# Immunogenicity without Immunoselection: A Mutant but Functional Antioxidant Enzyme Retained in a Human Metastatic Melanoma and Targeted by CD8$^+$ T Cells with a Memory Phenotype

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## Abstract

**Human melanomas can express unique tumor antigens, resulting from mutated proteins, and shared epitopes encoded for by normal genes, but these two classes of antigens have not been previously compared for immunogenicity and retention in metastatic cells. Here, we identified a new unique antigen generated by a point mutation in the peroxiredoxin 5 (*Prdx5*) gene in an HLA-A*0201$^+$ human metastatic melanoma lacking the wild-type allele. An antioxidant assay, with recombinant Prdx5 proteins, and evaluation of peroxide accumulation in transiently transfected cells, indicated that the mutant protein retained its enzymatic activity. The mutation in the Prdx5 protein did not generate a new HLA agretope but yielded an HLA-A*0201–restricted T cell epitope ($Prdx5_{110\text{-}119}$). By HLA-tetramer analysis, in a tumor-invaded lymph node, >50% of mutant Prdx5-specific CD8$^+$ T cells (frequency 0.37%/CD8$^+$) showed a CCR7$^{+/-}$ CD45RA$^-$ "$T_{CM}$" or "$T_{EM}$" phenotype, as found in Melan-A/MART-1–specific T cells (frequency 0.68%/CD8$^+$) in the same tissue. In agreement with their memory phenotype, the Prdx5-specific T cells readily expanded *in vitro* in mixed lymphocyte-tumor culture, as did the Melan-/MART-1–specific T cells. By immunohistochemistry of the invaded lymph node, the mutant Prdx5 protein was expressed in all neoplastic cells, in contrast with the heterogeneous expression of shared antigens as Melan-A/MART-1, gp100 and tyrosinase. Thus, a unique tumor antigen can be as immunogenic as the melanoma differentiation antigens but, in contrast to the latter, may be retained in all metastatic cells possibly as result of the relevant cellular function exerted by the mutated protein.** (Cancer Res 2005; 65(2): 632-40)

## Introduction

A number of tumor antigens recognized by CD4$^+$ and CD8$^+$ T cells and resulting from mutated proteins have been identified in murine and human tumors (1, 2). These mutations, when present only in the tumor in which they have been first identified, are truly tumor-specific and may yield unique tumor antigens (1, 2), which provide evidence for immune surveillance of genome integrity. Although unique tumor antigens may be difficult to exploit in therapeutic settings, nevertheless they represent attractive immunologic targets for studying the dynamics of the host-tumor interaction. On one hand, identification of a number of unique tumor antigens in long-term survivors (3–8), has suggested that this class of antigens is immunogenic and relevant to tumor rejection. On the other hand, some of the mutant proteins that generate unique tumor antigens can be critical for tumor cell survival, due to their involvement in basic metabolic pathways, or in regulation of cell cycle and apoptosis (3–12). In these instances, immunogenicity and function of the mutant protein will likely act as opposing selective forces for the maintenance of expression of the antigen along tumor progression. In fact, immunogenicity may promote the T cell–mediated response leading to emergence of antigen loss variants, where the mutant protein is no longer expressed. However, neoplastic cells that will survive in the host, during tumor progression, will attempt to maintain expression of the mutant protein, due to its relevant cellular function. Experimental evidence in murine models has indicated that the balance between immunogenicity and cellular function of mutant proteins may be tilted in favor of the latter attribute, thus preventing the emergence of antigen loss variants (13, 14).

The identification of new unique tumor antigens, expressed in a human tumor, may thus offer the opportunity to evaluate the function of the mutant protein, its immunogenicity compared with other shared antigens expressed by the same tumor, and to assess its maintenance or loss of expression in metastatic cells.

Here, we identify a new unique tumor antigen expressed in a human metastatic melanoma. The mutation that led to a missense amino acid substitution, occurred in the most recently identified member of the peroxiredoxin (*Prdx*) gene family named peroxiedoxin 5 (*Prdx5*; refs. 15–17). Prdxs are ubiquitous enzymes that control intracellular $H_2O_2$ levels by catalyzing its reduction into water (18, 19). Despite their biochemical similarities, Prdx proteins seem not to be functionally redundant (18, 19), since loss of expression of distinct Prdx family members has been shown to affect normal physiology and initiation/progression of pathologic conditions in a cell type–dependent fashion (18, 19). The relevance of these enzymes for normal cellular physiology is not only linked to their protective activity against oxidative stress, but it is even revealed by their role as modulators of $H_2O_2$-dependent signaling pathways activated by cytokine and growth factor receptors (18, 19).

Here we show that the missense substitution in one allele of *Prdx5*, occurring in a tumor lacking the wild-type (wt) allele, did not impair the antioxidant function of the mutant protein and yielded a 10-mer HLA-A*0201–restricted unique T-cell epitope

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targeted by $CD8^+$ T cells. Analysis of frequency, maturation stage and potential for *in vitro* expansion of Prdx5-specific T cells at tumor site, in comparison with T cells directed to shared melanosomal antigens, provided evidence for immunogenicity of the novel unique antigen. Moreover, expression of the mutant protein, along with that of the HLA-A2–restricting element, was maintained in all metastatic cells, in contrast to normal antigens of the melanocyte lineage.

## Materials and Methods

**Patients and Cell Lines.** The clinical course of patient 8959 has been already reported (20). Briefly, this patient (HLA typing: A*0201, A*1101/02, BW55, CW3, DRw11, DQw7) developed a lymph node (LN) metastasis (Me8959) 14 months after surgical removal of the primary tumor, and 1 month later, died from brain metastases. All primary and metastatic melanomas used in this study were established in culture in our laboratory from specimens of patients admitted for surgery to our Institute. Culture medium for melanoma, transformed embryonal kidney 293 (Invitrogen, Carlsbad, CA), and 174.CEMT2 (T2, American Type Culture Collection, Rockville, MD) human lines was RPMI 1640 (Cambrex Bio Science, Walkersville, MD), whereas for the mouse fibrosarcoma WEHI-164 clone 13 and the transformed African green monkey kidney COS-7 cells (kindly provided by Prof. T. Boon, Ludwig Institute for Cancer Research, Brussels, Belgium) was DMEM (Cambrex Bio Science). Culture media were supplemented with 10% FCS, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine, and 50 μg/mL penicillin/streptomycin. Normal human epidermal melanocyte lines (PromoCell, Heidelberg, Germany) were kept in melanocyte growth medium M2 (PromoCell). Isolation of lymphocytes from PBMC and LN, establishment of mixed lymphocyte-tumor cultures (MLTC), maintenance of PBMC-derived CTL clones, all $CD3^+$ $CD4^-$ $CD8^+$, T-cell receptor (TCR) $\alpha\beta^+$, was done as described (21).

**cDNA Library Construction and Screening.** A cDNA library was constructed as described (22) in the mammalian expression vector pcDNA3.1 (Invitrogen) starting from Me8959 polyadenylated RNA. Recombinant plasmids were electroporated into DH5-α *Escherichia coli*. DNA was extracted from pooled bacteria containing 100 cDNA clones in average and cotransfected using the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) with pcDNA3/HLA-A*0201 (22) into COS-7 cells grown at 80% confluence into 96 flat-bottomed well plates. CTL 181 was added 30 hours later and, after additional 24 hours, tumor necrosis factor-α released in the supernatant was measured on the sensitive WEHI-164 clone 13 cells with a colorimetric assay as described (22). The entire procedure was repeated to isolate individual plasmids encoding the antigen recognized by CTL clones. The cDNA insert was automatically sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA) with T7 forward and pcDNA3.1BGH reverse sequencing primers (Invitrogen). Computer search for sequence homology was done with FASTA EMBL-Heidelberg.

