flat-bottomed 96-well plate in 200 μl of culture medium containing 10 μM of P.polypeptide for 4 days, and plates were laid flat and restaged 14 days later for specific peptide reactivity against T2 cells pulsed with relevant or control (hgp100α2-m13) peptides. The most reactive wells were restimulated (as above), and 10 days later each subline was screened for peptide and tumor specificity by coculturing in duplicate 10^5 T cells with 10^5 antigen presenting cells (T2 or tumor cells)/well in U-bottomed 96-well plates for 24 h. Supernatants were assayed using cytokine ELISA kits from Endogen (Woburn, MA). mAb HB55 (IgG2a specific for HLA-DR molecules) and mAb W6/32 (IgG2a specific for HLA-A, B, and C molecules) were used in blocking experiments at concentrations of 20 μg/ml. Cytolysis experiments were performed and evaluated as described (12).

Results and Discussion

P.polypeptide mRNA Is Expressed in Multiple Melanoma Cell Lines and Is Melanocyte-specific. We sought to identify other melanosomal proteins for potential use in vaccine design. Identification of candidate antigens was facilitated because of the impact that mutation or deletion of an MDA can have on pigmentation. In addition, we used analysis of SAGE and EST databases to evaluate differential expression of known and potentially novel MDA that could potentially function as cancer vaccines. One candidate, designated P.polypeptide, is encoded on chromosome 15q11-q13 and appears to be an integral melanosomal membrane protein of 838 amino acids (M, 110,000) with 12 putative membrane-spanning regions. It shares an 83% similarity with the murine homologue, the pink-eyed dilution gene (14). Recent evidence has shown its involvement in the maintenance of intramelanosomal pH, and mutations associated with the locus include both Prader-Willi syndrome and ocular-cutaneous albinism II (15). P.polypeptide has been shown to affect the size, number, shape, and contents of melanosomes; and like MDA gp100, MART-1, tyrosinase, TRP-1, and TRP-2, it is crucial for normal pigmentation. Patients with defective P.polypeptide function have diffuse cutaneous and retinal hypopigmentation. Mice with mutations to the pink-eyed dilution gene exhibit changes that are phenotypically similar to their human counterparts (14).

To determine whether the melanomasomal bound P.polypeptide glycoprotein was expressed specifically by both melanoma cells and melanocytes, total RNA was prepared from nine established melanoma cell lines and four nonmelanoma cell lines. Using RT-PCR with P.polypeptide-specific primers, P.polypeptide mRNA was detected in all of the melanoma cell lines except 1102 Mel (Lane 7) and was not detected in control samples (SW-480, human colon carcinoma; MDA-231, breast carcinoma; 293 RCC, renal cell carcinoma; and 888F, fibroblast; Lanes 10–13). To determine whether P.polypeptide is expressed in other normal tissues, a Northern blot was performed using a full-length P.polypeptide probe (described above) with 13 normal tissue total RNA samples. As seen in Fig. 1B, hybridization was detected to the melanocyte mRNA (Lane 13; 3 Kb, including untranslated regions) but not to the other 12 normal tissue mRNA samples. All of the tissues were also positive for the β-actin probe. Our findings were consistent with reports published previously in which probes for P.polypeptide had been used to study specific gene expression in normal tissue samples.

Fig. 1. P.polypeptide expression is both melanoma- and melanocyte-specific. A. RT-PCR was performed with P.polypeptide and β-actin specific primers on nine melanoma cell lines (397, 526, 586, 624, 697, 1088, 1102, 1300 and SK23 Mel; Lanes 1–9), a breast carcinoma line (MDA-231; Lane 10), a colon carcinoma line (SW-480; Lane 11), a renal cell carcinoma line (293 RCC; Lane 12), and a fibroblast line (888F; Lane 13). Specific amplification of P.polypeptide was observed (701-bp product) for all melanoma cell lines except 1102 Mel cells (Lane 7) and all four nonmelanoma cell lines (Lanes 10–13). Control β-actin expression was observed for all samples. B. Northern blot demonstrates specific expression of P.polypeptide in melanocyte mRNA. Total RNA from 13 normal tissues (brain, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, stomach, thymus, uterus, and melanocyte; Lanes 1–13) was assayed with a full-length P.polypeptide 32P-labeled probe. Specific hybridization at 3 Kb was observed for melanocyte mRNA only (Lane 13). Both sets of experiments were performed twice with similar results. Control β-actin expression was observed for all samples.
1 Internet address: http://cgap.nci.nih.gov/Tissues/xProfiler.

Fig. 2. Identification of an HLA-A*0201-restricted epitope from P.polypeptide using A2K b transgenic mice. Mice were gene-gun immunized with DNA encoding P.polypeptide, and splenocytes were stimulated for 7 days in vitro with candidate peptides. Cultures were tested for specific peptide reactivity using T2 cells pulsed with a control peptide (gp100 209–217) or the specific peptide in question. T-cell cultures stimulated with 5 of the 10 peptides (ranked 1, 2, 4, 7, and 10) were found to induce specific reactivity and were restimulated with peptide pulsed syngeneic splenocytes. Eight days later, cultures were again tested against peptide pulsed T2 cells. Only one peptide (IMLCLAIAAV) was found to be specifically reactive and was used to stimulate human PBMC for additional epitope characterization. For both experiments, T cells and targets were cocultured in duplicate for 24 h, and supernatants were measured for IFN-γ secretion by ELISA. The initial screening assay was performed twice with similar results.

