Peptides with dual binding specificity for HLA-A2 and HLA-E are encoded by alternatively spliced isoforms of the antioxidant enzyme peroxiredoxin 5

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Keywords: splice variants, tumor antigen, MHC class Ia, MHC class Ib

Abstract

Peptides with dual binding specificity for classical HLA class I and non-classical HLA-E molecules have been identified in virus-encoded proteins, but not in cellular proteins from normal or neoplastic cells. Expression screening of a melanoma cDNA library with a CTL clone recognizing an HLA-A2-restricted tumor-specific epitope encoded by mutant peroxiredoxin 5 (Prdx5), a stress-inducible peroxidase, led to the identification of two alternatively spliced isoforms of the same gene. These isoforms, which lack the catalytic cysteine fundamental for enzymatic activity, showed widespread expression in neoplastic and normal tissues but were unstable at the protein level, being detectable, following transient transfection, only after lactacystin treatment to inhibit proteasomal degradation. Isoform-specific sequences which formed, respectively, as result of exon 1 splicing to either exon 3 or 4, encoded two distinct nonapeptides (AMAPIKTHL and AMAPIKVRL, not present in the full-length protein) with anchor residues for HLA-A2 and HLA-E molecules and able to stabilize HLA-A2 and HLA-E cell surface expression. HLA-E targets, loaded with these peptides, were not recognized by NK cells expressing CD94/NKG2A inhibitory or CD94/NKG2C activatory receptors. However, both peptides were recognized, although with low avidity, by HLA-E-restricted CD8+ CTL. The nonapeptide AMAPIKVRL was used to elicit HLA-A2-restricted CTL clones that killed peptide-pulsed lymphoblastoid cell lines and melanoma cells expressing the corresponding Prdx5 isoform. Our results suggest that alternatively spliced isoforms of Prdx5, through the generation of HLA-E- and HLA-A2-restricted peptides may be part of immune-mediated stress response contributing to the detection and elimination of damaged normal or neoplastic cells.

Introduction

In humans, MHC class I molecules comprise the classical (class Ia) HLA-A, -B and -C antigens and the non-classical (class Ib) HLA-E, -F and -G (1–4). HLA class Ia molecules are highly polymorphic and bind antigens in the form of 8–10 amino acid-long peptides, primarily generated from the degradation of a cytosolic pool of self or viral proteins through active proteolysis by the proteasome (2, 5, 6). Endogenous peptides presented by MHC class Ia molecules derive not only from proteins encoded by standard open-reading frames (ORFs), but even from proteins generated by different molecular mechanisms like cryptic promoters usage, alternative ORFs and alternative splicing (7). Additional sources of endogenous peptides are provided by post-translational events that involve protein splicing in the...
Immunological features of Prdx5 splice isoforms

Peroxiredoxin 5 (Prdx5) has peroxides and peroxynitrite (24/C0 stress being able to reduce hydrogen peroxide, alkyl hydroperoxides and paraoxon-1. We identified two alternatively spliced isoforms of Prdx5 being able to reduce hydrogen peroxide, alkyl hydroperoxides and paraoxon-1. The non-classical or HLA class Ib molecules are structurally similar to HLA class Ia molecules and from viral or stress-related proteins (9–14). HLA-E molecules are recognized by CD94/NKG2A inhibitory and CD94/NKG2C activating receptors expressed by NK cells and T cell subset (15). In addition, HLA-E can also function as a restriction element for some human CTL (16–18) including NK–CTL (19–22). Independently from the mechanism that generates the peptide, most previously identified T cell epitopes can either bind to a single HLA allele or, in some instances, show promiscuous binding to different alleles of the same class of HLA molecules. Peptides with dual binding specificity (e.g. to classical HLA-A2 class I and to non-classical HLA-E molecules) have been so far identified only for virally encoded proteins but not for cellular proteins (9, 11).

We have recently reported that a missense substitution in one allele of peroxiredoxin 5 (Prdx5), occurring in a melanoma (melanoma 8959) lacking the wild-type (wt) allele, yielded a 10-mer HLA-A*0201-restricted unique T cell epitope (Prdx5115L) targeted by CD8+ T cells (23). Prdx5 belongs to a family of stress-inducible peroxides that have an important role in cellular defense against oxidative stress being able to reduce hydrogen peroxide, alkyl hydroperoxides and peroxynitrite (24–28). Reduction of peroxide substrates occurs through a conserved cysteine residue within an active site pocket structure (26–28). In this study, we identified two alternatively spliced isoforms of Prdx5 being able to reconstitute the Prdx5115L epitope. As a result of alternative splicing, each splice isoform lacks the catalytic cysteine but encodes, in the region surrounding the novel exon–exon joining boundaries, a nonapeptide with dual binding ability for non-polymorphic HLA-E and polymorphic HLA-A2 molecules. Recognition of the nonapeptides by HLA-E-restricted CD8+ T cells (NK–CTLs) and by HLA-A2-restricted CTLs, but not by CD94/NKG2A inhibitory or CD94/NKG2C activatory receptors expressed by NK cells, suggests that a T-cell-mediated immune response against such epitopes may contribute to elimination of cells severely damaged by oxidative stress. This could occur in several pathologies and cancer (29).

