

Tumor-Associated Antigen Preferentially Expressed Antigen of Melanoma (PRAME) Induces Caspase-Independent Cell Death *In vitro* and Reduces Tumorigenicity *In vivo*

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Abstract

Preferentially expressed antigen of melanoma (PRAME) is expressed in a wide variety of tumors, but in contrast with most other tumor associated antigens, it is also expressed in leukemias. The physiologic role of PRAME remains elusive. Interestingly, PRAME expression is correlated with a favorable prognosis in childhood acute leukemias. Moreover, a high expression of PRAME seems to be predominantly found in acute leukemias carrying a favorable prognosis. On these clinical observations, we assumed that PRAME could be involved in the regulation of cell death or cell cycle. In this study, we show that transient overexpression of PRAME induces a caspase-independent cell death in cultured cell lines (CHO-K1 and HeLa). Cells stably transfected with PRAME also exhibit a decreased proliferation rate due, at least partially, to an elevated basal rate of cell death. Immunocytochemistry of a FLAG-tagged PRAME, *in vivo* imaging of an enhanced green fluorescent protein-tagged PRAME, and Western blotting after cell fractionation reveal a nuclear localization of the protein. Using a microarray-based approach, we show that KG-1 leukemic cells stably transfected with PRAME present a significant decrease of expression of the heat-shock protein Hsp27, the cyclin-dependent kinase inhibitor p21, and the calcium-binding protein S100A4. The expression of these three proteins is known to inhibit apoptosis and has been associated with an unfavorable prognosis in a series of cancers. Finally, repression of PRAME expression by a short interfering RNA strategy increases tumorigenicity of K562 leukemic cells in nude mice. We suggest that all these observations might explain the favorable prognosis of the leukemias expressing high levels of PRAME. (Cancer Res 2005; 65(16): 7348-55)

Introduction

During the last couple of years, several classes of human tumor-associated antigens (TAA) recognized by autologous CTLs have been identified (1). An antitumor CTL response can be induced by antigens encoded by genes overexpressed in tumors. Preferentially expressed antigen of melanoma (PRAME) was identified as a gene encoding an HLA-A24-restricted antigenic

peptide presented to an autologous tumor-specific CTL clone derived from a melanoma cell line called LB-33.MEL (2). PRAME encodes a putative protein of 509 amino acids, the function of which is still unknown. Whereas most normal tissues do not express PRAME, a very low expression was observed in testis, placenta, endometrium, ovary, and adrenals (1). PRAME expression was found in several tissues and cell lines derived from melanoma, lung, kidney, and head and neck carcinomas. Unlike the other TAAs, PRAME was also expressed in adult as well as pediatric acute leukemias: in 40% to 60% of acute lymphoid leukemia (ALL) and in more than 60% of acute myeloid leukemia (AML) at diagnosis (3, 4). Interestingly, a high expression of PRAME seems to be predominantly found in acute leukemias carrying a favorable prognosis: AML with the (8;21) translocation, AML-M3 with the (15;17) translocation, and childhood B-ALL with or without the (12;21) translocation (5). In addition, overexpression of PRAME seems to be associated with significantly higher rates of overall and disease-free survival and lower relapse rate, compared with patients with no or low PRAME expression (4, 6). At least in one case, it has also been shown that leukemic cells are able to process the PRAME protein, to present its peptide on their cell surface by HLA-A24, and to be lysed by cytotoxic cells (7). Whereas those lines of evidence seemed to link prognosis and immune response in PRAME-positive malignancies, the unusually high response rate of t(8;21) and t(15;17) AML to chemotherapy suggested that nonimmune mechanisms may also determine the response to xenobiotics. The current study aims at evaluating the contribution of PRAME in the regulation of cell proliferation, programmed cell death, and tumorigenicity.

Materials and Methods

Cell Lines

A panel of cell lines was chosen according to their basal level of endogenous PRAME expression: the Chinese hamster ovary (CHO) transformed fibroblasts CHO-K1 cell line and the acute myeloblastic leukemia cell line KG-1 express no or very low amounts of PRAME³ whereas the HeLa cervical carcinoma and K562 chronic myelogenous leukemia cell lines express significant amounts of the protein. All cell lines were purchased from American Type Culture Collection (Manassas, VA).

KG-1 and K562 cell lines were grown at 37°C in a 5% CO₂ humidified atmosphere in RPMI 1640 (Life Technologies, Inc., Carlsbad, CA). CHO-K1 and HeLa cell lines were grown in F12-Ham and DMEM media, respectively (Life Technologies). All media were supplemented with 100 mL/L FCS (Life Technologies) and 10 mL/L glutamine (Life Technologies).

Note: N. Tajeddine and J.-L. Gala contributed equally to this work.

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³ Unpublished data.

PRAME cDNA Cloning

PRAME-EGFP. cDNA encoding PRAME gene was kindly provided by Dr. P. Coulie. PRAME cDNA was PCR amplified and cloned into the plasmid pEGFP-N1 (Clontech, Palo Alto, CA), which encodes an enhanced green fluorescent protein (EGFP), to obtain the translation in PRAME-EGFP fusion protein. It was constructed by cloning a PCR product generated by using a full-length PRAME cDNA as template with the following primers: PRMHS-XH 5'-TCAGATCTCGAGGCCAGCCTAAGTCGCTTCAAA-3' and PRMHA-BA 5'-TAACCGGTGGATCCTTAGGCATGAAACAGGGGCACA-3'. These PRAME-specific primers were designed to generate a *Xho*I restriction site immediately upstream of the ATG initiation codon and a *Bam*HI restriction replacing the TGA stop codon. This PCR product was digested with *Bam*HI and *Xho*I, then cloned into pEGFP-N1 to generate pPRAME-EGFP, the plasmid carrying the full PRAME sequence fused in-frame to EGFP.

