PP2A activity is controlled by methylation and regulates oncoprotein expression in melanoma cells: A mechanism which participates in growth inhibition induced by chloroethylnitrosourea treatment

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Abstract. Protein phosphatase 2A (PP2A), an Akt pathway inhibitor, is considered to be activated by methylation of its catalytic subunit. Also PP2A downregulation was proposed to take part in carcinogenesis. Recently, PP2A activation was shown to be activated in response to DNA damage. To obtain further information on the role of PP2A in tumors and response to DNA damage, we investigated the relationship between PP2A methylation and activity, cell proliferation, Akt activation, c-Myc expression and PTEN activity in B16 melanoma cells untreated and after chloroethylnitrosourea (CENU) treatment. In untreated cells, okadaic acid, an antagonist of PP2A methylation, inhibited PP2A activity, stimulated cell proliferation, increased Akt activation and c-Myc expression. Xylulose-5-phosphate, an agonist of PP2A methylation, increased PP2A activity, decreased cell proliferation, Akt activation and c-Myc expression. However, both PP2A methylation modulators increased PTEN activity. During the response to CENU treatment, PP2A methylation and activity were strongly increased, Akt activation and c-Myc expression were decreased. However PTEN activity was increased. After tumor cell growth recovery, these modifications were moderately decreased. PP2A methylation was quantified and correlated positively with PP2A activity, and negatively with criteria for cell aggressiveness (cell proliferation, Akt activation, c-Myc expression). Based on these data, PP2A methylation status controls PP2A activity and oncoproteins expression and PP2A is strongly activated after CENU treatment thus partly explaining the growth inhibition in response to this agent. It follows that PP2A promethylating agents are potential candidates for anticancer drugs.

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Introduction

Among the major cellular proteins, protein phosphatase 2A (PP2A) is the most common phosphatase accounting for about 0.05-0.1% of the total cell protein content (1). PP2A is one of the major regulatory protein of the Akt/PKB pathway. PP2A inactivates Akt by dephosphorylation (1,2). PP2A has a regulatory role in the Akt/PKB pathway together with phosphatase and tensin homolog deleted on chromosome 10 (PTEN, also known as MMAC1). The Akt/PKB pathway is implicated in cell proliferation, protein synthesis, resistance to apoptosis and is often mutated in cancer (2-4). PP2A activity was shown to be dramatically decreased in tumor cells suggesting that PP2A dysfunction might contribute to cell transformation (5,6). Recently PP2A was shown to be activated in response to DNA damage thus inhibiting polo-like kinase 1 and inducing mitosis blockade (7). The response to DNA damage may also involve the Akt pathway through the inhibition of p53 leading to the transcriptional regulation of a variety of genes involved in cell cycle control and cell survival (8-10).

We previously reported that B16 melanoma cells responded to chloroethylnitrosourea (CENU)-induced DNA damage (11), by G_2/M cell cycle arrest, biopolar spindle blockade, glycolysis downregulation followed by adaptative and/or redifferentiation processes (12,13). This suggested the inhibition of the Akt/PKB pathway as a possible consequence of PP2A activation in response to DNA damage.

PP2A activity is regulated by posttranslational modification by phosphorylation or methylation (14). Phosphorylation on Tyr^{204} and Tyr^{307} of the C subunit can inhibit PP2A activity (1). However, PP2A activity mainly depends on methylation (1). PP2A is a trimeric serine/threonine phosphatase: the regulatory subunit B, is recruited by a C-A dimer composed of the catalytic subunit C (PP2AC), and the structural subunit A. The recruitment takes place when C gets carboxyl-methylated

on the terminal Leu^{309} and results in the assembling of the active PP2A trimer (1,5,15). PP2A methylation is considered to result from the opposing activities of a specific S-adenosyl-L-methionine (SAM)-dependent leucine carboxymethyltransferase and a specific carboxyl methylesterase, the regulation of which is not fully understood (16,17). Furthermore, there are conflicting reports on the relationship between PP2A methylation and activity (1).

The goal of this article was to get deeper knowledge on the role of PP2A in tumors and in response to DNA damage. We investigated the relationship between PP2A methylation, activity and function (Akt/PKB expression and activation and c-Myc expression), in B16 melanoma tumor cells at baseline and in response to CENU treatment.

We show that PP2A methylation status is tightly linked to PP2A activity, that PP2A is strongly activated during the primary response to DNA damage, and that PP2A regulates melanoma tumor cell proliferation through the expression and activation of Akt and c-Myc both at baseline and in response to DNA damage. Our data are in favor of the implication of PP2A methylation in the mechanistics of loss of aggressiveness of B16 melanoma tumor cells and of a potential interest of PP2A methylation as a target for anticancer drug development.