**Methods**

**Cell lines**

Human melanoma cell line 8959 (HLA typing: A*0201; A*1101/02, Bw55; CW3; DRw11; Dw7) and all primary and metastatic melanoma cell cultures were established from surgically removed specimens. Other cancer cell lines used were ovarian carcinoma lines IGROV1 and SK-OV3, lung carcinoma lines CALU-1 and SW48, Burkitt’s lymphoma Daudi, colorectal cancer lines CO115 and CO756 and breast carcinoma lines MCF-7 and MDA-MB-231. Cancer cell lines, the human peptide transporter (TAP)-deficient 174.CEM.T2 (T2; American Type Culture Collection, Rockville, MD) cell line, the human HLA class I negative erythroleukemia cell line K562, the murine peptide transporter-deficient T cell lymphoma RMA-S cell line, the β2-microglobulin- and HLA-E*01033 allele-transfected derivative of RMA-S (RMA-S/HLA-E; kindly provided by J. E. Coligan, National Institute of Allergy and Infectious Diseases, Rockville, MD) and HLA-A2* EBV-transformed lymphoblastoid cell lines (LCLs) were maintained in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES and antibiotics. The human HLA-E*01033-transfected derivative of K562 (K562/HLA-E) cell line was cultured in the same medium supplemented with 0.4 mg/ml G-418 (Calbiochem, San Diego, CA). 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) were maintained in DMEM (Lonza) supplemented as above. Normal human epidermal melanocytes were purchased from Promocell GmbH (Heidelberg, Germany) and were grown under the conditions recommended by the manufacturer. The human CD94/NKG2A* NK cell line NK92 (kindly provided by E. O. Long, National Institute of Allergy and Infectious Diseases) and the CD94/NKG2C* NK clone #15.1 were cultured in RPMI 1640 supplemented with 100 U/ml recombinant IL-2 (rIL-2) and 10% FCS. The NK clone #15.1 was obtained according to previously described methodology (30). Briefly, enriched NK cells were isolated from PBMCs of one healthy donor using the Human NK Cell Enrichment Cocktail-RosetteSep (StemCell Technologies Inc., Vancouver, BC), cultured on irradiated feeder cells in the presence of rIL-2 (100 U/ml Proleukin; Novartis, East Hanover, NJ) and PHA (1.5 ng/ml; Gibco, Paisley, GB) and cloned by limiting dilution.

Establishment and culture conditions of the HLA-A2-restricted CTL clone 181 derived from PBMC of patient 8959 and of HLA-E-restricted CD8+ T cell lines from PBMC of donors GF and DP have been previously described (20, 23, 31, 32).

**Antibodies and peptides**

mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2), gD5 (anti-β2 microglobulin), A6.136 (anti-HLA class I), 3D12 (anti-HLA-E, kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), Y9 (anti-CD94) and anti-HA high affinity (anti-HA, clone 3F10; Roche, Basel, Switzerland). PE-conjugated goat anti-mouse (BD Biosciences, San Jose, CA) or biotinylated goat anti-rat (Dako, Copenhagen, Denmark) mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2), gD5 (anti-β2 microglobulin), A6.136 (anti-HLA class I), 3D12 (anti-HLA-E, kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), Y9 (anti-CD94) and anti-HA high affinity (anti-HA, clone 3F10; Roche, Basel, Switzerland). PE-conjugated goat anti-mouse (BD Biosciences, San Jose, CA) or biotinylated goat anti-rat (Dako, Copenhagen, Denmark) mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2), gD5 (anti-β2 microglobulin), A6.136 (anti-HLA class I), 3D12 (anti-HLA-E, kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), Y9 (anti-CD94) and anti-HA high affinity (anti-HA, clone 3F10; Roche, Basel, Switzerland). PE-conjugated goat anti-mouse (BD Biosciences, San Jose, CA) or biotinylated goat anti-rat (Dako, Copenhagen, Denmark) mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2), gD5 (anti-β2 microglobulin), A6.136 (anti-HLA class I), 3D12 (anti-HLA-E, kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), Y9 (anti-CD94) and anti-HA high affinity (anti-HA, clone 3F10; Roche, Basel, Switzerland). PE-conjugated goat anti-mouse (BD Biosciences, San Jose, CA) or biotinylated goat anti-rat (Dako, Copenhagen, Denmark) mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2), gD5 (anti-β2 microglobulin), A6.136 (anti-HLA class I), 3D12 (anti-HLA-E, kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), Y9 (anti-CD94) and anti-HA high affinity (anti-HA, clone 3F10; Roche, Basel, Switzerland). PE-conjugated goat anti-mouse (BD Biosciences, San Jose, CA) or biotinylated goat anti-rat (Dako, Copenhagen, Denmark) mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2), gD5 (anti-β2 microglobulin), A6.136 (anti-HLA class I), 3D12 (anti-HLA-E, kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), Y9 (anti-CD94) and anti-HA high affinity (anti-HA, clone 3F10; Roche, Basel, Switzerland). PE-conjugated goat anti-mouse (BD Biosciences, San Jose, CA) or biotinylated goat anti-rat (Dako, Copenhagen, Denmark) mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2), gD5 (anti-β2 microglobulin), A6.136 (anti-HLA class I), 3D12 (anti-HLA-E, kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), Y9 (anti-CD94) and anti-HA high affinity (anti-HA, clone 3F10; Roche, Basel, Switzerland). PE-conjugated goat anti-mouse (BD Biosciences, San Jose, CA) or biotinylated goat anti-rat (Dako, Copenhagen, Denmark) mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2), gD5 (anti-β2 microglobulin), A6.136 (anti-HLA class I), 3D12 (anti-HLA-E, kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), Y9 (anti-CD94) and anti-HA high affinity (anti-HA, clone 3F10; Roche, Basel, Switzerland). PE-conjugated goat anti-mouse (BD Biosciences, San Jose, CA) or biotinylated goat anti-rat (Dako, Copenhagen, Denmark) mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (anti-HLA-A2), gD5 (anti-β2 microglobulin), A6.136 (anti-HLA class I), 3D12 (anti-HLA-E, kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), Y9 (anti-CD94) and anti-HA high affinity (anti-HA, clone 3F10; Roche, Basel, Switzerland). PE-conjugated goat anti-mouse (BD Biosciences, San Jose, CA) or biotinylated goat anti-rat (Dako, Copenhagen, Denmark) mAbs used in this study were W6/32 (anti-HLA class I), CR11-351 (
human immunodeficiency virus (HIV) (ILKEPVHGV) and heat shock protein 60 (Hsp60) (QMRPVSRL). All peptides were synthesized as described (33).