To explore the differential expression of P.polypeptide in greater detail we used the cDNA xProfiler tool for comparing two sets of EST libraries. In Set A, we included skin-based libraries (9 total libraries; 58,321 EST sequences), whereas in Set B we included libraries from all of the other tissues (352 total libraries; 2,874,415 EST sequences). Taken together, these data indicated that P.polypeptide was both a melanoma and melanocyte-specific protein.

Identification of an HLA-A*0201-restricted Epitope from P.polypeptide Using A2K b Transgenic Mice. Transgenic mice expressing chimeric human-murine MHC class I and II molecules have been used to identify a wide array of CD4+ and CD8+ T-cell epitopes, and A2-transgenic mice have been successfully used to study immune responses and tolerance to gp100, p53, and tyrosinase (16–18). To determine whether peptide fragments from P.polypeptide are presented in the context of HLA-A*0201 molecules, we used murine class I-restricted T-cell responses, and tolerance to peptide pulsed syngeneic splenocytes. Eight days later, cultures were again tested against peptide pulsed T2 cells. Only one peptide (IMLCLAIAAV) was found to be specifically reactive and was used to stimulate human PBMC for additional epitope characterization. For both experiments, T cells and targets were cocultured in duplicate for 24 h, and supernatants were measured for IFN-γ secretion by ELISA. The initial screening assay was performed twice with similar results.

P.polypeptide is a new tumor antigen
infiltrating T cells can be expanded that have specificity for each of these antigens. It may be important that we can find no evidence that tumor-infiltrating T cells can recognize P.polypeptide. Recent work has shown that T cells at the tumor site or draining lymph nodes may remain ignorant if antigen levels fail to reach an inducible threshold (20). Thus, P.polypeptide-specific T cells may be functionally ignorant in melanoma patients.

Given the wide but specific expression of P.polypeptide on melanocytic cells (based on RT-PCR and Northern blotting) and the results using different but comparable targets, thus demonstrating the generalizability of the finding.

A2-restricted nature of this epitope, this new MDA may be clinically useful in the development of therapeutic vaccines for patients with metastatic melanoma. P.polypeptide-specific CD8⁺ T cells were shown to recognize fresh melanoma samples (Fig. 3C), indicating sufficient expression in vivo and at the tumor site. Immunization of patients may activate otherwise ignorant T cells and enable them to recognize and lyse tumor cells. Recent work has shown that activation of T cells results in an increase in their apparent functional avidity enabling them to recognize antigen at doses that are >50-fold lower than seen in unactivated cells (21).

High levels of pre-existing T cells may not be necessary for a tumor-specific immune response, and an ignorant but inducible immune state of activation is likely to be more favorable for the antitumor response than an actively tolerized state. Because of the relative difficulty studying long-term tumor-bearing states in mice, these questions may be best addressed within the context of a therapeutic human cancer vaccine targeting P.polypeptide.

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Fig. 3. Human CD8⁺ T cells sensitized in vitro to P.polypeptide,427-435 recognize melanoma cells. A, CD8⁺ T-cell line TD P7/A2 3/3 are shown to recognize P.polypeptide,427-435 pulsed T2 cells at titrating (10⁻⁰–10⁻⁰.0001 μM) peptide concentrations but not gp100,209-217 (10 μM; empty diamond). B, T cells are then shown to recognize multiple HLA-A2⁺ P.polypeptide⁺ melanoma cell lines (562, 624, 697, 1088, 1300, and SK23 Mel) but not A2⁻ P.polypeptide⁺ melanoma cell line 1102 Mel nor A2⁻ P.polypeptide⁺ melanoma cell lines 397 and 586 Mel. Specific reactivity is seen against P.polypeptide,427-435 but not against control peptide HA,209-217 (both pulsed onto T2 cells at 10 μM). C, T cells are shown to recognize SK23 Mel cells and multiple darkly pigment A2⁺ fresh melanoma tumor samples (697, 1742, and 1752 Fr Mel) but not the A2⁻ darkly pigmented melanoma tumor sample 1720 Fr Mel nor the A2⁻ P.polypeptide⁺ 1088 EBV-B cell line. For all experiments, T cells and targets were cocultured in duplicate for 24 h, and supernatants were measured for IFN-γ secretion by ELISA. All experiments were performed three separate times with similar results using different but comparable targets, thus demonstrating the generalizability of the findings.

Fig. 4. P.polypeptide,427-435-specific CD8⁺ T cells recognize human melanoma cells in an MHC-class I-restricted fashion and are capable of lysing a human melanoma cell line. T cells were cocultured with targets in the presence of anticlass I (W6/32) and anti-HLA DR (HB55) mAbs. A, recognition of A2⁺ P.polypeptide⁺ melanoma cell lines (1300 and SK23 Mel) by TD P7/A2 3/3 was blocked by W6/32 but unaffected by HB55. Again no recognition was observed against the A2⁻ P.polypeptide⁺ 1088 EBV-B cell line. Recognition of DR4/gp100⁺ melanoma cell line 1102 Mel cells by the control gp100,209-217-specific, HLA-DRB1*0401-restricted, human CD4⁺ T-cell line (Te-22) was blocked by HB55 but not W6/32. As above, no recognition of DR4⁺ gp100⁺ 1088 EBV-B cells was observed. For both assays, T cells and targets were cocultured in duplicate for 24 h in the presence or absence of 20 μg/ml of mAb, and supernatants were measured for IFN-γ and granulocyte macrophage colony-stimulating factor secretion by ELISA. B, line TD P7/A2 3/3 specifically lyse A2⁺ human melanoma cell line SK23 Mel but not a control melanoma cell line, 397 Mel, in a 12 h ⁵¹Cr release assay. Experiments were performed twice with similar results using different but comparable targets, thus demonstrating the generalizability of the findings.

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