Materials and methods

Chemicals. Okadaic acid (OA) (Sigma, Saint Quentin Fallavier, France) was prepared as a 100-μM stock in dimethyl sulfoxide (DMSO, Sigma). Xylulose-5-phosphate (X5P) was a gift from Dr L. Hecquet (CNRS, UMR6504, Aubiere, France) and was prepared as a 3-mM stock in 0.9% NaCl. Goat serum used for PTEN activity determination was from Sigma. A member of the CENU drug family, cystemustine (N'-(2-chloroethyl)-N-(2-(methylsulphonyl)-ethyl)-N'-nitrosourea) (Orphachem, Clermont-Ferrand, France) was prepared as 5 mM solution in 0.9% NaCl. L-[$^{14}CH_3$]-S-adenosylmethionine ([$^{14}CH_3$]-SAM) (specific activity 55 mCi.mmol^{-1}) was purchased from Amersham Bioscience (Buckinghamshire, UK). L-[$^{13}CH_3$]-methionine was purchased from Euriso-Top (Gif sur Yvette, France). TRIzol® reagent was purchased from Sigma. The PP2A immunoprecipitation phosphatase assay kit was purchased from Upstate (Lake Placid, NY). PTEN ELISA kit was from Echelon Biosciences (Salt Lake City, UT).

Cell culture. The transplantable B16 (F1) melanoma cells originating from C57BL6/6J Ico mice were obtained from ICIG (Villejuif, France) and adapted to grow in culture. The cells were maintained as monolayers in culture flasks in Eagle's MEM-glutaMAX medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (Sigma), 1 mM sodium pyruvate, 4 μg/ml gentamycin, 200 mM glutamine, 1X non-essential amino-acid solution (Invitrogen) and vitamins (Invitrogen).

Cell treatments. B16 melanoma cells were treated by okadaic acid (OA) at 5 nM or by xylulose-5-phosphate (X5P) at 50 μM for 48 h, an antagonist (5) and an agonist (18) of PP2A methylation, respectively. After treatments, cells were harvested by trypsinisation, counted and stored at -80˚C until analysis. In another set of experiments, B16 melanoma cells were exposed to CENU (at day 0) for 2 h at 200 μM, the culture medium was then rinsed with PBS. For the investigation of global cellular methylations, CENU-treated B16 melanoma cells were incubated with 0.8 μCi/ml of [$^{14}CH_3$]-SAM. After 16 h of incubation, cells were harvested, counted and stored at -80˚C until analysis. Three independent experiments were performed for each conditions. For cell culture labelling with L-[$^{13}CH_3$]-methionine, ^{1}H-^{13}C NMR spectroscopy experiments were performed as previously described (19). In Fig. 1, cell number was determined in each group of cell culture (UN, OA and X5P). The effect of OA and X5P on cell proliferation was determined as follows:

$$\frac{\text{Number of treated cells x 100}}{\text{Number of UN cells}} - 100$$

[$^{14}CH_3$]-SAM incorporation in proteins. For methyl groups incorporation in cytoplasmic and nuclear proteins, cells were Dounce homogenized with pestle 'B' in a fractionation buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA) containing protease inhibitor mixture. Complete cell lysis was checked by microscopy with trypan blue. Then lysed cells were centrifuged (10,000 g for 10 min at 4˚C). Supernatant was kept as the cytosolic fraction. The pellet was carefully washed with lysis buffer and centrifuged (10,000 g for 10 min at 4˚C). The supernatant was added to the cytosolic fraction. The pellet containing nuclei was further disrupted by ultrasonication in fractionation buffer (3 times 15 sec in ice) and centrifuged (10,000 g for 10 min at 4˚C). The supernatant was kept as the nuclear fraction. Proteins from the cytoplasmic and the nuclear compartments were precipitated by adding 4 volumes of cold acetone to the cytoplasmic and nuclear fractions. The homogenates were vortexed, stored at -20˚C for 2 h and then centrifuged (14,000 g, 30 min at 4˚C). The pellet containing the precipitated proteins was washed twice in acetone. Then acetone was evaporated under nitrogen stream. The pellets were mixed with 1 M NaOH and protein concentration was determined. An aliquot of the protein solution was mixed with liquid scintillation cocktail (Packard, Rungis, France), the radioactivity was measured in a scintillation counter (Winspectral Wallac 1414). Radioactivity incorporation was expressed as the percentage of dpm in CENU-treated cells proteins compared to untreated cell dpm values. Data represent three independent experiments.

[$^{14}CH_3$]-SAM incorporation in DNA. DNA was extracted by the TRIzol® method according to the manufacturer's instructions and its concentration was determined at $\lambda = 260$ nm. Then, 5 μg of the dissolved DNA was mixed with liquid scintillation cocktail, and the radioactivity was measured in a scintillation counter. Radioactivity incorporation was expressed as the percentage of dpm in CENU-treated cells proteins compared to dpm values in untreated cells. Data represent three independent experiments.

Methylation of proteins assayed by ^{1}H-^{13}C NMR spectroscopy. Using a step-labeling experiment similar to that of the radioactivity section, but using stably labeled L-[$^{13}CH_3$]methionine

and ^{1}H-^{13}C NMR spectroscopy, we recently demonstrated post-translational methylation of proteins, mainly histones, in a B16 melanoma model (19). The technique was used to quantify the ratio of the signal of methylated residues in proteins (asymmetrical dimethyl-arginine, dimethyl-lysine and trimethyl-lysine) to the signal of methionine incorporated in proteins, in untreated and CENU-treated B16 melanoma cells. Because observed methylations mostly concerned histones, the proposed ratio represented the amount of nuclear protein methylations relative to cellular total proteins in tumor cells.