cDNA library screening, insert identification and sequencing
Construction of the cDNA library from RNA obtained by melanoma 8959, screening and identification of individual plasmids encoding the antigen recognized by CTL 181 have been described elsewhere (23). cDNA inserts were 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, Carlsbad, CA).

Total RNA isolation, reverse transcription—PCR and cloning of reverse transcription—PCR fragments
Total RNA was isolated from cultured cell lines or fresh specimens with the Tripure reagent (Roche) as described by the manufacturer and reverse transcribed (1 μg) into cDNA using the Superscript II enzyme (Invitrogen) and oligo (dT) (Invitrogen). The resulting cDNA, in a final volume of 20 μl, was used as the template for PCR amplification of full-length Prdx5 with forward 5’-AGGAGGCGGAAGTGAAGT-3’ and reverse 5’-ATTGCAGAAATCTGGCCAAC-3’ primers. The reaction mixture consisted of 25 μl containing 200 μM of each deoxynucleotide triphosphates, 0.4 μM of each primer and 0.6 U of HotMaster Taq polymerase (Eppendorf, Hamburg, Germany), 2.5 mM MgCl2 and 1 μl of cDNA. Amplification was conducted on a DNA thermal cycler (model 9700 Gene Amp PCR system; Applied Biosystems) according to the step-down PCR parameters (34), with minor modifications (35). The resulting fragments were resolved by gel electrophoresis, excised, purified with NucleoSpinExtract (Machinerey-Nagel, Hoerdt, France), cloned into pCR4 plasmid (TOPO TA Sequencing kit) and sequenced.  

Eukaryotic expression cloning of Prdx5 fragments and immunohistochemical analysis
The N-terminal HA-tagged (HA sequences in bold, Kozak sequence underlined) forward 5’-GCCACCATGTACCA-TACGACGTCCAGACTACGTGCCCAATCAAGGT-3’ primer in combination with the reverse 5’-GCCTAGAGCTGTGAGATGAT primer was used to amplify from pCR4 plasmids of wt Prdx5. Amplification was performed using Expand High Fidelity PCR System (Roche). Purified fragments were subcloned into pcDNA3.1/V5-His expression vector plasmid using the Eukaryotic TOPO TA Expression kit (Invitrogen) and sequenced. COS-7 cells were transfected with these expression vectors using Fugene (Roche) according to manufacturer instructions, incubated at 37°C for 36 h and then treated for 17 h with vehicle alone [dimethyl sulfoxide (DMSO)] or with the proteasome inhibitor lactacystin at 5 μg/ml ( Biomol International, Plymouth Meeting, PA) dissolved in DMSO. Cytospins were prepared and examined by immunostaining as described (23).