**Constructs and Peptides.** Full-length coding sequences and truncated variants including translated regions corresponding to amino acids 159-214 ($Prdx5^{159\text{-}214}$) or 183-214 ($Prdx5^{183\text{-}214}$) were amplified by 30-cycle stepdown reverse transcription-PCR (22) from cDNA obtained from Me8959 and PBMC. The forward primers that contained an ATG start codon with an appropriate Kozak consensus sequence (italicized) were:

F-1100: 5′-*GCCACCATG*GGACTAGCTGGCG (*Prdx5*)
F-1098: 5′-*GCCACCATG*GTGTCCATCTTTGGGAATCG ($Prdx5^{159\text{-}214}$)
F-1103: 5′-*GCCACCATG*AAGGAGACAGAC ($Prdx5^{183\text{-}214}$)

The reverse primers which contained the stop codon (italicized) was R-1102: 5′-GCC*TCA*GAGCTGTGAGATGAT. Amplification products were subcloned into pcDNA3.1/V5/His plasmid using the Eukaryotic TOPO TA cloning Kit (Invitrogen). Expression vectors were sequenced and transiently transfected with or without pcDNA3/HLA-*A*0201* (22) into COS-7 or 293 cells for functional or antioxidant assays. As a control, pcDNA3.1/*-gal* encoding -galactosidase was used (Invitrogen). Candidate HLA-A*0201–binding peptides were purchased from Primm (Milan, Italy). $^{51}Cr$-labeled target cells were preincubated at room temperature for 1 hour with various or fixed concentrations of peptides. CTL clones were than added and tested for cytotoxicity by standard $^{51}Cr$-release assay (21). Means and SD (that never exceeded 10%) were calculated from triplicates within each experiment and each experiment was repeated at least twice.

**Genomic DNA.** Genomic DNA, extracted according to standard protocols, was amplified by a 30-cycle stepdown PCR using primers F1127: 5′-TGAGCTTCCTAGTGGCCAAG and R1126: 5′-TCTCCCCACA-GACGACTCTC. A specific band of 720 bp was gel-purified and directly sequenced using the forward primer F-1125: 5′-CAGGGAGTCAGGAC-CAGGTA.

**Bacterial Expression and Purification of Prdx5.** *Prdx5,* without the 52 amino acid–long mitochondrial targeting sequence cleaved in the mature protein, was amplified by stepdown PCR, from plasmids containing the wt or mutant sequences using the primers described in ref. 15: 5′-CTGCA*CATATG*GCCCCAATCAAGGTG-3′ (*Nde*I site *italicized*) and 3′-GTTATAGTAGAGTGTCGAGACGC*CTTCTCG*CGGTCT-5′ (*Sap*I site *italicized*). The PCR products were digested with *Nde*I and *Sap*I and ligated into vector pTYB1 (IMPACT System, New England BioLabs, Beverly, MA). Plasmid inserts were sequenced using primers provided by the kit. The resulting constructs pTYB1-$Prdx5^{wt}$ (wt) or pTYB1-$Prdx5^{Leu}$ (mutant) were then used to transform *E. coli* ER2566 strain. Recombinant proteins were expressed after addition of 0.5 mM isopropyl -D-thiogalactopyranoside and purified onto chitin beads columns provided in the IMPACT System according to the manufacturer's instructions.

**Western Blotting.** Recombinant Prdx5 proteins and cellular proteins, obtained by lysing cells in ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 1 mmol/L EDTA, 1 mmol/L EGTA, 1mmol/L $NaVO_3$, and 1mmol/L NaF] containing protease inhibitor cocktail (Roche Applied Science) were quantified by the Bio-Rad Assay (Bio-Rad Laboratories, Hercules, CA). To achieve higher accuracy, recombinant Prdx5 proteins and escalating known amounts of a recombinant Prdx1 (Sigma-Aldrich, St Louis, MO) as reference standard, were stained, after SDS-PAGE with SYPRO Red (Amersham Biosciences, Buckinghamshire, United Kingdom). Fluorescent signal intensities were scanned by Typhoon 8600 Imaging System and were quantified by ImageQuant (both from Amersham Biosciences). Data were exported to Microsoft Excel to generate a standard Prdx1 curve and, by regression analysis, the protein concentration of both Prdx5 isoforms was calculated. Cellular proteins (15 μg) and recombinant $Prdx5^{Leu}$, $Prdx5^{wt}$ and thioredoxin (Calbiochem, Darmstadt, Germany), each at 10 ng, were electrophoresed and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). Immunoblotting was carried out with anti-Prdx5 (clone 44, 1:5,000, BD Biosciences, San Diego, CA) and anti-thioredoxin (1:4,000, Serotec, Oxford, United Kingdom) monoclonal antibody (mAb) or, on stripped membranes, with polyclonal rabbit anti-actin (Sigma-Aldrich, 1:2,000). Bound antibodies were visualized using SuperSignal West Dura Extended Duration Substrate enhanced chemiluminescence (Pierce, Rockford, IL) followed by autoradiography.

**Antioxidant Assay.** The ability of recombinant Prdx proteins to protect glutamine synthetase (GS, Sigma-Aldrich) from DTT/$Fe^{3+}$/$O_2$–mediated oxidative inactivation was monitored as described (23). The assay was conducted by adding Prdx5 proteins (1.5 μg) into a 100 μl inactivation mixture [10 mmol/L DTT, 3 μmol/L $FeCl_3$, and 50 mmol/L HEPES/HCl (pH 7)] containing 2.5 μg of GS. After incubation at 37°C for different time, 25 μL of the solutions were added to a γ-glutamyltransferase assay mixture [0.4 mmol/L ADP, 150 mmol/L glutamine, 10 mmol/L K-$ASO_4$, 20 mmol/L $NH_2OH$, 0.4 mmol/L $MnCl_2$, and 50 mmol/L HEPES/HCl (pH 7)], and incubated at 37°C for 15 minutes before the addition of 1 mL of stop mixture (55 g of $FeCl_3 \cdot 6H_2O$, 20 g of trichloroacetic acid, and 21 mL of concentrated HCl/liter). The absorbance resulting from the γ-glutamylhydroxamate-$Fe^{3+}$ complex was read at 540 nm.

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**$H_2O_2$ Levels.** Cellular $H_2O_2$ levels were determined using 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-$H_2$-DCFDA, Molecular Probes, Eugene, OR). This cell-permeable compound becomes fluorescent and remains trapped inside the cell upon oxidation (24). COS-7 and 293 cells were transfected with the appropriate expression vectors and incubated for 24 hours. The medium was then removed and replaced with HBSS without phenol red containing 5 μmol/L of CM-$H_2$-DCFDA. After incubation in the dark at room temperature for 15 minutes, cells were trypsinized, washed, and resuspended in medium containing a 100 μmol/L *tert*-butylhydroperoxide solution (tBHP, Sigma-Aldrich). After 5 minutes, the cells were washed thrice and their fluorescence immediately measured by a dual laser FACScalibur (BD Biosciences) using the CellQuest software.

**HLA Stabilization Assay.** T2 cells were incubated overnight at 37°C with different dilutions of each peptide in serum-free RPMI 1640 supplemented with 1 mg/mL of β2 microglobulin (Sigma-Aldrich). Cells were then washed to remove free peptides and stained with mAb CR11-351 followed by FITC-conjugated goat anti-mouse immunoglobulin G. Values are indicated as mean fluorescence intensities (MFI) of peptide-induced HLA-A*0201 expression after subtraction of the MFI of cells similarly treated but without added peptide.