PIRES-PRAME. pPRAME-EGFP was digested with *Xho*I and *Bam*HI then cloned into the pBK-CMV plasmid (Stratagene, La Jolla, CA) to generate pBK-PRAME. Then, the pBK-PRAME vector was digested by *Xma*I and *Bam*HI and cloned into the bicistronic vector pIRES-hrGFPII (Stratagene), which encodes a humanized recombinant green fluorescent protein (hrGFP) to generate pIRES-PRAME. In this construct, PRAME was tagged with a FLAG epitope at the COOH-terminal extremity to generate the PRAME-FLAG protein.

All the constructions were verified by sequence analysis.

Cell Transfection and Clone Selection

For transient transfection assay, the pPRAME-EGFP and pIRES-PRAME plasmids as well as the two control plasmids encoding only EGFP (pEGFP-N1) or hrGFP (pIRES-hrGFPII) were transfected into the CHO-K1 or HeLa cell lines with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) and into the KG-1 cell line with DMRIE-C (Invitrogen) following the instructions of the manufacturer.

For selection of stably transfected CHO-K1 and KG-1 cells, geneticin (Life Technologies) was added to the culture medium 48 hours after transfection at a concentration of 500 μ g/mL. After 5 weeks of selection by geneticin, these stably transfected cells were screened by real-time PCR for the expression of PRAME transcript.

Quantification of PRAME by Real-Time PCR

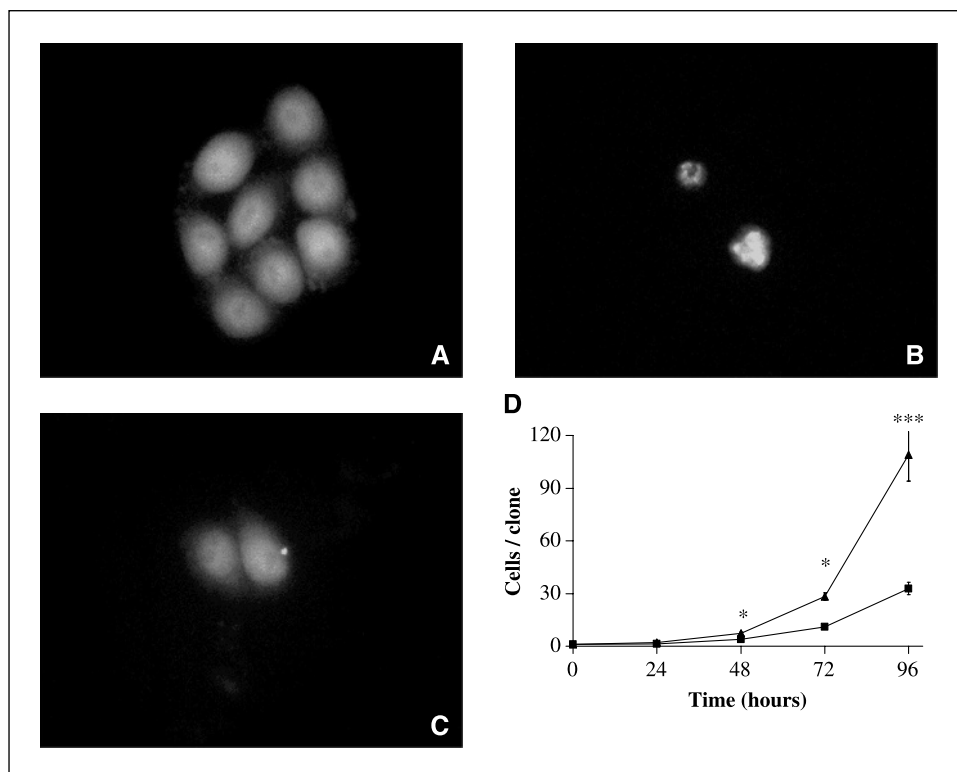
Primers and probes were all designed for Taqman amplification using Primer Express software (Perkin-Elmer Biosystems, Boston, MA). PRAME-specific primers and probe were designed according to GenBank sequence (accession no. NM_006115) as follows: forward primer 5'-CTCTATGTG-GACTCTTTATTTTCCTTAGA-3' (nucleotides 686-715), reverse primer 5'-CGAAAGCCGGCAGTTAGTTATT-3' (nucleotides 768-790), and fluorescent probe 5'-CTGGATCAGTTGCTCAGGCACGTGA-3' (nucleotides 722-746). The Abelson (Abl) housekeeping gene (GenBank accession no. U07563) was used to normalize PRAME expression. Primers and probe were as follows: forward primer 5'-AGCCATGGAGTACCTGGAGAAG-3' (nucleotides 68,824-68,845), reverse primer 5'-TTCTCCCCTACCAGGCAGTTT-3' (nucleotides 70,708-70,728), and fluorescent probe 5'-AACTTCATCCACAGA-GATCTTGCTGCCCC-3'. Each probe contained a 5'-terminal fluorescein reporter (6-carboxyfluorescein, FAM) and a 3'-terminal quencher (6-carboxy-tetramethyl-rhodamine, TAMRA).

Western Blot

Primary antibodies used in this study were anti-FLAG antibody, dilution 1:1,000 (Stratagene), anti-PRAME AH-151 antibody, dilution 1:200 (kindly provided by Dr. P. Coulie), anti-S100A4 antibody, dilution 1:500 (Dako, Glostrup, Denmark), anti-p21 antibody, dilution 1:500 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), and anti-Hsp27 antibody, dilution 1:200 (Santa Cruz Biotechnology).