Peptide-HLA molecule binding assays
Peptide binding was assessed by HLA-A2 or HLA-E stabilization assays according to previously published methods (19, 23) using T2 cells or K562, K562/HLA-E, RMA-S and RMA-S/HLA-E cell lines. Briefly, cells were re-suspended in serum-free medium at 1 × 10⁶ cells/ml. Where appropriate, peptide was added to the desired concentration. After an overnight incubation at 37°C (T2) or 26°C (K562 and K562/HLA-E; RMA-S and RMA-S/HLA-E), cells were washed with PBS to remove free peptides. Next, HLA surface expression was monitored after staining with either CR11-351 mAb for HLA-A2 or 3D12, W6/32 and A6.136 mAbs for HLA-E followed by appropriate PE-conjugated goat anti-mouse antibody. Analysis was done on a FACS Calibur cytometer (BD Biosciences). Results are reported either as FACS histograms or as percentage of increase in mean fluorescence intensity (MFI) calculated over the “no-peptide” control using the following formula [(test peptide MFI) – (no-peptide MFI)]/ (no peptide MFI) × 100.

Induction of peptide-specific CTL lines and clones
PBMC (1 × 10⁶/ml) from three HLA-A*0201-positive healthy donors were cultured in the presence of irradiated T2 as antigen-presenting cells (at 0.5 × 10⁶/ml) pulsed with the AMAPIVKRL peptide (10 μM) in complete medium supplemented with 60 IU/ml of rIL-2 (Novartis) and 5 ng/ml of IL-7 (R&D systems, Minneapolis, MN, USA). Restimulation was done after 10 days with irradiated T2 cells pulsed with the corresponding peptide in presence of rIL-2. T cell lines were cloned in round-bottom microwells under limiting dilution conditions as described (36).

Cytotoxicity assays
A standard 4 h [51Cr] radioisotope release assay was used. Before [51Cr] labeling, targets were incubated overnight at 26°C (RMA-S/HLA-E) or 37°C (LCL) in presence or absence of synthetic peptides. E:T ratios and peptide concentrations are stated in the text. Depending on the experiment, [51Cr]-labeled targets could be pre-incubated with anti-HLA-A2 mAb CR11-351 (10 μg/ml) or with anti-CD94 mAb Y9 (10 μg/ml) before addition of effectors. For NK cell assay, peptides were kept throughout the assay to assure higher levels of HLA-E expression.

TCR β-chain variable region usage
Nomenclature for TCR β-chain variable region (TCRβV) gene segments is according to the recommendations of the WHO—IUIS Subcommittee on TCR designation (37). cDNA obtained from CTL clones was amplified by step-down PCR using panels of TCRβV-specific forward primers and one reverse BC primer as described (38).

Results
Two different mRNA splice variants of a mutant Prdx5 encode the unique melanoma antigen recognized by CTL clone 181
Expression screening, in HLA-A*0201-transfected COS-7 cells, of melanoma 8959 cDNA library led to the identification of two additional plasmids able to induce tumor necrosis factor (TNF) release by the previously described Prdx5115L* specific CTL clone 181 recognizing the HLA-A2 restricted LLLDDLVSI110–119 peptide encoded by a mutant Prdx5.
gene (see 23). These plasmids, indicated in Fig. 1(A) as pcDNA3.1_cDNA 510 and pcDNA3.1_cDNA 375, contained smaller cDNA inserts than that encoding for full-length mutant Prdx5 (pcDNA3.1._Prdx5Leu). Nucleotide sequence analysis of these cDNA inserts indicated that they were exon-skipping variants generated by alternative splicing of the Prdx5 gene (Fig. 1B). Both isoforms, which also possess the sequence encoding the mitochondrial localization domain at their N-terminus (Fig. 1B and C), are identical to full-length Prdx5 (GeneBank AF110731) in the first 159 nucleotides, whereas downstream a deletion encompasses the entire exon 2 (Prdx5D2) or the entire exons 2 and 3 (Prdx5D2,3) sequences (Fig. 1B and C). Coding sequences of both isoforms, in frame with the first ATG and the stop codon, did not include the catalytic conserved cysteine (bold underlined in Fig. 1C). Beside exons 4 and 6, exon 5, with the point mutation leading to leucine for serine substitution, is maintained in all isoform, thus explaining the retention of the epitope recognized by CTl 181 (italic underlined in Fig. 1C, with the mutant residue in bold).

Alternative splicing of Prdx5 gene occurs in normal and neoplastic cells of different histologic types

Reverse transcription (RT)–PCR analysis using primers which amplify full-length human Prdx5 was used to assess expression of the two alternatively spliced variants in cDNA derived from mRNA obtained from 12 melanomas and 4 melanocyte cell lines as well as from neoplastic cell lines of different histologies (IGROV1, SK-OV3, CO115, CO756, SW48, CALU-1, DAUDI, MCF-7, MDA-MB231), from PBMC and from normal specimens of ovary and skin. All these samples showed, in addition to a dominant product of the expected 642 bp for full length Prdx5, two smaller bands at of 510 and 375 bp (Fig. 1D, for representative results on melanomas, melanocytes and PBMC). Sequence analysis of RT–PCR amplification fragments (from PBMC) subcloned into pCRII sequencing vector confirmed that the higher molecular weight product corresponded to full-length wt Prdx5, whereas the two smaller fragments corresponded to wt Prdx5D2 and to wt Prdx5D2,3 (data not shown).