Total cell protein extracts and protein concentration. Intact cells were lysed in a lysis buffer (50 mM Tris HCl pH 8.0, 100 mM NaCl) containing protease inhibitor mixture (Roche, Mannheim, Germany) by ultrasonication (3 times 15 sec in ice). After centrifugation (14,000 g for 10 min at 4˚C), the supernatant was kept at -80˚C until analysis. Protein concentration was determined with Commassie Blue (Invitrogen) at λ = 595 nm with bovine albumin serum as standard.

Western blotting. Proteins from total cells were subjected to SDS-PAGE on 10% SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-NC membrane (Millipore, Saint Quentin en Yvelines, France). The membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 4% non-fat milk for 60 min and probed with antibodies against non-methylated PP2AC (1:2,500), methylated PP2AC (PP2AC-Met+) (1:250), PP1 (1:2,000) (Upstate), Akt (1:2,000), phosphorylated Akt or P-Akt (Ser^{473}) (1:1,000), PTEN (1:2,000), phosphorylated PTEN or P-PTEN (Ser^{380}/$Thr^{382/383}$) (1:2,000) (Cell Signaling, Danvers, MA), c-Myc (1:200) (Santa Cruz Biotechnology, Le Perray en Yvelines), and ß-tubulin (1:2,500) (Sigma) overnight at 4˚C and washed three times with TBST. The membrane was probed with horseradish peroxidase-conjugated secondary antibodies (Upstate) at 1:2,000 (PP2AC-Met+, PP1, Akt, P-Akt, PTEN, P-PTEN, c-Myc) or 1:2,500 (PP2AC, ß-tubulin) for 1 h at room temperature and washed with TBST three times. The immunoblots were visualized by enhanced chemiluminescence (Amersham Bioscience). Densitometrical measurement of the band of interest was done using the Quantity One software (Bio-Rad). Normalization was done using ß-tubulin densitometrical values. All the results shown are representative of three independent experiments.

PP2A in vitro protein phosphatase assay. A PP2A immunoprecipitation phosphatase assay kit (Upstate) was used to detect PP2A activity according to the manufacturer's instructions. In brief, PP2A was immunoprecipitated with a monoclonal anti-PP2A antibody and protein A-Sepharose beads in lysis buffer. PP2A-bound beads were washed with phosphatase assay buffer and then with pNPP serine/threonine assay buffer (50 mM Tris HCl, 100 mM $CaCl_2$, pH 7.0; Upstate). Diluted phosphopeptide (K-R-pT-I-R-R) in serine/threonine assay buffer (250 μM) was added and then incubated for 15 min at 30˚C. After centrifugation, 25 μl of supernatant was transferred to an assay plate, and 100 μl of Malachite Green phosphate detection solution was added for 15 min incubation at 30˚C. The relative absorbance was measured at λ = 630 nm. Results are from two independent experiments that were performed in duplicate.

PTEN in vitro lipid phosphatase assay. An ELISA assay was used to detect PTEN activity according to the manufacturer's instructions. This kit allows to measure the transformation of $PI(3,4,5)P_3$ into $PI(4,5)P_2$ by PTEN lipid phosphatase activity by spectrophotometric detection. PTEN was immunoprecipitated using protein A-Sepharose beads with anti-PTEN antibody (Cell Signaling) in lysis buffer for 2 h at 4˚C. The beads were washed three times with TBS and once with PTEN enzyme reaction buffer (10 mM HEPES, 150 mM NaCl, 10 mM DTT, pH 7.2). Then, 100 μl of PTEN enzyme reaction buffer was added and the beads were transferred to an assay plate where the substrate ($PI(3,4,5)P_3$) was coated in the bottom of each well. After incubating the plate for 1 h at 37˚C, the beads were discarded and the wells were washed three times with 200 μl of TBST. Then, the $PI(4,5)P_2$ detector complex was prepared by dissolving one detector pellet in TBS containing 1% goat serum (TBSGS) (10.5 ml of TBSGS and 10.5 μl of co-detector solution). Then 100 μl of $PI(4,5)P_2$ detector complex were added by well and the plate was incubated for 1 h at room temperature under gentle agitation. The detector complex was discarded and the wells were washed three times with TBST. The secondary antibody (260 μl of enzyme-linked antibody mixed in 10.25 ml of TBSGS) was added (100 μl/well). The plate was incubated for 1 h at room temperature under gentle agitation and the wells were washed three times with TBST. Then, 100 μl of TMB were added per well and the plate was incubated at room temperature until the color development was sufficient for photometric analysis. The reaction was stopped with 50 μl per well of 0.5 M sulfuric acid. The absorbance in each well was read with a plate reader at λ = 450 nm. Results are from two independent experiments that were performed in duplicate.

Statistical analysis. In all experiments, data are given as mean ± standard deviation (SD). Comparison between groups was performed using the Mann-Whitney U test and $p<0.05$ was considered as significant. Linear regression was used to show correlation between PP2A methylation, PP2A activity and the retained criteria of aggressiveness (proliferation rate, P-Akt and c-Myc expression).

Results

Okadaic acid inhibits PP2A methylation and activity, and increases Akt and c-Myc in B16 melanoma cells. PP2A activity is regulated by the methylation of its catalytic subunit. In turn PP2A regulates the Akt/PKB pathway, which is involved in cell proliferation, protein synthesis, resistance to apoptosis (2). This pathway is often hyperactivated in cancer (3).