**TCR Usage.** Nomenclature for *TCR* gene segments is according to the recommendations of the WHO-IUS Subcommittee on TCR Designation (25). cDNA obtained from CTL clones was amplified by stepdown PCR using panels of TCRAV- or BV-specific forward primers and one reverse AC or BC primer (26). Amplified fragments were gel purified. Their complementarity-determining region 3 (CDR3) was directly sequenced as described (26).

**Tetramer Staining and Flow Cytometry.** HLA-A*0201 PE-labeled tetramers containing peptides from Melan-A/MART-$1_{26-35}$ (modified sequence carrying Ala at position 2), $gp100_{154-162}$, $gp100_{209-217}$, $tyrosinase_{368-377}$, and $Prdx5_{115leu}$ were purchased from ProImmune Ltd. (Oxford, United Kingdom) and used as described (27). All other reagents, unless otherwise specified, were purchased from BD Biosciences. Briefly, T cells were stained for 15 minutes with tetramers (1:200 of the stock solution) at 37°C and then stained for 30 minutes on ice with allophycocyanin coupled anti-CD8 mAb. To detect CCR7, staining was done with IgM anti-CCR7, followed by biotin-conjugated rat anti-mouse IgM and then by Cy-Chrome–conjugated streptavidin. After this incubation period, the cells were washed twice and stained for 20 minutes in ice with CD8-APC and CD45RA-FITC and immediately analyzed (27). To detect TCRBV expression, T cells were incubated for 30 minutes in ice with mAbs directed to BV8S1-S2 (Serotec, Raleigh, NC), washed once, stained with FITC-conjugated goat anti mouse secondary immunoglobulin G (1:30, Jackson ImmunoResearch, West Grove, PA) for 30 minutes in ice, incubated with PE-labeled tetramers in ice, washed twice and finally fixed in PBS with 1% formalin. Intracellular staining of melanoma cells was done on fixed (ethanol and acetone v/v) and permeabilized (HBSS plus 0.1% Triton X-100) cells with anti-Prdx5 (1:200) or anti-vimentin (VIM 3B4, 1:50, Chemicon, Hampshire, United Kingdom) mAbs followed by the FITC-conjugated secondary Ab. All data were acquired by a dual laser FACScalibur (BD Biosciences) using the CellQuest software.

**Immunohistochemical Analysis.** Immunohistochemical analysis of tissue sections and cytospins was done as described (20, 28). Primary mAbs were anti-Melan-A/MART-1 (A103, 1:50, Novocastra, Newcastle upon Tyne, United Kingdom), anti-gp100 (HMB45, 1:25, Dako, Copenhagen, Denmark), anti-tyrosinase (T311, 1:50, NeoMarkers, Fremont, CA) and anti-Prdx5 (1:200). After primary antibodies incubation overnight at 4° followed by biotinylated goat anti-mouse immunoglobulin G (Dako, 1:100) addition, the slides were covered with streptavidin-horseradish peroxidase (1:300; Dako) and visualized with the use of Sigma Fast 3,3′-diaminobenzidine tablet set (Sigma-Aldrich).

## Results

**A Mutant Antioxidant Enzyme, Expressed in a Melanoma Lacking the wt Allele, Is Recognized by Different Autologous $CD8^+$ HLA-A2–Restricted CTL Clones.** Among CTL clones derived from PBMC of a patient with metastatic melanoma (patient 8959), several recognized HLA-A*0201–restricted shared melanoma-associated antigens (29). Others, like CTL 22, 121, and 181 lysed in HLA-A*0201–restricted fashion the autologous Me8959 cells even if tested after a few *in vitro* passages, but neither of 10 HLA-A*0201–matched primary or metastatic melanoma lines, nor normal HLA-$A*0201^+$ melanocytes (data not shown). TCR repertoire analysis followed by sequencing of the CDR3 indicated that the three CTL clones, although all of independent origin, shared usage of the same variable (BV8) and constant gene (*BC2*) segments in their β chain (data not shown). CTL 22 and 121, but not 181 that expressed AV8 and was not sequenced, also displayed an identical AV region (AV3), identical J regions in both the α and β chains (BJ2S3 and AJ55) and quite similar CDR3 rearrangements (data not shown). Screening of a cDNA library, constructed with Me8959 poly(A)+ mRNA allowed the identification of an 800-bp-long cDNA clone (cDNA 78) able to transfer the expression of an antigen recognized by all three CTL clones, when cotransfected with pcDNA3.1/*HLA-A*0201* into COS-7 cells (data not shown). cDNA 78 contained a poly(A) tail, a polyadenylation signal and its sequence matched the coding sequence of *Prdx5* (Genbank/EMBL accession no. AF110731; ref. 15) except for one C-T nucleotide change located in exon 5. This change resulted in a Ser to Leu substitution at position 115 of the translated polypeptide (Fig. 1*A*; numbering according to Declercq et al. 17). We next compared the full-length *Prdx5* coding sequences derived from cDNA of Me8959 and autologous PBMC. In all sequences analyzed, 10 from PBMC and 10 from Me8959, PBMC expressed always the wt TCG codon, whereas the tumor only harbored the TTG codon (data not shown). Thus, a mutation took place in the neoplastic cells that apparently did not harbor, or did not express the wt *Prdx5* allele. To dissect the latter mechanism, a genomic 720-bp fragment, containing exon 5 sequences of *Prdx5* was amplified from a frozen suspension of fresh Me8959 cells and from autologous PBMC and sequenced. The electropherograms shown in Fig. 1*B* indicated that only *Prdx5* allele present in Me8959 was the mutant isoform, whereas as expected, DNA derived from PBMC displayed the wt sequence. The crystallographic structure of Prdx5 has been recently published and file coordinates deposited at the RCSB Protein Data Bank under accession no. 1hd2 (17). Figure 1*C* displays a closer view of the Prdx5 structure around the wt Ser115 residue. This residue lies close to a stretch of hydrophobic amino acids whose side chains come in proximity of the aromatic ring of a benzoate molecule. In the Prdx5 molecule expressed by Me8959 cells, the presence of Leu115 instead of Ser115, could confer further hydrophobicity to this region. Additional HLA-A*0201 melanoma lines expressing Prdx5 were analyzed for the presence of Leu115 but none of them was recognized by CTL clone 181 in a $^{51}Cr$ release assay and all displayed the wt sequence (data not shown). An anti-Prdx5 mAb recognizing both mutant and wt isoforms was then used to estimate, by flow cytometry, the Prdx5 levels in this panel of melanomas, in Me8959 and in normal cultured melanocytes. Figure 1*D* shows that Me8959 has constitutive expression, in all cells, of mutant Prdx5, the only isoform present. All additional lines were also positive for Prdx5 expression (Fig. 1*E*, ○). Prdx5 levels in melanomas, as assessed by MFI values (Fig. 1*E*, □), were heterogeneous but in general lower than that displayed by normal melanocytes. MFI value of Me8959 (Fig. 1*E*, ■), did not differ from that displayed by a subset of melanomas with wt Prdx5.