After trypsinization, cells were harvested, rinsed twice with ice-cold PBS, and counted with a hemocytometer. Ten million cells were resuspended in hypotonic lysis buffer containing 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 μ mol/L DTT, and 1% protein inhibitor cocktail (Sigma, St Louis, MO). For subcellular localization experiments, cytoplasm and nuclei were separated with the CellLytic Nuclear Extraction Kit (Sigma) as described by the protocol of the manufacturer. The extracts were diluted in Laemmli buffer [Tris 50 mmol/L (pH 6.8), SDS 2%, bromophenol blue 0.01%, glycerol 10%, β -mercaptoethanol 5%] and heated at 95°C for 3 minutes. Samples were electrophoresed on 10% SDS-polyacrylamide gels and transferred on 0.2 μ m nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were saturated in "milk block" [Tris 20 mmol/L, NaCl 137 mmol/L, Tween

Figure 1. Effects of overexpression of EGFP and PRAME-EGFP fusion protein on CHO-K1 cells. A-C, CHO-K1 cells were transfected with pEGFP-N1 vector (A) or PRAME-EGFP vector (B and C). Exposure time was about 200 ms in A and B and about 30 seconds in C. Magnification, $\times 63$. D, number of cells per clone was counted in CHO-K1 cells stably expressing EGFP ($\blacktriangle$) or PRAME-EGFP ($\blacksquare$). *, $P < 0.05$; ***, $P < 0.001$.



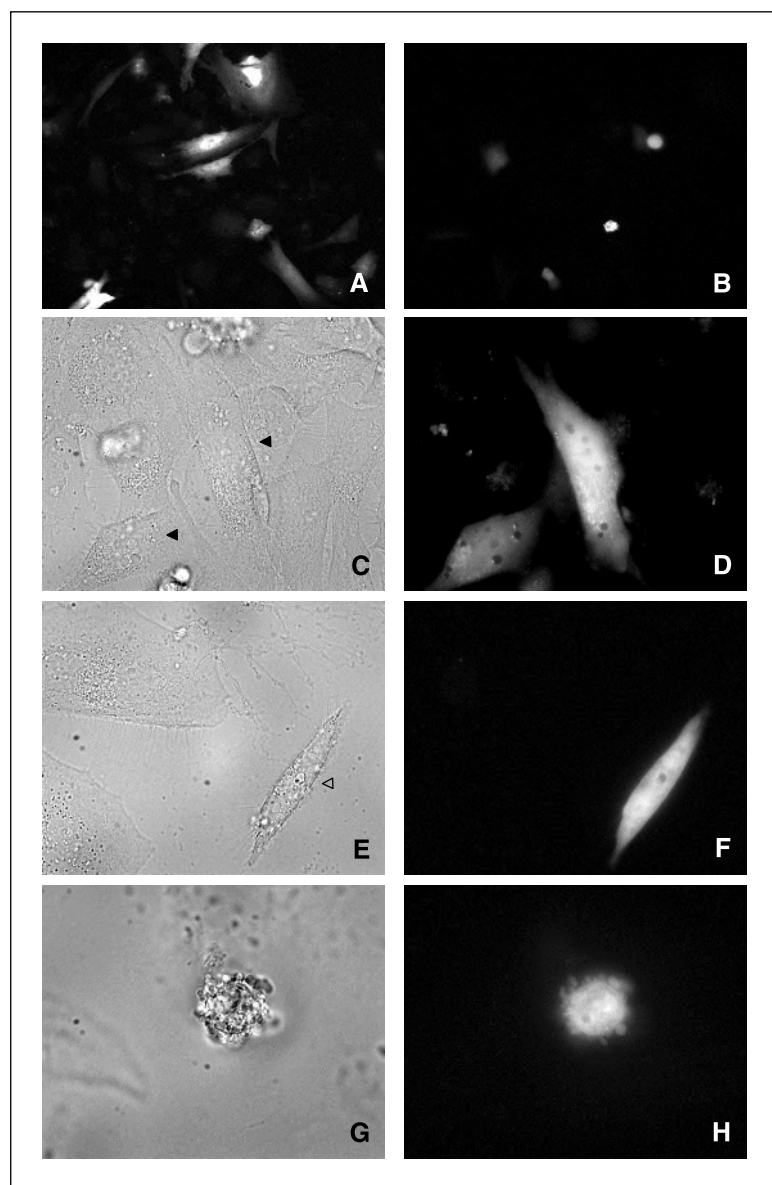


Figure 2. Morphologic changes after transfection with pIRES-hrGFP or pIRES-PRAME in HeLa cells. A, C, and D, HeLa cells transfected with pIRES-hrGFP; B, E, F, G, and H, HeLa cells transfected with pIRES-PRAME. A and B, cells were visualized with FITC filters (Ex/Em, 485/510) at low magnification ($\times 20$). C, E, and G, images were achieved at high magnification ($\times 63$) with bright-light illumination. D, F, and H, the same fields as in C, E, and G, respectively, but were captured with FITC filters. Note that pIRES-hrGFP-transfected cells (C, closed arrow) were not morphologically different than untransfected cells. On the contrary, pIRES-PRAME-transfected cells (E, open arrow) severed support attachments, showed cytoplasmic vacuolization, or were then disintegrated into apoptotic bodies (G and H).