OA, at low dose (5 nM), is a PP2A antagonist and demethylating agent (5). At this dose, OA has been reported to increase cell proliferation (20). In our experiment, OA at 5 nM slightly stimulated cell growth (+30%, $p<0.05$) when compared to untreated cells (UN) (Fig. 1A). As shown in Fig. 1B, OA partially inhibited methylation of PP2AC few days after exposure. OA treatment did not alter the expression

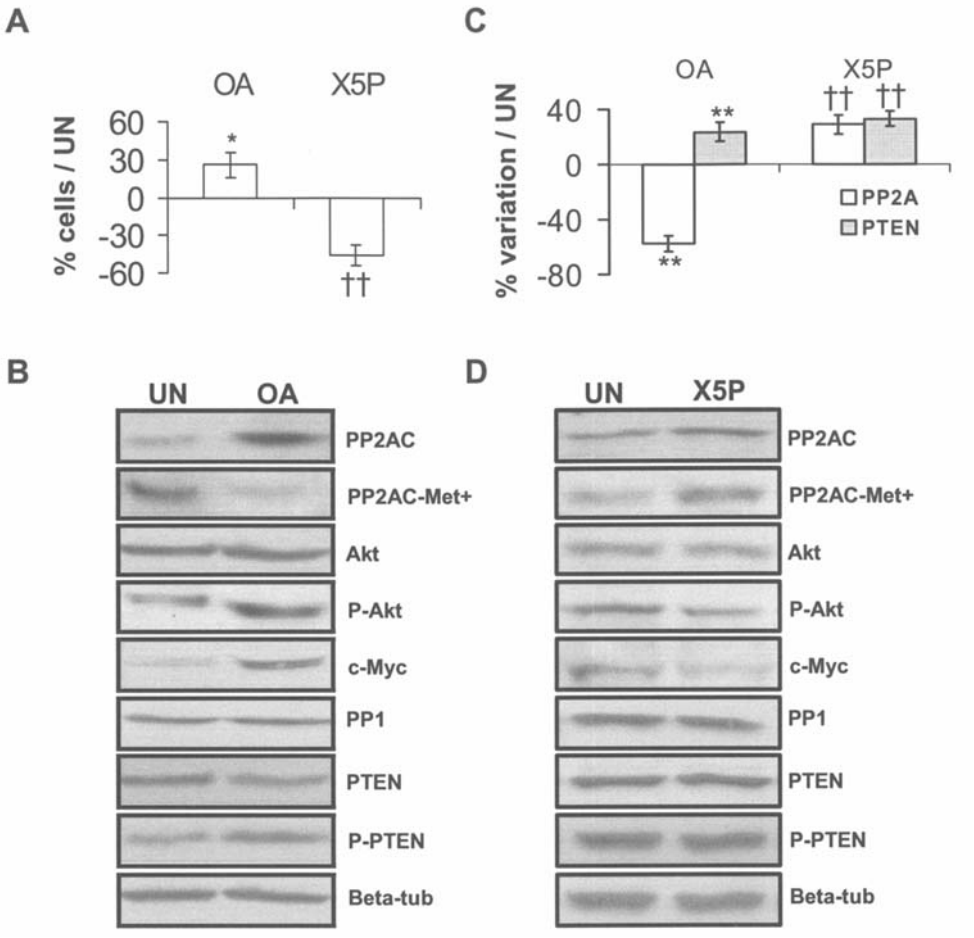

Figure 1. Effect of an antagonist (OA) and an agonist (X5P) of PP2A methylation on untreated B16 melanoma cell proliferation, phosphatase and oncoprotein expression and phosphatase activities. A, OA treatment stimulated cell proliferation whereas X5P treatment inhibited cell growth. B, Western blot analysis of OA treatment on the expression of non-methylated PP2AC (PP2AC), methylated PP2AC (PP2AC-Met+), Akt, P-Akt, c-Myc, PTEN, P-PTEN, PP1 and ß-tubulin. C, Effect of OA and X5P treatments on PP2A and PTEN activities. D, Western blot analysis of X5P treatment on the expression of non-methylated PP2AC (PP2AC), methylated PP2AC (PP2AC-Met+), Akt, P-Akt, c-Myc, PTEN, P-PTEN, PP1 and ß-tubulin. A, Cells were treated either by OA at 5 nM or by X5P at 50 μM for 48 h; at the end of the treatment, cells were harvested and counted. B and D, Equal protein loading was verified by Ponceau S staining of the membrane and by ß-tubulin expression. All the experiments are representative of three independent experiments. C, PP2A and PTEN activities were determined as described in Materials and methods. Columns, mean of 2 independent experiments; bars, SD. $^{*}p<0.05$, OA vs UN; $^{**}p<0.01$, OA vs UN; $^{††}p<0.01$, X5P vs UN.

of protein phosphatase 1 (PP1) and Akt expression but increased the relative amount of the activated form of Akt (phosphorylated Akt, P-Akt) and c-Myc expression (Fig. 1B). The relative amount of the active form of PTEN was decreased whereas the inactive form of PTEN, phosphorylated PTEN (P-PTEN), was increased, as shown in Fig. 1B. Then, we measured PP2A and PTEN activities. OA decreased PP2A activity by >50% (Fig. 1C) and, surprisingly, increased PTEN lipid phosphatase activity by 25% (Fig. 1C). The low dose of OA used in our experiment selectively targeted PP2A because PP1 expression was not modified (Fig. 1B). Taken together, these results confirm the tumor-promoting effect of OA through PP2A demethylation (21).