**Mutant Prdx5 Retains Antioxidant Activity.** To directly evaluate the function of mutant ($Prdx5^{Leu}$) versus wt ($Prdx5^{wt}$) protein, they were expressed in *E. coli*, purified, and quantified

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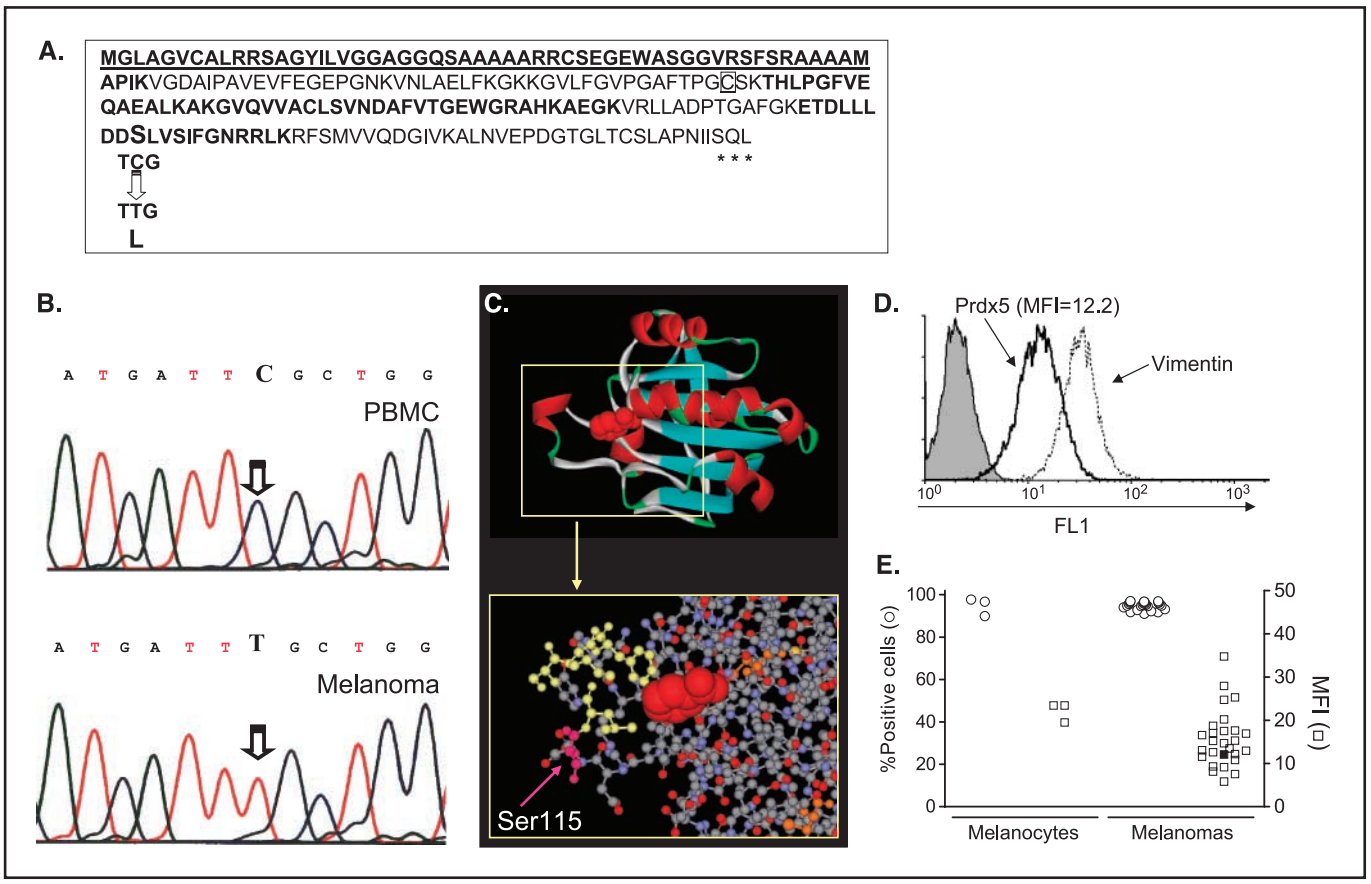


**Figure 1.** Identification of mutant Prdx5 as a melanoma antigen. *A,* amino acid sequence of cDNA 78 is identical to that corresponding to *Prdx5* (Genbank/EMBL accession no. AF110731) except for a Ser (*TCG*) to Leu (*TTG*) substitution (*arrow*). Alternate bold and regular fonts mark the six exons. The catalytic Cys residue, conserved in all Prdx, is boxed. Features of Prdx5 are the mitochondrial (*underlined*) and peroxisomal (*) targeting sequences. *B,* electropherograms of a partial exon 5 sequence of *Prdx5* gene amplified from genomic DNA of fresh Me8959 and of autologous PBMC. The nucleotide that differs among the two sequences is evidenced. *C,* ribbon modeling of Prdx5, built with DS Viewer Pro from the crystallographic file of Prdx5 as deposited at the RCSP Protein Data Bank (accession no. 1HD2; ref. 17). *Inset,* wt Ser115 residue (*pink*, modified in Leu115 in the mutant protein) and adjacent hydrophobic residues (*yellow*). Cys (*orange*) involved in intramolecular disulfide intermediate formation following oxidation; benzoate molecule (*red*). *D,* intracellular flow cytometry of mutant Prdx5 in Me8959. Cells stained by FITC goat anti-mouse or anti-vimentin mAb are, respectively, the negative (*grey histogram*) and positive control (*dotted histogram*). *E,* intracellular levels of Prdx5 in melanoma and melanocytes. For each cell line, analyzed by flow cytometry, data are plotted on two different *Y* axes as either % positive cells (○, *left y axis*) and as mean fluorescence intensity (□, *right y axis*). ■, MFI of Prdx5 expressed in Me8959.

to identical concentration. By Western blot analysis, anti-Prdx5 mAb recognized both purified recombinant Prdx5 proteins, but not a control 15-kDa thioredoxin protein, whereas an anti-thioredoxin mAb recognized thioredoxin, but neither form of Prdx5 (Fig. 2*A*). These results established the identity of both recombinant Prdx5 that appeared as a single band of an estimated size of 17 kDa (data not shown) and excluded that the mutant residue could interfere with antibody recognition. $Prdx5^{Leu}$ and $Prdx5^{wt}$ were then compared for their ability to prevent GS inactivation induced by a low concentration of $H_2O_2$ produced by a cell-free oxidation system, a method used to evaluate antioxidant activity of the Prdx family members (23). To this end, GS was incubated for different times in the inactivation mixture with $Prdx5^{Leu}$, $Pdx5^{wt}$ (1.5 μg/each), or with EDTA (1 mmol/L) as control for oxidative inactivation prevention (23). As shown in Fig. 2*B*, both Prdx5 exerted a protective effect since, after 20 minutes, 63% and 79% of GS activity could be recovered in presence of $Prdx5^{wt}$ and $Prdx5^{Leu}$, respectively. To provide further support for the retained function of the mutant Prdx5 protein, we transiently transfected COS-7 or 293 cells with expression vectors encoding either $Prdx5^{Leu}$ or $Prdx5^{wt}$ or a -gal protein as negative control. Compared to endogenous levels, Prdx5 was over expressed in *Prdx5* transfected cells as verified by Western blot and fluorescence-activated cell sorting analysis (Fig. 3*A* and *B*). Transfected cells were allowed to incorporate CM-$H_2$-DCFDA indicator dye (24) and exposed to a peroxide (100 mol/L tBHP) for 5 minutes. This treatment resulted in a rapid increase of CM-$H_2$-DCFDA fluorescence in β-gal-expressing COS-7 (MFI = 323) and 293 cells (MFI = 328), reflecting the increased oxidative status of the cells promoted by tBHP (Fig. 3*C*). Such CM-$H_2$-DCFDA fluorescence increase was markedly reduced by expression not only of the wt but even of the mutant Prdx5 protein (compare MFI values in β-*gal*– versus *Prdx5*-transfected cells in Fig. 3*C*). Interestingly, in both cell types, expression of $Prdx5^{Leu}$ resulted in an increased protection from oxidative stress, compared with $Prdx5^{wt}$ (compare MFI values in wt and mutant *Prdx5* transfected cells in Fig. 3*C*). Since transfection was not associated with higher levels of mutant proteins (as shown in Fig. 3*A* and *B*), these results suggest that the mutant protein may be even more active than the wt one in its antioxidant activity.