20 0.05% (pH 7.6), and skimmed milk 5%] for 1 hour at room temperature. Then, these were incubated with the primary antibody overnight at 4°C. After incubation with the secondary antibody coupled to peroxidase (dilution 1:20,000), peroxidase was detected with Western Lighting Chemical Reagent + (Perkin-Elmer) on enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom) hyperfilm.

PRAME Subcellular Localization

For microscopic analysis, cells were plated on glass coverslips and were observed with a Zeiss Axiovert S-100 microscope (Zeiss, Oberkochen, Germany). GFP was detected by acquisition of fluorescence (Em, 510 nm) images of cells excited at 485 nm (objective 63 $\times$, water immersion, numerical aperture 1.2). Individual images were acquired with a Zeiss AxioCam camera.

For PRAME-FLAG visualization by immunocytochemistry, cells were fixed in 4% paraformaldehyde for 15 minutes at 4°C and permeabilized with 0.2% Triton/PBS for 10 minutes. PRAME-FLAG was detected with a monoclonal anti-FLAG antibody (dilution 1/400; Stratagene), a biotinylated anti-mouse goat immunoglobulin G, and Texas red-conjugated avidin following the instructions of the manufacturer (Vector Laboratories, Burlingame, CA).

Cell Proliferation Assay

Cells transfected with the pPRAME-EGFP plasmid or with a control plasmid were sorted by flow cytometry (FACSCalibur, Beckton Dickinson, Palo Alto, CA) to retrieve 100,000 EGFP positive cells, which were then plated on a T75 flask. The cell numbers of at least 10 clones were counted at 24, 48, 72, and 96 hours.

Measurement of Cellular DNA Content

Seventy-two hours after transfection, cells were harvested, washed in PBS, fixed in 70% ethanol, again washed in PBS, and incubated in a staining mixture containing 50 μ g/mL propidium iodide, 0.1 mg/mL RNase A, and 0.05% Triton X-100 for 40 minutes at 37°C. After washing in PBS, cells were analyzed by flow cytometry. To selectively analyze cells expressing transfected cDNA, only cells manifesting a high level of GFP fluorescence were counted.

Caspase Activation Assay

Caspase activation was detected 72 hours after transfection with pIRES-hrGFP or pIRES-PRAME plasmids by using the fluorochrome inhibitor of polycaspases SR-VAD-FMK (Immunochemistry Technologies, Bloomington, MN) following the instructions of the manufacturer. Briefly, the cells were stained with SR-VAD-FMK at 37°C, trypsinized, washed, cytospun, and

analyzed by fluorescence microscopy. Only cells exhibiting EGFP fluorescence signal were counted. SR-VAD-FMK measurement was done by acquisition of fluorescence images (Em, 645 nm) of cells excited at 560 nm (objective Achromplan 40×). For each experiment, at least 200 cells were counted.

Microarray Assay

Microarray assay was done on a DualChip human general (Eppendorf Array, Namur, Belgium). The chip contains a range of genes involved in DNA damage, oxidative stress, cell signaling, cell proliferation, DNA repair, transcription, apoptosis, and inflammation. Total RNA was isolated using the RiboPure Kit. Quantification and integrity of RNA were assessed by the Agilent 2100 Bioanalyser and RNA 6000 LabChip Kit (Agilent Technologies, Palo Alto, CA). Synthesis of labeled cDNA and hybridization using biotinylated-labeled cDNA were made following the instructions of the manufacturer. Hybridization was detected by using a cyanin-3 streptavidin conjugate (Amersham Pharmacia Biotech, Buckinghamshire, England) and quantified with a confocal laser scanner (ScanArray 4000XL, Perkin-Elmer Life Sciences; ImaGene 4.1 software, BioDiscovery, El Segundo, CA). The fluorescence intensity of each DNA spot (average intensity of each pixel present within the spot) was calculated using local mean background subtraction. A signal was accepted if the average intensity following background subtraction was at least 2.5 times higher than the local background. Each experiment was repeated six times. The

intensity values of the control and test DNA spots were averaged and used to calculate the intensity ratio between the reference and the test samples. Data were normalized using both internal standard and house keeping genes, as described (8). Ratios outside the 95% confidence interval were determined to be significantly different following pIRES-PRAME transfection.

PRAME Short Interfering RNA Design

PRAME-short interfering RNA (siRNA) was purchased by Invivogen (Toulouse, France) and had the following sequence: 5'-TTCATCACGTGCCT-GAGCAAC-3'. It was cloned in a psiRNA-h7SKblast1 expression vector (Invivogen). As control, we used a siRNA targeted to green fluorescent protein (GFP-siRNA). Control and PRAME-siRNA-encoding plasmids were transfected into K562 cells. Cells were selected by blasticidin during at least 6 weeks. PRAME silencing was confirmed by real-time PCR and Western blot.

Tumorigenicity in Nude Mice

Approximately 10^7 transfected K562 cells resuspended in 200 μ L of a serum-free culture medium/Matrigel mixture (1:1) were injected s.c. into the left flank of female BALB/c nude mice. Tumor-bearing mice were sacrificed after 3 weeks and tumor masses were excised and weighed.

Statistical Analysis

Data are presented as means $\pm$ SE. Student's *t* test was used to determine statistical significance.