Xylulose-5-phosphate increases PP2A methylation and activity and decreases Akt and c-Myc in B16 melanoma cells. X5P, at 50 μM, was reported to be an agonist of PP2A methylation *in vitro* (18). At this dose, X5P strongly decreased cell growth of B16 melanoma cells (-46%, $p<0.01$, Fig. 1A). As shown in Fig. 1D, the methylation of PP2AC was increased, but PP1 expression was not affected neither the Akt expression. However, after 48 h of treatment with X5P, P-Akt levels and c-Myc expression were decreased (Fig. 1D). PTEN and P-PTEN relative amounts were not altered. X5P increased the activity of PP2A by 30% and the activity of PTEN by 35% (Fig. 1C).

Untreated B16 melanoma cells showed an accumulation of P-Akt, Akt and c-Myc proteins in comparison with normal human melanocytes (data not shown). These oncoproteins have been reported to play a key role in the abnormal proliferation of melanoma tumor cells (2,3).

Data using PP2A methylating and demethylating agents demonstrate that PP2A methylation status controls tumor cell proliferation and oncoprotein expression and activation.

Methylation disorders in response to DNA damage induced by chloroethylnitrosourea. We next investigated PP2A methylation in tumor cells in response to DNA damage. This model was established by treating B16 melanoma cells with chloroethylnitrosourea (CENU). CENU treatment is known to induce DNA damage and global DNA hypomethylation

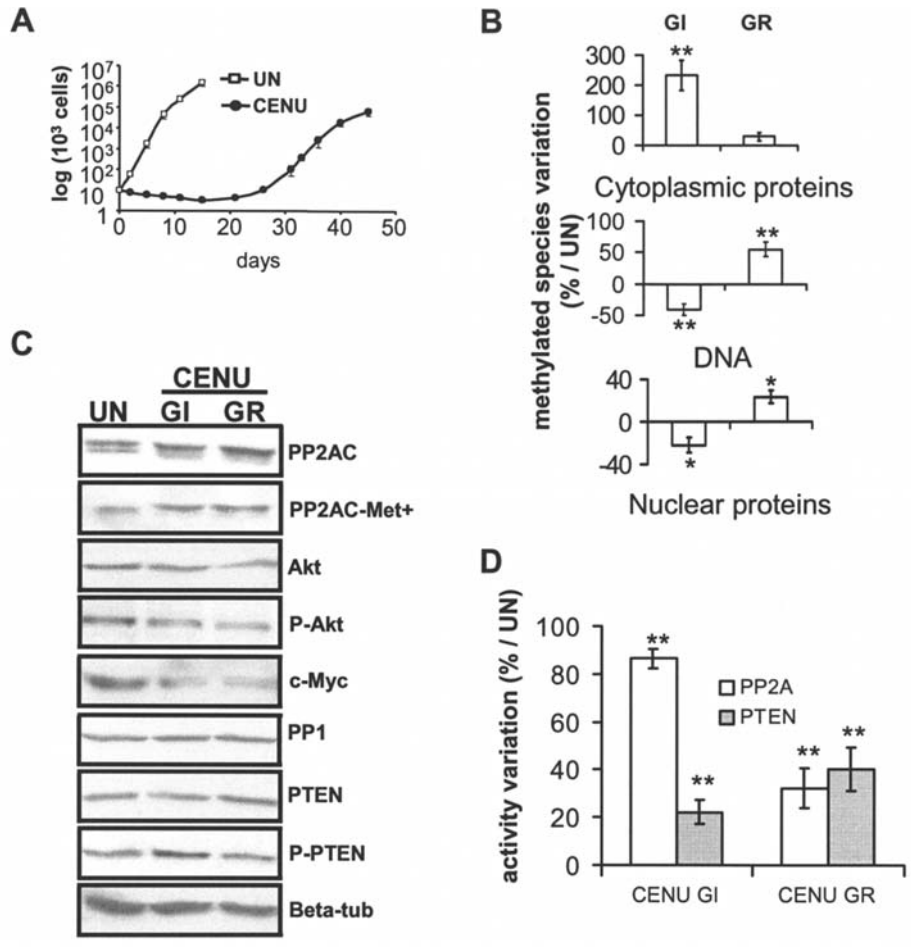

Figure 2. Response of B16 melanoma cells to CENU treatment on cell proliferation, global protein and DNA methylation, phosphatase and oncoprotein expression and phosphatase activities. A, Cell proliferation curves. B16 melanoma cells were treated by CENU (200 μM, 2 h) at day 0. Cell proliferation was followed for 45 days. B, Impact of CENU treatment on cytoplasmic and nuclear protein methylation and on DNA methylation. Methyl group incorporation in CENU-treated cells was determined as described in Materials and methods. Columns, mean of 3 independent experiments; bars, SD. C, Western blot analysis of the effect of CENU treatment on the expression of non-methylated PP2AC (PP2AC), methylated PP2AC (PP2AC-Met+), Akt, P-Akt, c-Myc, PTEN, P-PTEN, PP1 and ß-tubulin. Equal protein loading was verified by Ponceau S staining of the membrane and by ß-tubulin expression. Results are representative of three independent experiments. D, Effect of CENU treatment on PP2A and PTEN activities. PP2A and PTEN activities were determined as described in Materials and methods. In graphics from B and D, variables are the variations with respect to UN, normalized to UN. Columns, mean of two independent experiments; bars, SD. $^{*}p<0.05$, CENU vs UN; $^{**}p<0.01$, CENU vs UN.