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**Identification of the Antigenic Peptide.** To evaluate whether the mutation in *Prdx5* was relevant to the generation of the CTL epitope, COS-7 cells were transfected with expression vectors containing full-length or fragmented *Prdx5* cDNA amplified from either Me8959 (*Prdx5*$^{Leu}$) or PBMC (*Prdx5*$^{wt}$) along with pcDNA3/HLA-*A*0201* and tested for recognition by CTL 181. Only constructs containing the mutant sequence retained the ability to stimulate tumor necrosis factor-α release by CTL 181, and the immunogenic peptide mapped in a 24-amino-acid sequence that included the mutant Leu residue (Fig. 4*A*). Three potential HLA-A*0201–binding nonamers or decamers ligands were identified in this region using available algorithms (30). The wt and the mutant peptides including positions 109 to 117, 110 to 119, and 111 to 119 of Prdx5 were then pulsed, at 1 μmol/L on an HLA-A*0201–positive melanoma that does not express the mutant Prdx5 (Me18732). The decamer peptide LLLDD*L*LVSI$_{110\text{-}119}$ (thereafter named Prdx5$_{115L}$), with the sixth residue being Leu115, was recognized in both tumor necrosis factor-α release and cytotoxicity assays, whereas all other peptides were not (data not shown). Dose-response curves, similar for all CTL clones recognizing mutant Prdx5, indicated that the mutant decamer was recognized at concentration lower than 100 pmol/L, whereas no reactivity was observed against the normal peptide (Fig. 4*B*, for the pattern of lysis of CTL 181) even at concentrations up to 10 μmol/L (data not shown). The mutant as well as the corresponding wt peptides displayed similar profiles in the HLA-A2 stabilization assay, indicating, in accordance with their predicted identical binding scores, that they could bind with similar affinities to this allele (Fig. 4*C*). Taken together these results indicate that the Ser to Leu mutation in Prdx5 of Me8959 generates a new T-cell epitope and not a new agretope.

**Analysis of T-Cell Response to the Novel Unique and to Shared Melanoma Antigens in the Same Patient.** T-cell frequency to differentiation antigens of the melanocyte lineage and to the unique Prdx5$^{Leu}$ antigen were compared in lymphocytes from a tumor-invaded LN (TILN) of patient 8959 by HLA-A*0201 tetramer analysis. Soluble HLA-A*0201 tetramers containing the Prdx5$_{115L}$ peptide stained specifically CTL clone 181 (Fig. 5*A*). CD8$^+$ T cells from PBMC of HLA-A*0201$^+$ healthy donors or melanoma patients contained <0.1% of T cells staining with the Prdx5-specific tetramer (Fig. 5*B*: *lanes 1* and *2*). In fresh, uncultured TILN of patient 8959, 0.37% of CD8$^+$ T cells were specific for Prdx5$_{115L}$ (Fig. 5: *B*, *lane 3*, ●; *C*, *bottom left*; and *D*, *lane 1*, ●) in comparison to <0.1% in TILN from two additional HLA-A*0201 melanoma patients (Fig. 5*B*, *lane 3*, ○); 57.8% of Prdx5$_{115L}$-specific T cells also expressed the BV8 region shared by anti-Prdx5$_{115L}$ CTL clones isolated from PBMC (data not shown). These data indicate that both local and a systemic immunity developed in patient 8959 to this HLA-A*0201–restricted epitope and that this response was dominated by the expansion of Prdx5$_{115L}$-specific T-cell populations expressing an identical TCRBV. In the same fresh TILN, the frequency of CD8$^+$ T cells directed to Melan-A/MART-1$_{26\text{-}35}$ was 0.68% (Fig. 5: *C*, *bottom right*; and *D*, *lane 1*, □), whereas frequencies to gp100$_{154\text{-}162}$, gp100$_{209\text{-}217}$, and tyrosinase$_{368\text{-}377}$ were <0.2% (data not shown). The maturation stage of CD8$^+$ T cells present in TILN and specific for Prdx5$_{115L}$ and Melan-A/MART-1$_{26\text{-}35}$ was evaluated through their expression of CCR7 and of CD45RA. The data shown in Fig. 5*C* indicate that 56% and 81% of T cells specific for Prdx5$_{115L}$ and Melan-A/MART-1$_{26\text{-}35}$, respectively, showed a differentiated CCR7$^{+/-}$CD45RA$^-$ (i.e., $T_{CM}$ or $T_{EM}$) phenotype. Moreover, culture *in vitro* for 2 weeks in autologous MLTC induced marked outgrowth of Prdx5$_{115L}$- and Melan-A/MART-1$_{26\text{-}35}$–specific CD8$^+$ T cells at comparable levels (7.5% for Melan-A/MART-1$_{26\text{-}35}$ and 8.9% for Prdx5$_{115L}$; Fig. 5*D*, *lane 2*). A predominant expression (84%) of TCRBV8-expressing T cells was found among Prdx5$_{115L}$ tetramer–positive cells (data not shown). In contrast, repeated MLTC stimulation, for up to 6 weeks, of lymphocytes from an autologous tumor-free LN led to remarkable expansion of T cells specific for Melan-A/MART-1$_{26\text{-}35}$, whereas those specific for Prdx5$_{115L}$ constituted only 1.3% of CD8$^+$ T cells (Fig. 5*D*, *lane 3*). Taken together, these data indicate that Prdx5$_{115L}$-specific T cells with a differentiated phenotype are present in metastatic deposits at frequency similar or higher than T cells recognizing shared melanosomal antigen and are responsive to antigen stimulation *in vitro* to a similar extent as T cells directed to the differentiation antigen Melan-A/MART-1.

**Immunohistochemical Analysis of Metastatic Lesions.** Intracellular staining of Me8959 cells with anti-Prdx5 mAb (as shown in Fig. 1*D*) had indicated expression of the mutant protein in all neoplastic cells. Staining on the same cell line with mAbs directed to gp100, tyrosinase, and Melan-A/MART-1 melanosomal antigens instead indicated that different proportion of cells were negative for the latter antigens (data not shown). To assess the antigenic profile of Me8959 in deeper detail, serial sections from the LN metastasis were analyzed by immunohistochemistry. COS-7 cells either untransfected or transiently transfected with

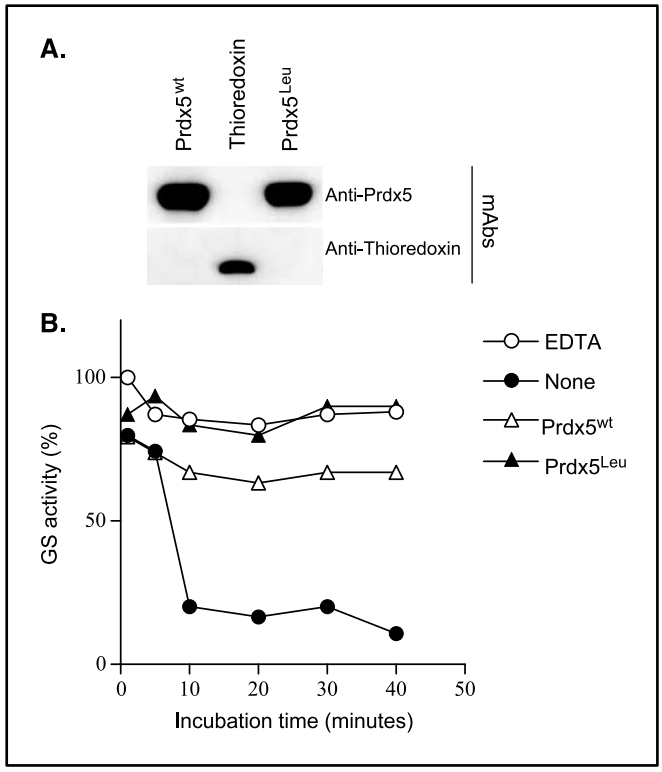


**Figure 2.** Isolation and functional activity in a cell-free assay of recombinant Prdx5 proteins. *A,* Western blot analysis of Prdx5 recombinant proteins (mutant and wt) as well as unrelated control protein (thioredoxin) with anti-Prdx5 and anti-thioredoxin mAbs. *B,* time-dependent GS protection activities of wt and mutant Prdx5 proteins as described in Materials and Methods.