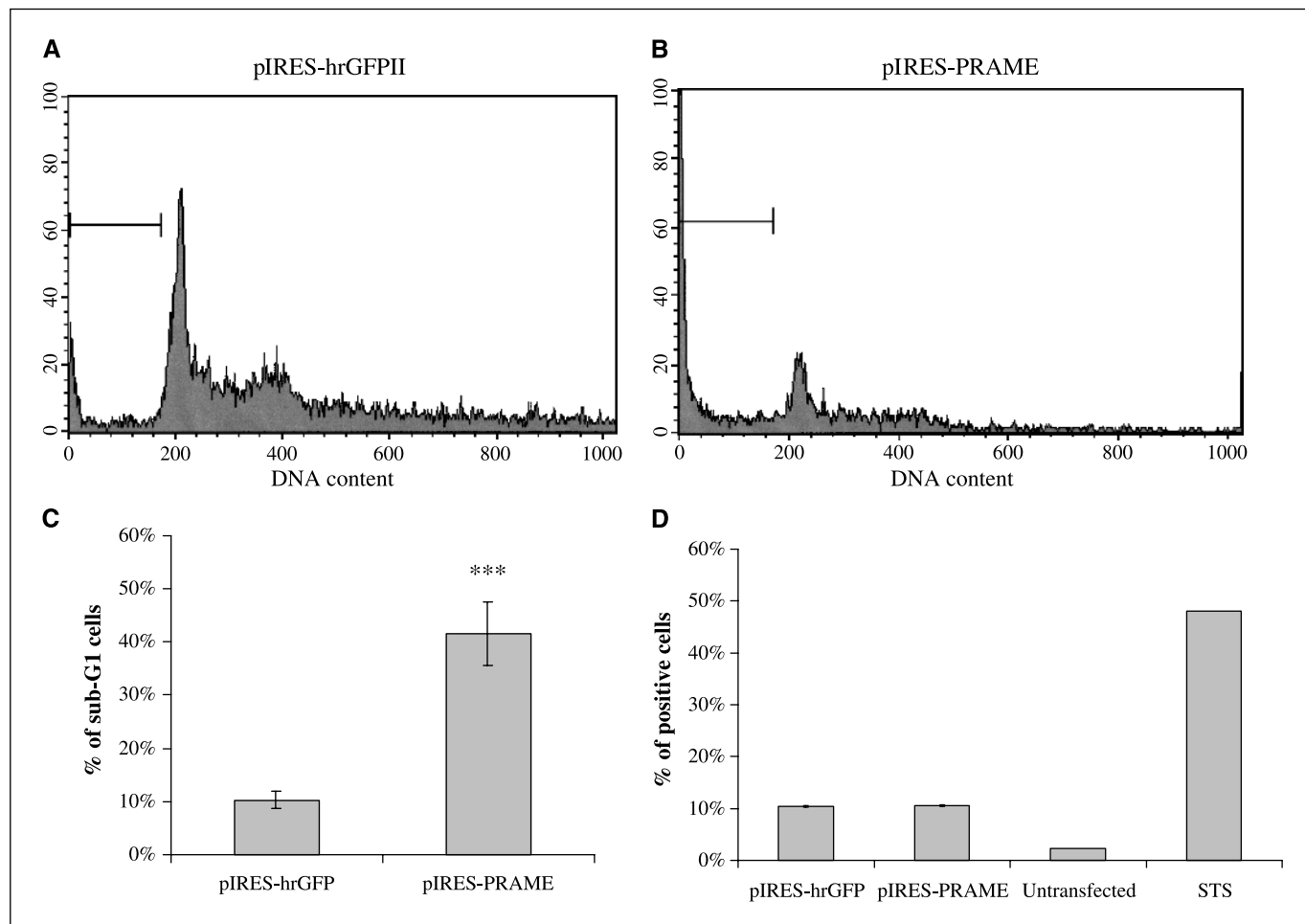


Figure 3. Caspase-independent cell death induced by overexpression of PRAME. *A* and *B*, DNA content was measured by propidium iodide staining 72 hours after transfection of CHO-K1 with pIRES-hrGFPII (*A*) or pIRES-PRAME (*B*). Only cells presenting a strong GFP signal were counted. *C*, percentage of cells with sub-G₁ DNA content in GFP-positive cells transfected with pIRES-hrGFPII or pIRES-PRAME. Columns, mean of five experiments; bars, SE. ***, $P < 0.001$. *D*, caspase activity measured by SR-VAD-FMK in CHO-K1 cells 72 hours after transfection with pIRES-hrGFPII or with pIRES-PRAME (columns, mean of three experiments; bars, SE). As a control, untransfected CHO-K1 cells were treated for 15 hours with staurosporine 1 μ M (STS).