(11). In response to this treatment, it was previously shown that proliferation evolved in two phases, a growth inhibitory (GI) and growth recovery (GR) phases (Fig. 2A) (13). Cell morphology, cell cycle distribution, nuclear aberrations, DNA damage and metabolism alterations of these cells during the GI and the GR phases have been previously reported (11-13,22-24). These cells demonstrated decreased aggressiveness and redifferentiation during both phases: accumulation in G_1 phase of the cell cycle during the growth GR phase, increased pool of PUFA, acquired metabolic alteration (phospholipid, methionine, energetic metabolism), decreased pool of glutathione (11-13,22-24).

It has been reported that the methylation of PP2A catalytic subunit was the most important methyl group consumer among the cellular proteins (14,17,25). To see whether PP2A methylation in our model correlated with DNA and global protein methylation disorders, we performed pulse-chase labeling experiments using S-adenosyl-L-[$^{14}CH_3$]methionine and L-[$^{13}CH_3$]methionine (Fig. 2B). During the GI phase, there was an increase in the incorporation of methyl groups into cytoplasmic proteins (2-fold, vs UN cells) (Fig. 2B, top), whereas methyl incorporation into DNA was decreased by 40% ($p<0.01$, Fig. 2B, middle). Data obtained by NMR spectroscopy analysis showed a decrease in the incorporation of methyl groups into nuclear proteins (Fig. 2B, bottom). During the GR phase, there was a slight increase of cytoplasmic protein methyl incorporation (Fig. 2B, top) but an increase in DNA methyl incorporation (+55%, $p<0.01$) and nuclear proteins (+25%, $p<0.05$) (Fig. 2B, middle and bottom). Taken together, these data show that CENU-induced DNA damage induces methylation disorders in DNA and proteins, and that PP2A methylation follows global cytoplasmic protein methylation (see below).

DNA damage induced by CENU increases PP2A methylation and activity and decreases Akt and c-Myc. Fig. 2C shows that, during the GI phase, PP2AC was more expressed and that its methylation was increased in comparison with UN cells. During the GR phase, PP2AC was even more methylated than in UN cells (Fig. 2C).

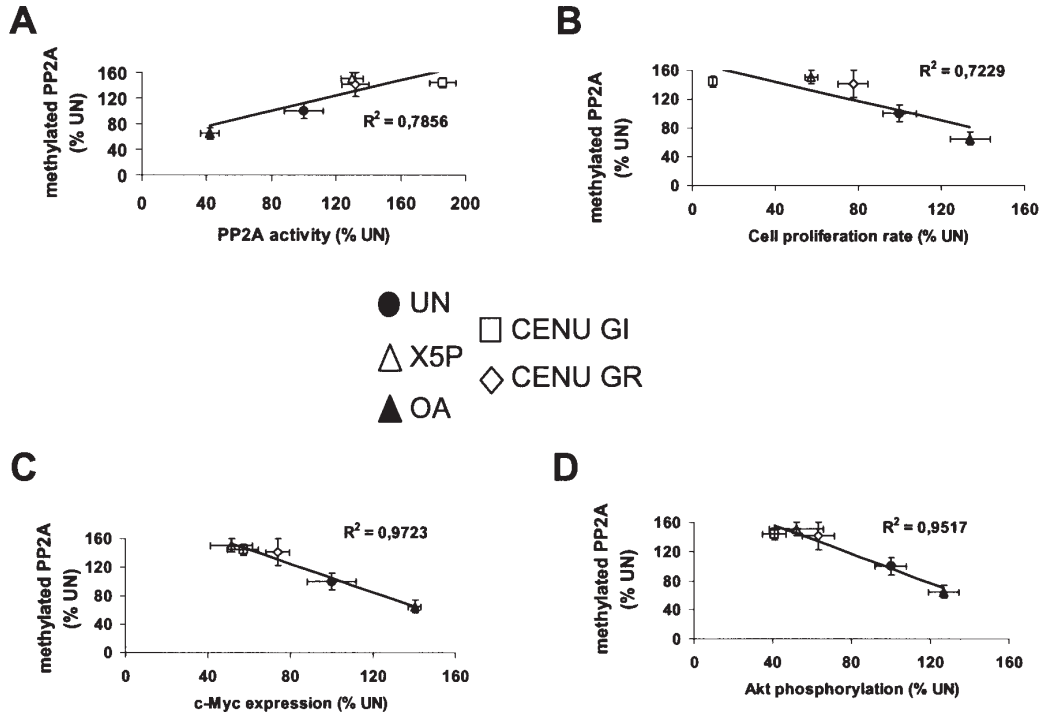

Figure 3. Relationship between PP2A methylation, PP2A activity and criteria of tumor cell aggressiveness. A, PP2A activity; B, Cell proliferation rate (expressed in percentage of UN); C, Phosphorylation of Akt (expressed in percentage of UN); D, c-Myc expression (expressed in percentage of UN). All criteria of aggressiveness (B-D) were highly and negatively correlated to PP2A methylation (expressed in percentage of the methylated form of UN). In the graphics, variables are the percentage of UN values.