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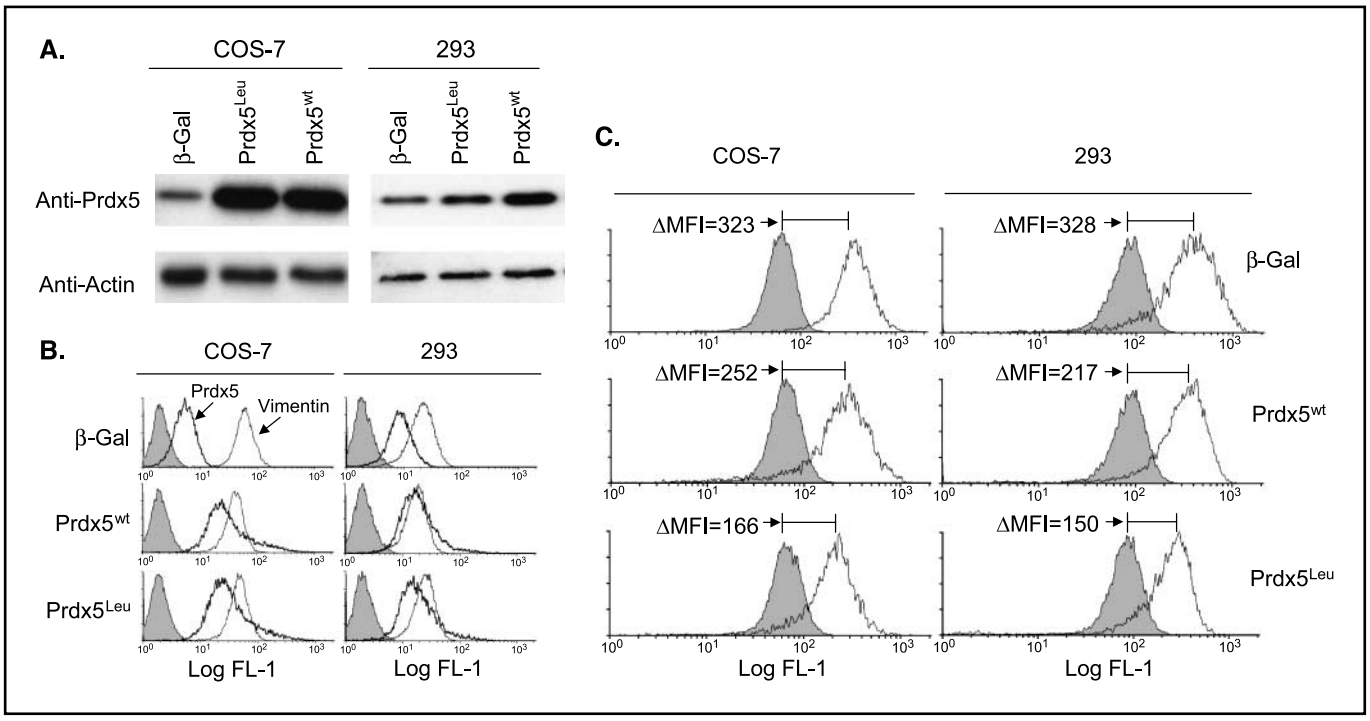


**Figure 3.** Functional activity of mutant Prdx5 when expressed in cells. pcDNA3.1 expression plasmids encoding $Prdx5^{Leu}$, $Prdx5^{wt}$, or β-Gal were transiently transfected into COS-7 and 293 cells. Overexpression of Prdx5 was assessed by (*A*) Western blot analysis or (*B*) by flow cytometry in permeabilized cells. Control mAbs were (*A*) anti-actin or (*B*) anti-vimentin. *C,* transiently transfected cells, incubated with the oxidation sensitive indicator CM-$H_2$-DCFDA, were treated (*empty peaks*) *peaks*) or not (*grey peaks*) for 5 minutes with 100 μmol/L of tBHP, and then analyzed by flow cytometry. Δ*MFI*, difference in mean fluorescence intensity between cell fluorescence in presence or absence of tBHP.

expression vector encoding mutant Prdx5 provided the specificity control for immunostaining with the anti-Prdx5 mAb (Fig. 6*A*). In the tissue section, all neoplastic cells, of both epithelioid and spindle cell morphology, were stained by this mAb (Fig. 6*B*). This homogeneous staining was in contrast with the frequent loss of expression of melanosomal antigens Melan-A/MART-1, gp100,

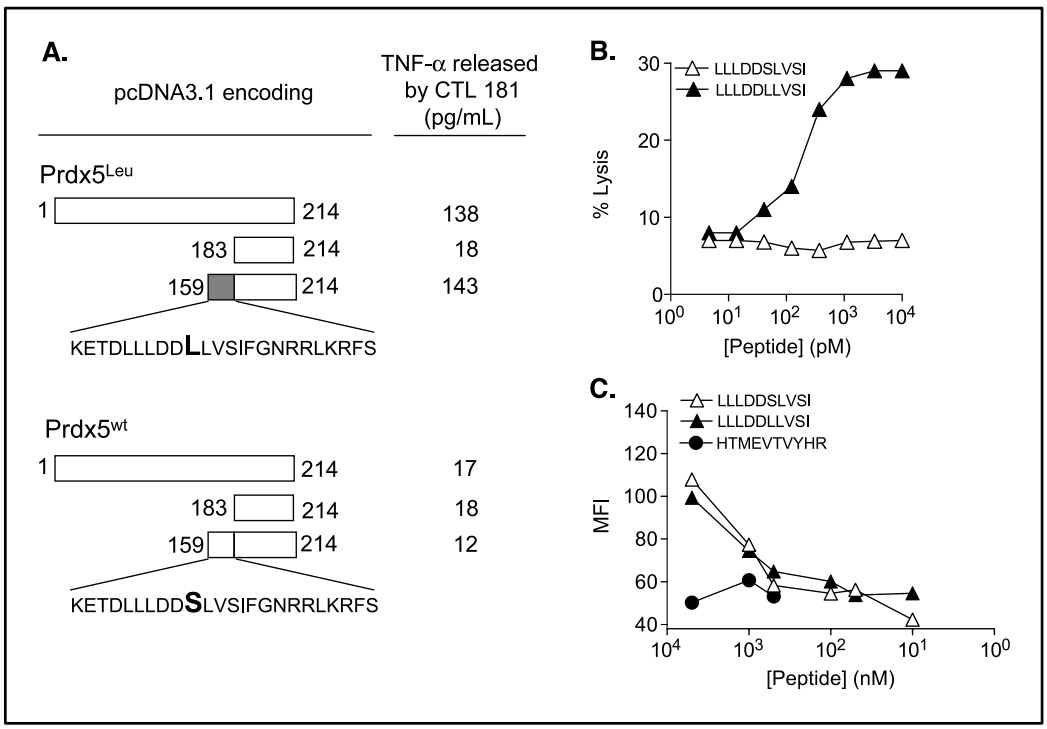


**Figure 4.** Identification of the antigenic Prdx5 epitope. *A,* expression vectors encoding the indicated full-length/subfragments of wt/mutant *Prdx5* cDNA were cotransfected into COS-7 together with pcDNA3/*HLA-A*0201*. TNF-α released by CTL 181 following recognition of transfectants was measured. The minimal 24-amino-acid sequence containing the epitope recognized by CTL 181 (*grey shading*) and the corresponding nonantigenic normal sequence are shown on *left*. Amount of TNF-α released by CTL 181 when cocultured with Me8959 was 125 pg/mL, whereas <15 pg/mL were released in response to both COS-7 and COS-7/*A*0201* cells. *B,* $^{51}$Cr-labeled HLA-A*0201$^+$ Me18732 cells were tested for lysis by CTL 181 (at E/T ratio of 10:1) after preincubation with different concentrations of the mutant or the wt Prdx5 peptides. Lysis of Me18732 in the absence of peptide was 4.5% and of Me8959 was 28.7%. *C,* binding of wt (△) or mutant (▲) Prdx5 peptides, or of the unrelated HLA-A*6801–restricted gp100/pMel17$_{182\text{-}191}$ (●) peptide (1, 2) to *HLA-A*0201* allele on T2 cells was assessed by flow cytometry. Results expressed as MFI for HLA-A2 expression.