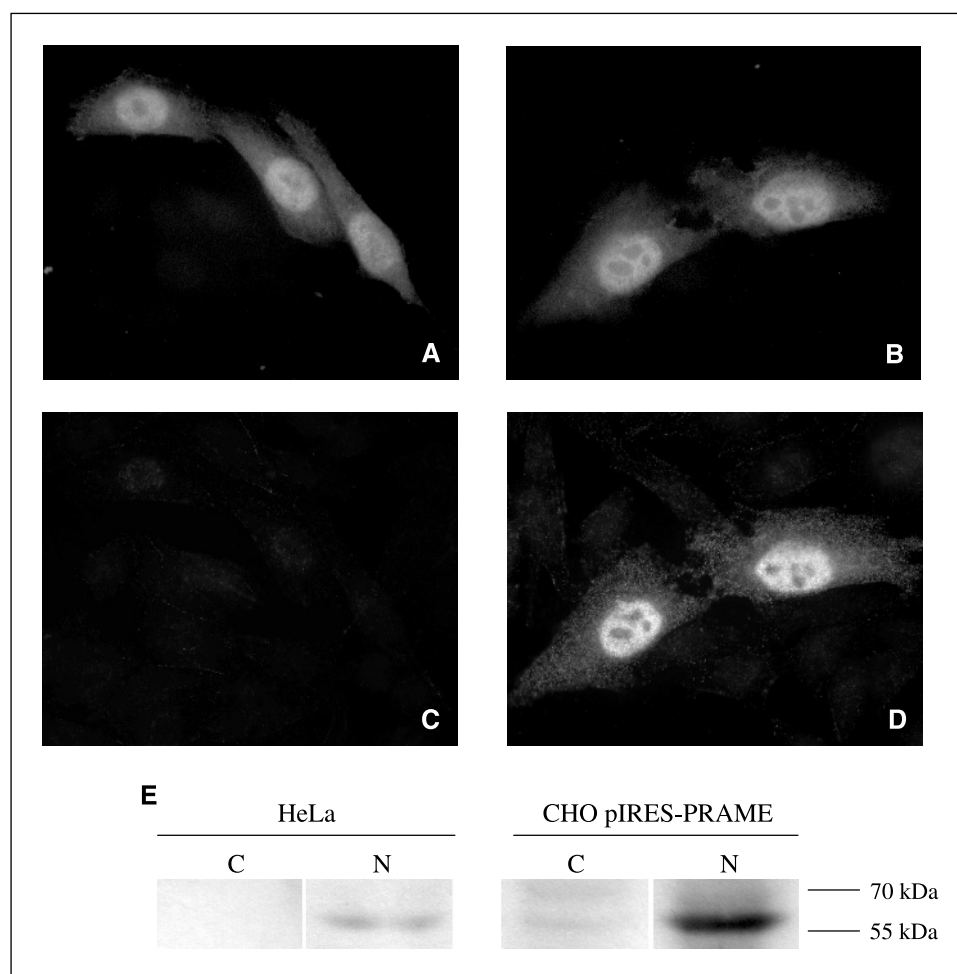


Figure 4. Nuclear localization of PRAME. Immunocytochemistry experiment in CHO-K1 cells transfected with pIRES-hrGFP or pIRES-PRAME. *A* and *B*, cells were visualized with FITC filters to detect the GFP protein in pIRES-hrGFP-transfected cells (*A*) or in pIRES-PRAME-transfected cells (*B*). *C* and *D*, cells were visualized with Texas red filters (Ex/Em, 560/645) to detect the presence of PRAME protein using an anti-FLAG antibody (see Materials and Methods) in pIRES-hrGFP-transfected cells (*C*) or in pIRES-PRAME-transfected cells (*D*). Magnification, $\times 63$. *E*, subcellular localization of PRAME was analyzed by Western blotting with an anti-PRAME antibody after cell fractionation of HeLa cells expressing PRAME natively and of CHO cells transfected with pIRES-PRAME (*C*, cytosolic fraction; *N*, nuclear fraction).

Results and Discussion

PRAME-EGFP sublocalizes in the nucleus and inhibits cell proliferation. The computational analysis of the primary sequence of PRAME reveals one mitochondrial targeting sequence at its NH₂-terminal end and five nuclear localization signals (PSORT II, Kenta Nakai, Tokyo, Japan). This prompted us to study PRAME subcellular localization by overexpressing a PRAME-EGFP fusion protein in CHO-K1 cells. We chose to insert PRAME at the NH₂-terminal end of EGFP to preserve its mitochondrial targeting sequence. Forty-eight hours after transfection with the control plasmid, healthy clones strongly expressed control EGFP in the cytosol as well as in the nucleus (Fig. 1*A*). Surprisingly, we did not observe any clone strongly expressing the PRAME-EGFP fusion protein. In highly fluorescent cells, a dramatic altered morphology and the trypan blue exclusion method were suggestive of cell death, which was also in line with the observation of many fluorescent cellular debris and floating fluorescent cells (Fig. 1*B*). Similar results were obtained with HeLa cells (data not shown). However, clones expressing very low amounts of PRAME-EGFP, all of small size, could be found occasionally. In these cells, PRAME-EGFP accumulated into the nucleus or the perinuclear region (Fig. 1*C*). These clones were selected during 6 weeks with geneticin to obtain a polyclonal population overexpressing PRAME-EGFP. The proliferation rate of such population was significantly lower than that of control EGFP-expressing cell (Fig. 1*D*). This observation might result either from a blockade of cell cycle or from an increasing basal rate of cell death or both.

Overexpression of PRAME-FLAG induces caspase-independent cell death. Despite the interest of this observation, we suspected that the quite large EGFP fused to PRAME could modify the normal function or the targeting of the latter. To exclude such an extraneous effect, we introduced the PRAME gene in a bicistronic vector. This strategy allowed the expression of PRAME to be monitored at a single cell level due to expression of hrGFP on the same transcript. PRAME was nevertheless tagged with a short FLAG epitope to allow its localization by immunocytochemistry.

HeLa cells transfected with pIRES-PRAME showed a dramatically altered morphology when compared with pIRES-hrGFP-transfected