During the GI phase (Fig. 2C), there was a decreased expression of Akt and c-Myc. Akt activation was diminished as represented by the fall of P-Akt level. In the meanwhile, there was a slight increase of PP1 expression and a strong increase of PTEN phosphorylation as shown in Fig. 2B. During the GR phase, most of these alterations remained. However there was a decrease of PTEN phosphorylation in comparison to UN cells, suggesting an activation of PTEN lipid phosphatase activity (Fig. 2C). PP1 protein level returned to base line expression during the GR phase (Fig. 2C).

PP2A and PTEN phosphatase activities were measured. Both PP2A and PTEN activities were increased, by 85% and 20% ($p<0.01$) during the GI phase and by 35% and 40% ($p<0.01$) during the GR phase, respectively (Fig. 2D, top). Altogether these data show that the response to CENU-induced DNA damage is in part mediated by the increase of PP2A methylation and activity.

Correlation between PP2A methylation status, PP2A activity and oncoprotein expression in B16 melanoma. There is a constant need to discover biomarkers for the evaluation of tumor cell aggressiveness, response to treatment and prediction of the response. We thus tried to correlate PP2A methylation status (expressed in percentage of PP2A methylation of UN) to PP2A activity and to the following parameters: rate of proliferation, Akt phosphorylation, and c-Myc expression (also expressed in percentage of expression of UN cells). For PP2A methylation status, Akt phosphorylation and c-Myc expression, we measured the relative intensity of each band from the Western blotting and normalized it with measured

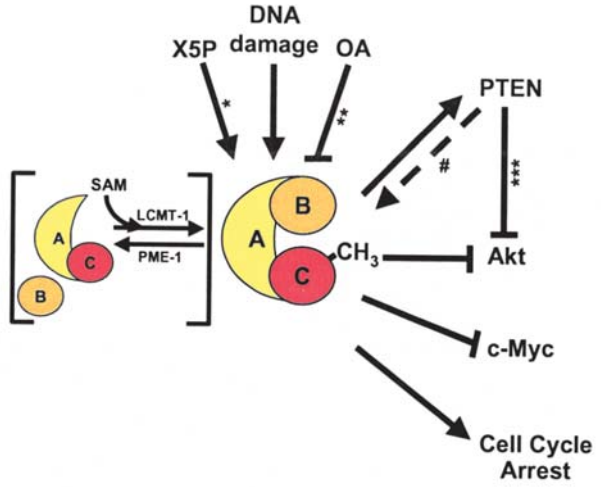

Figure 4. Interpretation and findings on the regulation of PP2A activity and function by methylation. X5P treatment increases PP2A methylation. OA treatment inhibits PP2A methylation. At the biochemical level, PP2A methylation results from the opposing effects of specific leucine methyltransferase (LCMT-1) or protein phosphatase methylesterase (PME-1). DNA damage induces PP2A methylation. Full lines, data from this work; asterisks, data strongly supported by the literature [*(18); **(20); ***(30)]; #hypothetical interaction between PP2A and PTEN (see Discussion).

ß-tubulin relative intensity. Regression analysis presented in Fig. 3, showed positive and significant linear relationships between PP2A methylation and PP2A activity ($R^2 = 0.7856$) in all used models and negative and significant linear relationships between PP2A methylation and tumor cell aggressiveness

criteria (proliferation rate, $R^2 = 0.7229$, $p<0.05$; P-Akt, $R^2 = 0.9517$; $p<0.01$, c-Myc, $R2 = 0.9723$, $p<0.05$).

Discussion

This report demonstrates that in B16 melanoma cells: i) PP2A methylation status is tightly linked to PP2A activity; ii) PP2A regulates melanoma tumor cell proliferation through the downregulation of oncoproteins; and iii) PP2A is strongly activated during the primary response to DNA damage. In addition we show that PP2A methylation status is indicative of the degree of tumor cell aggressiveness.

There are conflicting results on the relationship between PP2A methylation and activity. A decrease of PP2A activity with increased PP2A methylation has been reported (26). However, these authors acknowledged that it was uncertain whether the direct cause of PP2A decreased activity was due the carboxyl-methylation itself. Other reports state that methylation was necessary for PP2A activation (5,6,17). Our data show that PP2A methylation was strongly correlated with PP2A activity. This is in agreement with the bulk of literature data (5,6,17,25). Moreover, the amplitude of PP2A activity variations that we found were in agreement with another report stating an increase of PP2A activity between 31 and 54% when purified catalytic subunit of PP2A from breast cancer cells where exposed to a mixture of methyltransferases (27).

PP2A methylation was shown to be strongly decreased in tumor cells (5,6). However, the link between PP2A methylation and oncoprotein expression has not been established yet. PP2A has multiple molecular targets (5), among which the protein kinase Akt. The Akt/PKB pathway is implicated in cell proliferation, protein synthesis, resistance to apoptosis and is often mutated in cancer (2,3). PP2A is one of the major regulatory protein of the Akt/PKB pathway. PP2A inactivates Akt by dephosphorylation (1,2,28).