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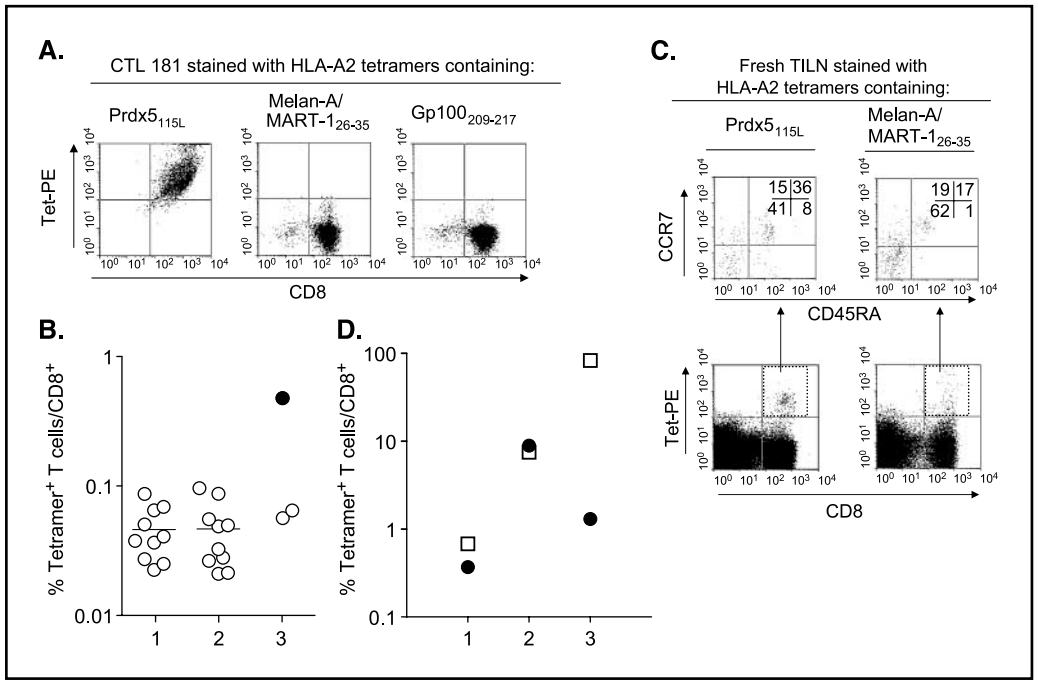


**Figure 5.** Enumeration and maturation phenotype of $CD8^+$ T cells recognizing $Prdx5_{115L}$ and Melan-A/$MART\text{-}1_{26\text{-}35}$ in TILN of patient 8959. *A,* CTL 181 was incubated with PE-labeled HLA-A*0201 tetramers containing the indicated antigenic peptides. *B,* $CD8^+$ T cells directed to $Prdx5_{115L}$ were quantified in PBMC of HLA-A*0201 healthy donors (*lane 1*: $n = 10$, ○), in PBMC (*lane 2*: $n = 10$, ○) and TILN (*lane 3*: $n = 2$, ○) of HLA-A*0201 melanoma patients as well as in TILN of patients 8959 (*lane 3*; ●). T cells were stained with anti-$CD8^{PerCP}$ and HLA-A*0201/ $Prdx5_{115L}$ tetramers. *Symbols,* percentage of $tetramer^+$ T cells/ $CD8^+$. *C,* expression of CCR7 and CD45RA on $CD8^+$ in TILN of patient 8959 recognized by PE tetramers containing either $Prdx5_{115L}$ or Melan-A/$MART\text{-}1_{26\text{-}35}$ as analyzed by four-color flow cytometry. *D,* enumeration of T cells recognizing $Prdx5_{115L}$ (●) or Melan-A/$MART\text{-}1_{26\text{-}35}$ (□) in TILN either fresh (*lane 1*) or cultured for 2 weeks in autologous MLTC (*lane 2*) or in tumor-free LN cultured for 6 weeks in autologous MLTC (*lane 3*).

and tyrosinase in different areas of the same lesion. As shown in Fig. 6*B*, the spindle cell component of the tumor that positively stained for Prdx5 was completely negative for all three melanoma markers. Antigen loss in the epithelioid component involved 10% and 30% of the cells for gp100 and tyrosinase and 60% of the cells for Melan-A/MART-1. Interestingly, freshly isolated neoplastic cells from the same metastatic lesion, previously analyzed by flow cytometry for expression of HLA-A2 antigens (20), showed that 92% of the cells retained expression of this *HLA* allele that acts as restricting element for recognition of mutant Prdx5 and of several normal antigens of the melanocyte lineage (1, 2).

## Discussion

This study reports the identification of a new unique tumor antigen recognized by HLA-A*0201–restricted CTL and generated in a human melanoma by a point mutation in the *Prdx5* gene, a recently identified member of the *Prdx* gene family encoding antioxidant enzymes (15–19). The C-T nucleotide change, found in the *Prdx5* gene constitutes a hallmark of UV-induced DNA damage (31). Interestingly, such change has been found to represent the genetic alteration underlying the generation of four HLA class I–restricted (1, 2) and two class II–restricted (1, 2) unique melanoma antigens. Altogether, these findings suggest an important role for the same DNA damaging agent (UV radiation) in the generation of the antigenic repertoire in transformed cells of the melanocyte lineage.

Although Prdxs are among the most abundant proteins in erythrocytes and can represent up to 0.8% of the soluble proteins in other mammalian cells (19), this class of enzymes has not been previously shown to contribute to the generation of tumor antigens. The mammalian *Prdx* gene family is composed by six members that are divided in three subgroups in accordance to the number of cysteines (Cys). They share the same catalytic mechanism, characterized by a first step in which a conserved $NH_2$-terminal Cys residue is oxidized to Cys-sulfenic acid by a peroxide (19). Following oxidation, there is the formation of a disulfide intermediate then reduced by thioredoxin and other physiologic reductants (16, 18, 19). The crystal structure of Prdx5 in reduced form indicates that the active site pocket of Prdx5 is a typical thioredoxin fold composed of four stranded -sheets flanked by three α-helices (17). Leu115 found in mutant Me8959 is close to an α helix (α5) that is typical of Prdx5 solely (17) and includes several hydrophobic residues whose side chains are located in the vicinity of a benzoate aromatic ring (17). It can be hypothesized that the additional hydrophobic Leu115 residue, generated by the mutation, may not adversely affect the enzymatic activity of Prdx5, but could even contribute to stabilize the interaction of the benzoate molecule with the active site. In agreement with this possibility, the mutant recombinant $Prdx5^{Leu}$ protein was at least as active as the wt enzyme, when tested in a cell-free system by the GS protection assay. Moreover, in transiently transfected COS-7 or 293 cells, the mutant Prdx5 was even more active than the wt enzyme, in terms of its ability of neutralizing oxidative stress. These findings suggest that melanoma cells harboring only the mutated *Prdx5* gene may need to retain it to ensure the relevant antioxidant activity of the corresponding protein.