Fig. 1. Identification of exon-skipping isoforms of mutant Prdx5. (A) TNF production. In all, 1500 cells of CTL 181 were added to COS-7 cells transiently co-transfected with the indicated expression vectors. TNF released in the supernatant after overnight coculture was tested by measuring the cytotoxicity on the sensitive WEHI-164 clone 13 cells with a colorimetric assay, as described (35). Each value represent the mean (±SD) of triplicates. (B) Schematic representation of the different isoforms. The 5′ donor gt and 3′ acceptor ag are in lower case and underlined (39). The mutant nucleotide present in exon 5 sequence is represented as a triangle. (C) The deduced amino acid sequence is displayed using the single-letter code. Both exon-skipping isoforms lack the catalytic N-terminal cysteine (C, bold underlined) fundamental for antioxidant activity of the protein. The mitochondrial targeting sequence is in bold. The antigenic epitope recognized by CTL 181 and shared among all isoforms is italic underlined with the mutant amino acid in bold. (D) Ethidium bromide-stained 1% agarose gel of Prdx5 RT–PCR products from representative normal and neoplastic human samples. The samples were human PBMC, normal human epidermal melanocytes (NHEM), lanes 1–5 human primary melanomas and lanes 6–9 human metastatic melanomas. Arrows delineates the 642-bp Prdx5, 510-bp Prdx5D2 and 375-bp Prdx5D2,3.
Proteasome-dependent degradation of Prdx5 alternatively spliced isoforms

Alternatively spliced mRNA isoforms have been recently described for Prdx5 gene, but such variants did not yield translated products and attempts to produce recombinant proteins failed due to protein misfolding (39). Recognition by CTL 181 of peptides derived from mutant Prdx5A2 and Prdx5A2.3 isoforms indicated that translation of these isoforms could occur. Nonetheless, to determine whether alternative splicing could affect protein stability, COS-7 cells were transfected with expression vectors encoding wt Prdx5 and wt Prdx5 splice isoforms, modified with an HA tag to allow protein identification by HA-specific mAb. COS-7 cells either mock-transfected or transiently expressing wt Prdx5 provided the negative and positive controls for immunostaining with the anti-HA mAb (Fig. 2A–D). Neither of the two isoforms could be detected by anti-HA mAb in cytosplins of transfected COS-7 cells (Fig. 2E and G). However, treatment of transfected COS-7 cells with proteasome inhibitor lactacystin led to their stabilization and recognition by anti-HA mAb (Fig. 2F and H). Identical results were obtained by transfecting expression vectors encoding mutant Prdx5 isoforms. These results provide evidence that the translated forms of exon-skipping variants are unstable being subjected to proteasome-dependent degradation.

Peptides derived from junctional sequences of Prdx5 splice isoforms stabilize HLA-A2 and HLA-E cell surface expression

The translated ORF of both variants (either wt or mutant), but not of full-length Prdx5, encoded at positions 52–60 (Fig. 1C) of the novel junctional regions, nonapeptides predicted by SYFPEITHI (http://www.syfpeithi.de/home.htm) and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) algorithms (40, 41) as being optimal binders to HLA-A and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) predicted by SYFPEITHI (http://www.syfpeithi.de/home.htm) (Table 1). In addition, based on the ranking scores predicted by the algorithm available at http://www.cbs.dtu.dk/services/NetCTL/, which integrates prediction of MHC class I binding, proteasomal C-terminal cleavage and TAP transport efficiency (42), each peptide was the second top-ranking peptide in the amino acid sequence of each isoform. Furthermore, the same peptides even displayed an HLA-E permissive motif possessing a methionine at position 2 and a leucine at position 9 in the carboxyl-terminal position (43) (Table 1). Binding of synthetic peptides corresponding to the predicted sequences was then evaluated on HLA-A2* and on HLA-E* targets by flow cytometry. Incubation of TAP-deficient HLA-A2* T2 cell line with the AMAPIKTHL or AMAPIKVRL peptides stabilized the expression of HLA-A2, as evidenced by the increased MFI values (Table 1). This increase was similar to that observed using as positive peptide the 10-mer HLA-A2-restricted Prdx5115L epitope (LLLLDDLVSIL) (23). No increase was induced using the HLA-A*6801-restricted gp100/pMel17182–191 (HTMEVTVYHR) peptide (44) derived from the differentiation antigen gp100 used as negative control. Binding of the peptides to HLA-E was assessed using K562/HLA-E. Incubation with the cancer–testis MAGE-1 peptide (EADPTGHSY), unable to bind HLA-E, did not enhance the minimal background levels of HLA-E detected by W6/32 or HLA-E-specific mAb 3D12 on K562/HLA-E cells (Fig. 3). Loading with peptides derived from Prdx5A2 (AMAPIKTHL) and Prdx5A2.3 (AMAPIKVRL) stabilized HLA-E surface expression (Fig. 3) similarly to the loading with HLA-E-binding peptides HLA-Cw3 (VMAPPRTLIL), HLA-A2 (VMAPPRTLVL), HLA-A1 (VMAPPRTLILL), HCV core (YLPRPRGPRP) and Hsp60 (QMRPVSVR). Incubation of HLA-E-negative K562 cells with these peptides did not result in enhanced W6/32 or 3D12 staining as measured by FACS analysis (data not shown). In contrast to these peptides, the corresponding peptide derived from full-length Prdx5 (AMAPIKVGD) was unable to stabilize HLA-E molecules on K562/HLA-E cells (Fig. 3).