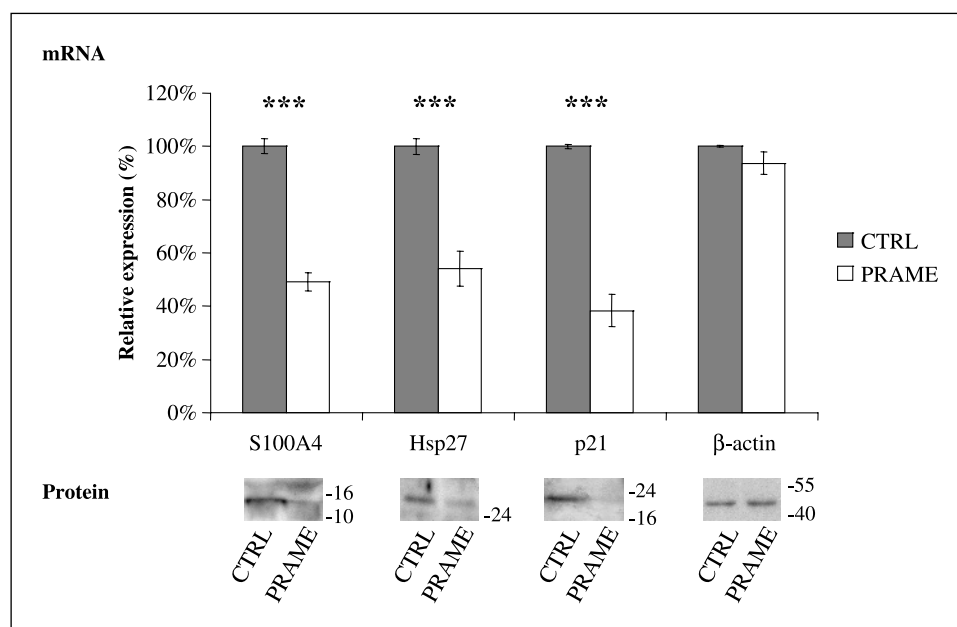
Table 1. Effect of PRAME overexpression on cell cycle in KG-1 cell line

	Sub-G ₁ (%)	G ₀ -G ₁ (%)	S (%)	G ₂ -M (%)
pIRES-hrGFP	4.4 $\pm$ 0.2	49.9 $\pm$ 0.9	22.8 $\pm$ 1.1	17.2 $\pm$ 0.9
pIRES-PRAME	7.7 $\pm$ 1*	43.8 $\pm$ 4.3*	24.8 $\pm$ 1.2	16.8 $\pm$ 1

NOTE: Cellular DNA content was evaluated after propidium iodide staining using fluorescence-activated cell sorting analysis.

**P* < 0.05 versus pIRES-hrGFP. Mean $\pm$ SE of four experiments.

Figure 5. Microarray analysis of pIRES-hrGFP or pIRES-PRAME overexpressing KG-1 cells. Histogram shows relative mRNA amounts of S100A4, Hsp27, p21, and β -actin in pIRES-hrGFP- and pIRES-PRAME-transfected cells (top). Arbitrarily, gene expressions in pIRES-hrGFP transfected were fixed at 100%. Note that expression of housekeeping genes such as β -actin was not significantly different in pIRES-hrGFP- and pIRES-PRAME-transfected cells. mRNA data were confirmed by Western blotting (bottom). ***, $P < 0.001$.



cells (Fig. 2). pIRES-PRAME-transfected cells severed their attachments to the support, with loss of filipodia, and showed cytoplasmic vacuolization and blebs (Fig. 2E and F). At a later stage, transfected cells disintegrated into apoptotic bodies (Fig. 2G and H). On the same coverslip, untransfected cells showed a normal morphology. We observed similar morphologic changes in CHO cells transfected with pIRES-PRAME.

Cell cycle and programmed cell death were studied by DNA content analysis. As shown in Fig. 2A, a significant increase in the percentage of cells with a hypodiploid (sub- G_1) DNA content was observed in HeLa and CHO cells overexpressing PRAME in comparison with controls, suggesting a higher basal level of programmed cell death (Fig. 3A-C). This cell death was caspase independent because, using a fluorescent caspase detection assay, no activation of caspases was observed 72 hours after transfection with the pIRES-PRAME plasmid (Fig. 3D). Concomitantly, the inhibition of caspases by the pan-caspase inhibitor z-VAD-FMK did not reduce the proportion of hypodiploid cells after transfection with pIRES-PRAME (data not shown).

Using an anti-FLAG antibody, we confirmed the nuclear targeting of PRAME by immunocytochemistry (Fig. 4A-D). The nuclear targeting of PRAME was also confirmed by cell fractionation and Western blot with an anti-PRAME antibody in CHO cells transfected with pIRES-PRAME and in HeLa cells natively expressing PRAME (Fig. 4E).

We then studied KG-1 leukemic cells stably transfected with pIRES-PRAME and selected with geneticin during at least 5 weeks. Quantitative real-time PCR showed that this selected population overexpressed PRAME by about 1,000 times more in comparison with cells transfected with pIRES-PRAME. For comparison, it is interesting to note that in bone marrow from healthy donors, PRAME is expressed at the same level as in KG-1 cells, whereas it can be expressed up to 40,000 times more in bone marrow from AML patients.⁴ We analyzed the spontaneous amount of cell death

by measuring the DNA content. The pIRES-PRAME stably transfected cells exhibited a significant increase in the percentage of hypodiploidy compared with the pIRES-hrGFP-transfected population. The percentage of cells in G_0/G_1 phase was significantly decreased in pIRES-PRAME-transfected cells whereas the number of cells in S and G_2 -M phases was not different between the two groups (Table 1).

Stable expression of PRAME in leukemic cells down-regulates S100A4, Hsp27, and p21. Using a microarray-based approach, we analyzed the gene expression profile of KG-1 cells stably transfected with pIRES-hrGFP or pIRES-PRAME. We found that overexpression of PRAME induced a significant decrease of transcription of the cyclin-dependent kinase inhibitor p21, the heat shock protein Hsp27, and the EF-hand calcium-binding protein S100A4. By Western blot, expression of the three proteins was also decreased (Fig. 5).