We found that PP2A methylation induced by an agonist (X5P) decreased cell proliferation, Akt activation and c-Myc expression. Conversely, the PP2A demethylation by an antagonist (OA) stimulated cell growth and oncoprotein activation or expression. Furthermore, activating PP2A also increased PTEN. The regulatory role of Akt signaling pathway is shared with PTEN whose role in carcinogenesis has been documented (29,30). The implication of PP2A in the regulation of PTEN activation is reported herein for the first time in a tumor cell line although it was recently reported in response to viral infection (31). The increased expression of the active form of PTEN and the increase in the methylation of PP2A strongly suggested a downregulation of the Akt pathway.

The real mechanism involving PTEN activation by PP2A remained unclear. However, from our results and the data from the literature, we can suggest that there might be an interaction between PTEN and PP2A. Both PTEN and PP2A are regulators of the Akt pathway and their cellular localization is very close. PTEN can be phosphorylated on multiple Thr and Ser sites (32,33). Some of these sites could be potential substrates for PP2A. Moreover PTEN was reported to express a protein tyrosine phosphatase activity (34,35). Then activated PP2A could dephosphorylate PTEN which would in turn dephosphorylate PP2AC on Tyr^{307} allowing PP2A to be more active. Thus, methylating PP2A would lead two tumor suppressor proteins to be activated.

Our data showed that PTEN activity was increased in response to OA treatment despite the decrease of PP2A activity. It has been shown recently that PTEN ubiquitination was necessary for its nuclear localization and function (36). Moreover, in neuronal cells, it was reported that inhibiting PP2A methylation increased the levels of ubiquitinated proteins such as tau protein (37,38). Thus, inhibiting PP2A methylation might allow PTEN nuclear translocation and ubiquitination.

CENU is a well known DNA damaging agent that preferentially targets guanine at the O^6 position (11). An important part of DNA damage lesions induced by alkylating agents, such as CENU, are repaired by methylguanine-N-methyltransferase (MGMT). Nevertheless, untreated B16 melanoma cells do not express this DNA repair protein. But it was shown that fotemustine, another nitrosourea, induced the re-expression of the MGMT gene (39). Moreover, the inactivation of Akt following DNA damage could lead to the activation of p53 (40) which was shown to upregulate MGMT expression (41). This might have taken part in growth recovery of CENU-treated B16 melanoma cells.

During this phase CENU-treated cells also exhibited signs of melanocytic differentiation and decreased aggressiveness. This differentiated phenotype was characterized by an increase in cellular polyunsaturated fatty acid (PUFA) content, probably in response to the activation of phospholipases A_2 (13). PUFA are precursors for *de novo* synthesis of ceramide, which are PP2A activators (42), and may participate in the activation of PP2A in response to CENU treatment.

In a recent study, PP2A was shown to participate in the response to doxorubucin or γ-irradiation by dephosphorylating Polo-like kinase-1, thus inducing cell cycle arrest in the G_2 phase (7). Our findings of PP2A methylation increase in response to CENU treatment is consistent with previous reports showing that CENU treatment induced a G_2 phase cell cycle arrest in B16 melanoma cells (11,13).

The Akt pathway is implicated in cell proliferation, and cell survival (2-4). Moreover, Akt activation was shown to protect from G_2 arrest induced by methylating agent, temozolomide (43). We found a downregulation of Akt and a G_2 arrest (13) in response to CENU treatment. These results are in agreement with the activation of PP2A induced by CENU treatment. The adjunction of OA during the GI phase induced by CENU treatment completely abrogated the activation of PP2A and its consequences (inactivation of Akt, decreased expression of c-Myc) and decreased the duration of the GI phase (data not shown).

Also, according to the work of Lee *et al* (17) on methylated species, PP2A methylation status should mainly reflect the activity of the specific methylesterase. The response to DNA damage would principally involve methylesterase activity thus PP2A demethylation. The increased methylation of nuclear proteins could follow the partial inactivation of Akt by PP2A. It was shown that the phosphorylation of EZH2, a histone methyltransferase, by Akt decreased its ability to trimethylate its substrate, lysine 27 in histone H3, resulting in the derepression of silenced genes, such as oncogenes (44).

By inactivating Akt, PP2A would let histone H3 be methylated. This would in turn silence oncogenes or allow a tumor suppressor gene to be expressed.

The genomic region of the c-myc gene is very sensitive to alkylating agents (45). c-Myc is involved in tumor cell proliferation, cell survival, and regulates glucose metabolism (46). We found a downregulation of c-Myc in response to DNA damage induced by CENU treatment. This is consistent with the report that PP2A induced the proteasome-dependent degradation of c-Myc (47). In addition c-Myc donwnregulation was linked to low glutathione level (48), in agreement with our previous report of decreased cell glutathione content in response to CENU treatment (13).

A summary of our molecular findings is given in Fig. 4: PP2A methylation agonist and antagonist modulate PP2A activity and oncoprotein expression. DNA damage induces PP2A methylation which inactivates Akt, downregulates c-Myc and activates PTEN.

In conclusion, this study demonstrated that, in B16 melanoma cells: i) PP2A methylation status is tightly linked to PP2A activity; ii) PP2A regulates melanoma tumor cell proliferation through the downregulation of oncoproteins; and iii) PP2A is strongly activated during the primary response to DNA damage. In addition PP2A methylation status and activity correlate to the degree of tumor cell aggressiveness. It follows from these data that agents with PP2A pro-methylating activity might be candidates for anticancer drug development.

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