The Ser to Leu substitution found at position 115 of the mutant Prdx5 protein of Me8959 generated a new T cell epitope ($Prdx5_{115L}$) recognized by T cells with high affinity. Furthermore, in agreement with reports on T cells recognizing several unique antigens (6–8, 10), even the mutant Prdx5-specific response seemed characterized by TCR dominance, as indicated by the prevalence of T cells (at tumor site or in MLTC or in CTL clones from peripheral blood) that expressed the same TCRBV8 region.

Available data indicates that unique tumor antigens expressed by human tumors may have significant immunogenicity (5, 7). In a lung carcinoma, 0.4% of CD8+ circulating T cells recognized an HLA-A2–restricted mutant malic acid (7) and an even higher frequency

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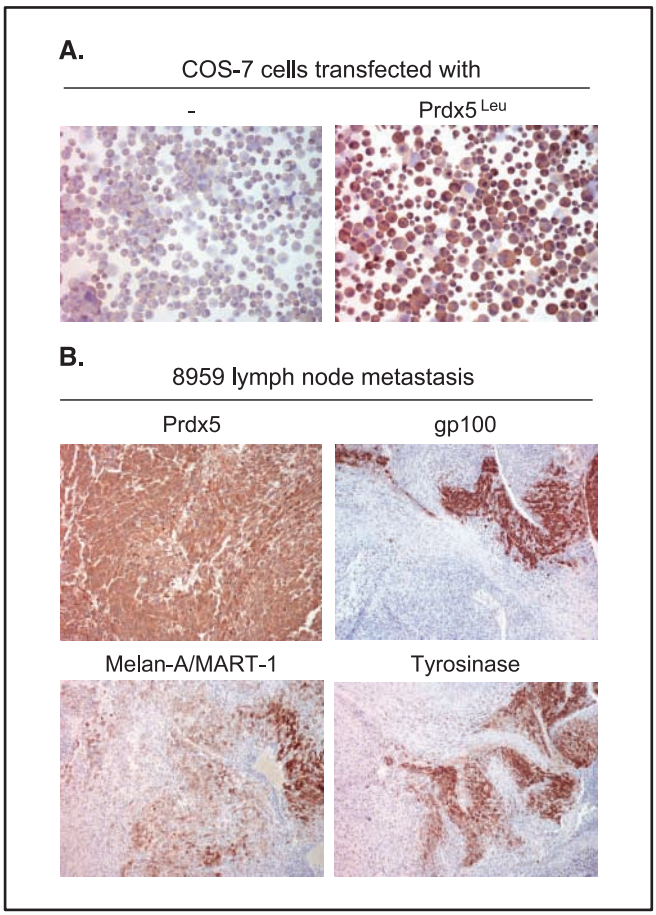


**Figure 6.** Immunohistochemical analysis for tumor antigen expression. *A,* cytospins of COS-7 cells, either mock transfected (−), or transfected with expression vector encoding mutant Prdx5 ($Prdx5^{Leu}$), were stained with anti-Prdx5 mAb which recognize both mutant and wt Prdx5. *B,* consecutive sections of the paraffin-embedded LN metastasis from patient 8959 were stained with Mabs to Prdx5, gp100, Melan-A/MART-1, and tyrosinase. Original magnification ×100 except for COS-7 cells (×200).

(1.2%) was found for circulating T cells directed to an HLA-A28–restricted mutant helicase epitope in a melanoma patient (5). Assessment of frequency of T cells recognizing unique mutant peptides at tumor site has however never been done previously. This issue could be addressed in our study, revealing that uncultured TILN of patient 8959 contained 0.37% of $CD8^+$ cells directed to the $Prdx5_{115L}$ epitope, in contrast to <0.1%/$CD8^+$ in HLA-$A2^+$ normal donors or melanoma patients. These data suggest that accumulation/clonal expansion of $Prdx5_{115L}$-specific T cells took place in the antigen$^+$ HLA-$A2^+$ neoplastic tissue of patient 8959. The evaluation of the T cell lines, selected by coculture of TILN T cells with Me8959, provided further evidence for immunogenicity of this unique antigen, as a remarkable $Prdx5_{115L}$-specific T cell expansion (~9%/$CD8^+$) could be achieved after a 2-week MLTC. Fresh PBMC were no longer available for comparison of frequency with TILN. Nevertheless, analysis of an existing 1-week-old MLTC, generated from peripheral blood indicated that mutant Prdx5-specific T cells were present at a frequency of 0.55%/$CD8^+$ (data not shown). Interestingly, limiting dilution analysis for frequency of antitumor T cells in peripheral blood lymphocytes of patient 8959, had previously documented a high frequency of T cells directed to HLA-A2–retricted antigens not shared with melanocytes (21). Thus, presence of mutant Prdx5-specific T cells in periphery confirm the previous data and indicate a systemic reactivity to the unique antigen.

The expression of both melanosomal and nonmelanosomal antigens by Me8959 (29) allowed us to directly compare in the same patient, *ex vivo* frequencies and phenotype of T cells directed to the unique mutant Prdx5 and to shared melanocyte differentiation epitopes, which are frequently presented in association with HLA-A2 (1, 2). This comparison has not been previously possible, since the known class I–restricted mutant peptides yielding unique antigens in melanoma were restricted, with a single exception, to alleles other than *HLA-A2* (1, 2). In TILN, $Prdx5_{115L}$-specific T cells were not as represented as Melan-A/MART-1–specific T cells, but showed a higher frequency compared with tyrosinase- or gp100-specific T cells. However, a large fraction of $Prdx5_{115L}$- and Melan-A/MART-1–specific T cells expressed a memory (either $T_{CM}$ or $T_{EM}$) phenotype, in support of antigen-driven T-cell maturation at tumor site.

Mutant, nonredundant proteins, essential for cell survival and/or for maintenance of the malignant phenotype, are likely to be retained in progressive tumors, in particular when encoded by genes that have lost their nonmutant counterpart. This has been documented in immunogenic murine fibrosarcomas (14). In these progressively growing tumors, the antigens were identified as mutant ribosomal components, fundamental for normal protein synthesis, and neither tumor retained the wt allele (14). Interestingly, recent reports on outcome of Prdx1, Prdx2, and Prdx6 inactivation in mice, by homologous recombination, have indicated that these enzymes are important for cell survival, and tumor suppression, in a cell context–dependent fashion (32–34). Although similar information is not available for Prdx5, it has been reported that overexpression of human Prdx5 could significantly prevent cell death after tBHP-dependent oxidative challenge, supporting the relevance of the antioxidant role of this gene (35). In further support of a relevant role of mutant Prdx5, we found that the mutant Prdx5 was expressed in all cells of the metastatic lesion, in contrast with marked heterogeneity of expression for other differentiation antigens in the same neoplastic tissue.

Finally, it seems reasonable to assume that unique tumor antigens, as the mutant Prdx5, may be difficult to exploit in the therapeutic setting, as either recombinant protein or peptides, due to the intensive and time-consuming effort needed to identify even a single antigen in each patient. Nevertheless, further attempts should be made aiming at the identification of determinants that, as mutant Prdx5, may be, at the same time, immunogenic and resistant to immunoselection. These determinants would represent ideal candidates for immune intervention approaches that do not require prior knowledge as to the molecular identity of the target antigen. Either vaccination with dendritic cells loaded with autologous tumor-derived antigens or adoptive immunotherapy with *ex vivo* activated T cells in MLTC might provide effective means of boosting immunity to epitopes as the one described in this study.

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