The S100A4 protein is specifically expressed in murine and human metastatic tumor cells (9). Its expression in nonmetastatic murine and human cell lines results in a more malignant phenotype (10). Moreover, a high level of S100A4 is correlated with a poor prognosis in several types of cancer (reviewed in ref. 11). However, the mechanisms that associate S100A4 expression with tumor progression and development of metastasis remain incompletely understood. S100A4 may exert its effects on metastasis progression by regulating the expression of the endopeptidases matrix metalloproteinases (MMP; ref. 12). Indeed, the degradative activity of MMPs can result in cancer progression by affecting tumor angiogenesis, tumor growth, and/or metastasis (13). S100A4 also induces the activation of MMP-2 and MMP-9 (14), two enzymes involved in the invasive behavior in ALL and AML cell lines (13). Based on these biological evidences, the favorable prognostic effect of PRAME in AML and ALL could be explained by a down-regulation of S100A4 and a consecutive reduction of activation of MMPs.

Alternatively, it has been reported that S100A4 sequesters and disables the tumor suppressor p53 (15). The down-regulation of S100A4 could therefore abrogate its inhibiting effect on p53, thus favoring apoptosis. However, this is still a matter of controversy (16).

⁴ Unpublished observations.

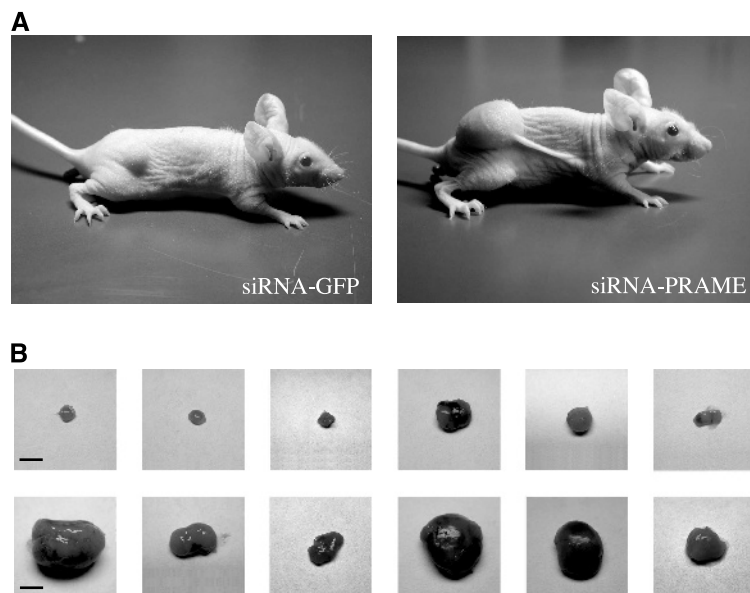


Figure 6. Tumorigenicity of PRAME knockdown leukemic cells in nude mice. A, examples of nude mice injected with control siRNA-transfected cells or PRAME-siRNA-transfected cells. B, excised tumors of nude mice 3 weeks after injection with control siRNA-transfected cells (top) or PRAME-siRNA-transfected cells (bottom); bar, 0.5 cm. C, tumor weights are quantified.

Hsp27 is a small heat shock protein found in normal and cancer cells. This protein is a molecular chaperone and inhibits key effectors of the apoptotic pathways such as apoptosis and caspases (for review, see ref. 17). Breast cancer, ovarian cancer, prostate adenocarcinoma, osteosarcoma, endometrial cancer, and leukemias express an increased level of Hsp27 when compared with nontransformed cells. Increased expression of Hsp27 in these malignant diseases seems to be correlated with oncogenesis and chemotherapy resistance, presumably due to its capacity to disable apoptosis. In particular, expression of Hsp27 in AML has been associated also with chemotherapy resistance (18).

Overexpression of PRAME in KG-1 cell line also down-regulates the transcription of the tumor suppressor p21. This observation is surprising because p21 is an inhibitor of cell proliferation. However, recent data have shown that p21 was implicated in both pro-apoptotic and antiapoptotic response to chemotherapy (for review, see ref. 19). Especially in AML, p21 expression is significantly correlated to resistance to chemotherapy and constitutes an independent factor of unfavorable prognosis (20, 21). *In vitro*, p21 expression protects AML blasts against etoposide-induced apoptosis (22).

Thus, down-regulation of these three proteins subsequent to the overexpression of PRAME could contribute to improve the prognosis of PRAME-expressing acute leukemias.

Down-regulation of PRAME promotes tumorigenicity of leukemic cells in nude mice. To investigate the potential implication of PRAME in tumorigenicity, PRAME-siRNA-transfected leukemic K562 cells were injected into nude mice. We confirmed by real-time PCR and Western blotting that PRAME was down-regulated more than 30 times in PRAME-siRNA-transfected cells. We observed that PRAME-siRNA-transfected cells proliferated

more rapidly *in vivo* than GFP-siRNA-transfected cells. The tumor weight was significantly higher in mice injected with PRAME-siRNA than in mice injected with GFP-siRNA-transfected cells (661.5 ± 236.6 mg versus 59.9 ± 25.7 mg; $n = 6$; $P = 0.009$) (Fig. 6). These results show that PRAME down-regulation promotes proliferation of leukemic cells *in vivo*.

In conclusion, this study shows that overexpression of PRAME in cultured cells induces a caspase-independent cell death responsible, at least partially, for a slower proliferation rate. Moreover, transfected KG-1 cells expressing levels of PRAME in the range observed in leukemic patients present a significant decrease in the transcription of S100A4, Hsp27, and p21. Finally, down-regulation of PRAME by siRNA promotes tumorigenicity of leukemic cells in nude mice *in vivo*. We suggest that these observations could explain the good prognosis of PRAME-expressing childhood acute leukemias.

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