AMAPIKTHL and AMAPIKVRL pulsed on K562/HLA-E cells are not recognized by inhibitory or activatory CD94/NKG2 receptors

The inhibitory C-type lectin receptor CD94/NKG2A specifically interacts with HLA-E resulting in inhibition of NK activity. In order to address whether enhanced HLA-E expression of K562/HLA-E loaded with peptides from Prdx5 splice variants could inhibit lysis mediated by a CD94/NKG2A+ NK cells. K562/HLA-E cells were incubated overnight at 26°C with either AMAPIKTHL or AMAPIKVRL or with the VMAPRTLIL peptide as positive control and tested as targets in a [51Cr] release assay (Fig. 4). K562/HLA-E cells were killed by CD94/NKG2A+ NK92 cell line but were protected from killing when incubated with VMAPRTLIL (Fig. 4A and B). Pre-incubation of NK cells with anti-CD94 mAb abolishes inhibition of cytolysis demonstrating recognition of HLA-E-VMAPRTLIL complexes by the inhibitory CD94/NKG2A receptor (Fig. 4B). Incubation of K562/HLA-E with either AMAPIKTHL or AMAPIKVRL had no effect on the cytotoxic function of NK effectors and cytotoxicity was not altered by the presence of anti-CD94 mAb (Fig. 4C and D). Although not shown, identical results were obtained using CD94/NKG2A+ NK cell clones as effectors. In addition, neither peptide, when incubated in excess over the protective VMAPRTLIL peptide, was able to interfere with CD94/NKG2A-mediated recognition by restoring lysis of K562/HLA-E (data not shown). We further investigated whether K562 cell transfectants expressing HLA-E molecules loaded with Prdx5 isoform-derived nonamers could be recognized by NK cells expressing a functional activatory CD94/NKG2C receptor. To this end, K562/HLA-E cells incubated with either AMAPIKTHL or AMAPIKVRL were tested as targets in a [51Cr] release assay with the CD94/NKG2C+ NK cell clone #15.1 as effector. Peptide-loaded K562/HLA-E were not killed more efficiently than control target cells (i.e. not pulsed K562/HLA-E). In addition, lysis of peptide pulsed K562/HLA-E was not inhibited on antibody-mediated masking of CD94 (data not shown). Taken together, these results suggest that HLA-E/Prdx5 isoform-derived peptides do not engage inhibitory or activatory CD94/NKG2 receptors on NK cells.

AMAPIKTHL and AMAPIKVRL allow recognition of RMA-S/HLA-E by HLA-E-restricted CD8+ T cell lines

HLA-E binds not only CD94/NKG2 receptors but even acts as an antigen presenting molecule for a subset of TCR b/β CD8+ T cells, named NK–CTLs (19–22). We therefore
Fig. 2. Lactacystin treatment stabilizes Prdx5 splice isoforms. Immunocytochemical staining of COS-7 cells expressing HA-Prdx5 (C and D), HA-Prdx5Δ2 (E and F) and HA-Prdx5Δ2,3 (G and H). Cells, including untransfected COS-7 (A and B), were incubated with or without 5 μg/ml lactacystin for 17 h and stained with the anti-HA mAb.
investigated whether AMAPIKTHL and AMAPIKVRL peptides could be recognized by NK−/C0 CTLs derived from two different healthy donors (GF and DP). These TCRα/β+ CD8+ effectors cells were previously shown to recognize, with high avidity, in an HLA-E-restricted fashion, both the VMAPRTLIL and the VMAPRTLVL peptides (20), but did not recognize other

Table 1. Predicted HLA-A2- and HLA-E-binding peptides and HLA-A2 stabilization

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Sequence</th>
<th>HLA-A2-binding scores predicted by BIMAS</th>
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aPercent increase in MFI was calculated over the empty T2 cells (no peptide) using the formula: \( \frac{(\text{MFI of test peptide}) - (\text{MFI of no peptide})}{(\text{MFI of no peptide})} \times 100.\)

Fig. 3. AMAPIKTHL and AMAPIKVRL enhance HLA-E surface expression. K562/HLA-E cells were incubated or not overnight at 26°C with the indicated peptides (200 μM). Cells were then stained with mAbs 3D12 (IgG1) and W6/32 (IgG2A). Cell surface expression of HLA-E is indicated as bold profiles for K562/HLA-E peptide-pulsed cell and as thin profiles for untreated K562/HLA-E cells. Numbers in each histogram refer to MFI values of peptide-pulsed cells. Dotted profiles refer to K562/HLA-E peptide-pulsed cells stained with control mouse IgG.
HLA-bound peptides loaded at saturating amounts on the murine TAP-2-deficient RMA-S/HLA-E cell line (20). Also for these experiments, murine RMA-S/HLA-E were used as targets. In the absence of peptide loading, these cells do not display surface expression of HLA-E as assessed by flow cytometry (Fig. 5A). After pulsing RMA-S/HLA-E overnight at 26°C with either AMAPIKTHL or AMAPIKVRL at saturating amounts (200 µM), HLA-E surface expression was induced (Fig. 5A) and killing of targets by NK/CTL cell lines increased (Fig. 5B). Taken together, these data indicate that peptides derived from Prdx5 isoforms can be recognized by CD8+ HLA-E-restricted CTL.

HLA-A2-restricted AMAPIKVRL-specific CTL clones recognize HLA-A2-positive targets expressing the corresponding Prdx5 isoform

To evaluate whether these peptides could be naturally produced by intracellular processing, we used AMAPIKVRL-loaded T2 cells to first derive AMAPIKVRL-specific CTL lines from three HLA-A2* donors. One out of these CTL lines was cytotoxic for LCL targets pulsed with the inducing cognate peptide (data not shown). CTL clones, generated by limiting dilution from this CTL line, were then tested at an E:T cell ratio of 10:1 on HLA-A2+ peptide-loaded targets and on HLA-A2* melanoma 8959 cells from which Prdx5 and its isoforms were identified. Four CTL clones were able to recognize specifically the peptide when exogenously added or endogenously presented by HLA-A2* cells. T cell receptor repertoire analysis indicated that these clones were not sister clones but were independent since they used different TCRBV regions (BV21, clone 5; BV2, clone 18; BV13.5, clone 30 and BV5, clone 52). As shown in Fig. 6(A), these independent clones were cytotoxic for AMAPIKVRL peptide-pulsed LCL cells, but not for LCL cells unpulsed or pulsed with an irrelevant HLA-A2-binding peptide. In addition, they were able to lyse melanoma 8959 (Fig. 6B) and the lysis was HLA-A2 restricted being inhibited by anti-HLA-A2 mAb. Collectively, these data indicate that the AMAPIKVRL peptide can be naturally presented by cells which express the Prdx5Δ2,3 splice isoform.

Discussion

We here identified, in virtue of their ability to generate the unique immunogenic epitope (Prdx5115L) recognized by an autologous melanoma-specific HLA-A2-restricted CTL clone (23), two exon-skipping isoforms of the gene encoding the anti-oxidant enzyme Prdx5. The generation of the same epitope by mutant full-length and exon-skipping isoforms indicated that, in patient 8959, all Prdx5 products contributed to the strong immunogenicity of the tumor (23). In addition, the splicing event, each isoform encoded a dual binding peptide specific for non-polymorphic HLA-E and polymorphic HLA-A2 molecules.

Prdx5 belongs to a six-membered family of peroxidases that efficiently reduce peroxide substrates through a conserved cysteine residue within an active site pocket structure (24–28). In addition to protect living organisms from peroxide toxicity, they also regulate hydrogen peroxide signaling (24, 25). Over-expression or depletion of Prdx5 has been reported to influence cell viability (45–46). The human Prdx5 gene is localized on chromosome 11q13 and is composed by six exons and five introns (39). The mRNA encoding full-length Prdx5 contains two ATG initiation codons, giving a short or a long Prdx5 form (27, 45–48). The short form of Prdx5 (17 kDa) is translated from its second AUG codon, contains a weak peroxisomal targeting signal and is localized...
to the cytosol, the peroxisomes and the nucleus. Several alternatively spliced mRNA transcripts corresponding to this form have been recently identified, but no evidence could be obtained in favor of their translation (39). The long full-length human Prdx5 form is translated from the first AUG codon and contains an N-terminal 50 amino acids sequence which targets the 17-kDa protein to mitochondria which are major sources for generation of reactive oxygen species (27, 45/28).

CTL recognition of the Prdx5 115L peptide derived from either Prdx5Δ2 or Prdx5Δ2,3 indicated that the mRNA encoding these isoforms could be translated. However, in order for these translated products to be detected, treatment with the proteasomal inhibitor lactacystin was required. Such results, coupled to the lack of the conserved cysteine residue in the NH2-terminal region that is fundamental for catalytic activity of all peroxiredoxins (26–28), indicate that these Prdx5 splice isoforms are not functional and are unstable being rapidly degraded by the multicatalytic proteasomal protease complex. These isoforms can therefore be added to the list of defective ribosomal products identified by their ability to provide MHC ligands for T cell recognition (8) and such finding led to the hypothesis that they could fulfill a different, immunological role.

So far, a number of peptides derived from leader or nonleader sequences of proteins involved in stress response have been reported as capable of binding to HLA-E (12–14). This MHC class molecule has very limited polymorphism and preferentially binds nonamer peptides derived from the signal sequence of other class I molecules (4, 9). HLA-E can interact with CD94/NKGA or CD94/NKG2C heterodimers expressed at the cell surface of NK cells resulting, respectively, either in inhibition or in triggering of NK cell-mediated cytotoxicity and cytokine production (15, 49). In addition, recent data indicate that a CD8+ CTL subset, named NK-CTL, can recognize HLA-E (19–22). As result of the splicing events, Prdx5 isoforms encode specific peptides that could be presented by HLA-E. Functional assays ruled

Fig. 5. AMAPIKTHL and AMAPIKVRL induce HLA-E surface expression and render RMA-S/HLA-E cells susceptible to lysis by HLA-E-restricted CD8+ CTL. (Left panel) RMA-S/HLA-E pulsed with the indicated peptides were analyzed by flow cytometry for cell surface HLA-E expression with A6.136 mAb (IgM, anti-HLA class I, gray profiles) or with control mouse IgM (empty profiles). Numbers in each histogram refer to MFI values of cells stained with A6.136 mAb. (Right panel) RMA-S/HLA-E cells were incubated in presence or absence of saturating amounts (200 µM) of the indicated peptides, labeled with [51Cr] and analyzed for susceptibility to lysis by HLA-E-restricted CD8+ T cell lines derived from donors GF and DP at the E:T cell ratio of 10:1. Each value represents the mean of triplicates and the standard deviation did not exceed 5% in the cytotoxicity assays.
out engagement of HLA-E complexed with peptides derived from either isoform by CD94/NKG2A inhibitory or CD94/NKG2C activatory receptors. Lack of NKG2A-mediated NK inhibition indicated that, differently from HCV and HIV HLA-E-bound peptides (11, 50), complexes composed by HLA-E and Prdx5 splice peptides were not functionally relevant for down-regulation of NK cell activity. Prdx5 splice peptides behave more similarly to the peptide derived from the Hsp60 leader sequence that did not interact with CD94/NKG2 receptors. This Hsp60 peptide, however, could interfere with HLA-E-mediated protection by competing with class I peptides (13), whereas no such effect could be exerted by Prdx5 splice peptides. All together, our data exclude that HLA-E-bound Prdx5 splice peptides could provide a mechanism to either inhibit or enhance NK cell cytotoxicity in peptide-dependent manner. Both peptides were, however, recognized, although at low avidity, by HLA-E-restricted CD8+ CTL generated from healthy donors (20). In addition, we obtained evidence for natural processing and presentation of one of these Prdx5 peptides by melanoma cells in the context of HLA-A2. It can therefore be speculated that, when cells become subjected to over-oxidation, up-regulation of Prdx5 and splice-variants occur concomitantly with increased availability of splice variants-derived peptides. Through their ability to bind non-polymorphic HLA-E as well as polymorphic HLA class Ia antigens (like HLA-A2), they might lead to T-cell-mediated recognition of stressed cells. Indeed up-regulation of Prdx5 expression has been observed under human pathological conditions such as osteoarthritis and tendon degeneration (51, 52). In melanoma, Prdx5 can be up-regulated by different forms of cellular stress (an 8-fold increase upon exposure of melanoma cells to either hydrogen peroxide or TNFα, data not shown). Melanoma cell lines can also express HLA-E molecules although these HLA molecules are mainly retained inside the cell (53). IFNγ treatment allows the transfer of HLA-E to the membrane probably by supplying HLA-E-binding peptides, derived from the signal sequence of newly produced classical MHC class I and HLA-G molecules (53). Non-protective HLA-E-binding peptides, such as those derived from the Hsp60 signal sequence (13) or even by alternatively spliced Prdx5 isoforms, may be induced in stress conditions and could contribute to HLA-E stabilization leading to NK- or CTL-mediated recognition of tumor cells.

In conclusion, the Prdx5 gene can generate multiple mRNA and corresponding protein isoforms. Full-length Prdx5, through their hydrogen peroxide reducing function, protects cells from endogenous and environmental stresses. Alternatively spliced Prdx5 variants appear instead involved in immune regulation. When endogenous and environmental oxidative stress levels alter normal cellular processes and induce cell damage, as it might occur in several pathologies and cancer, dual binding peptides derived from Prdx5 variants may activate HLA class Ia- and class Ib-restricted T cells, thus contributing to the elimination of stressed cells.

**Funding**

Associazione Italiana per la Ricerca sul Cancro; Istituto Superiore di Sanità; Ministero dell’Istruzione, dell’Università e della Ricerca (MIUR-PRIN 2005, project 2005063335_004); FIRB-MIUR project-RBLA039LSF-001; Ministero della Salute: Ricerca Oncologica-Project of integrated program 2006–2008.

**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>Hsp60</td>
<td>heat shock protein 60</td>
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<td>K562/HLA-E</td>
<td>HLA-E*01033-transfected derivative of K562</td>
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<td>LCL</td>
<td>lymphoblastoid cell line</td>
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<td>MFI</td>
<td>mean fluorescence intensity</td>
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<td>ORF</td>
<td>open-reading frame</td>
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References


