The MicroRNA 15a/16–1 Cluster Down-regulates Protein Repair Isoaspartyl Methyltransferase in Hepatoma Cells

IMPLICATIONS FOR APOPTOSIS REGULATION

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Background: Protein carboxyl-O-methyltransferase (PCMT1) repairs isomerized proteins and prevents apoptosis. PCMT1 may act as an effective modulator of apoptotic cell death, with a potential role in proliferative/degenerative disorders.

Results: PCMT1 is down-regulated by microRNAs 15a/16–1. Its silencing results in conspicuous BclxL isomerization and increased susceptibility to apoptosis.

Conclusion: MicroRNA 15a/16–1 cluster can modulate PCMT1: a repair methyltransferase with antiapoptotic activity.

Significance: PCMT1 may act as an effective modulator of apoptotic cell death, with a potential role in proliferative/degenerative disorders.

Asparaginyl deamidation, a spontaneous protein post-biosynthetic modification, determines isoaspartyl formation and structural instability and biological function of BclxL from deamidation-related functional impairment. The role of PCMT1 as an isoAsp-repairing enzyme has been demonstrated. PCMT1 activity has been demonstrated in various models, such as the human erythrocyte, where an increase in protein methylesterification monitors a state of increasing deamidation associated with cell age in circulation (16) or is accelerated by pathological states (17, 18). Moreover, protein deamidation/isomerization is increased 4–8-fold in PCMT1 knockout mice compared with the levels detected in the wild-type animals, causing brain damage and fatal epileptic seizures (19). More recently, evidence indicated that PCMT1

Protein deamidation occurs, under physiological conditions, at susceptible asparaginyl residues, which are flanked, on the carboxyl side, by small nonbulky residues, such as Gly, Ala, or Ser, or Thr (1, 2) yielding a L-aspartyl residue into L-aspartyl, thus “repairing” the isopeptide bond and preventing the accumulation of conformationally altered, dysfunctional proteins, like tautomer, collagen degradation of isoaspartyl, the pathogenesis of age-related ocular diseases, like macular degeneration (10). The enzyme promotes the conversion of the abnormal L-isoAsp residue into L-aspartyl, thus “repairing” the isopeptide bond and preventing the accumulation of conformationally altered, dysfunctional proteins, like tautomer, collagen degradation of isoaspartyl, the pathogenesis of age-related ocular diseases, like macular degeneration (10).

This article has been retracted by the publisher. The actin immunoblots in Figs. 2A, 2B, and 2C were reused in Figs. 3A, 3B, and 3C, respectively. Lanes 1, 3, and 5 of the PCMT immunoblot in Fig. 3B were reused in lanes 1, 3, and 5 of the PCMT immunoblot in Fig. 3C. The first lane of the PCMT immunoblot from Fig. 5 was reused in lane 1 of the PCMT immunoblot in Fig. 4. The PARP immunoblot in Fig. 7A was inappropriately manipulated. The bands in Fig. 9A were duplicated. Fig. 9C was spliced. The BclxL immunoblot from Fig. 10A was duplicated in Fig. 10B. The Pro-Caspase 3 and Cleaved PARP immunoblots from Fig. 10A were inappropriately manipulated. The PCMT, BclxL, and Cleaved PARP immunoblots from Fig. 10B were inappropriately manipulated.
may be also endowed with the ability of regulating partitioning of certain proteins between two possible structures: (Asx)-containing (active) or isoAsp (deamidated and inactive). Co-transfection with a PCMT1 carrying vector prevents apoptosis induced by Bax, in neuronal cell lines (20). Moreover, BclxL, an antiapoptotic member of the Bcl2 protein family, contains two labile asparaginyl sites, which are deamidation-prone (i.e. positions 52 and 66). Deamidated BclxL is no longer active to block pro-apoptotic proteins, thus leading to cell death (4, 21, 22, 24). In fact, cell damage induces BclxL deamidation as a mechanism to commit cells to apoptosis. During cell stress a transient intracellular alkalization takes place causing BclxL deamidation/isomerization, as alkaline pH conditions increase the tendency of labile asparaginyl residues to deamidate (25). Consistently, reduced levels of BclxL deamidation were detected in hepatocellular carcinomas compared with normal liver tissue and this has been linked to the typical apoptosis resistance of such transformed cells (26). In our previous work (27) we demonstrated the antiapoptotic action of PCMT1 and proposed a mechanism of action, based on the ability of this methyltransferase to maintain the structural integrity of some crucial antiapoptotic proteins, including BclxL, through methylation and repair of their dysfunctional L-isoaspartyl-containing derivatives.

Over the last decade, microRNAs have been involved in a number of cell processes, including differentiation and development as well as tissue reshaping and apoptosis. In particular, the microRNA 15a/16–1 cluster is involved in the regulation of both apoptosis and tissue reshaping during liver disease, hepatocarcinoma development as well as tissue reshaping and apoptosis. In particular, microRNA-dependent PCMT1 regulation in hepatocarcinoma cells, particularly as related to microRNA 15a/16–1 (26, 30). In particular, we investigated the role of microRNAs on PCMT1 overexpression (qiCycler iQ Real time PCR. The amplification mix consisted 1 µl of cDNA, 0.3 µM of each primer, 12.5 µl of a 2x QuantiTect SYBR Green Master mix QuantiTect SYBR Green (Qiagen), and H2O/diethyl pyrocarbonate for a final volume of 25 µl. GAPDH (as internal reference) primers were: GAPDH sense, 5’-TTGG-TATCGTGGAAGGACTCCATG-3’; and GAPDH antisense, 5’-CAGTAGAGGCGGATGTGTTTC-3’.

The QuantiTect Primer Assay mixture (Qiagen), containing specific sense and antisense primer pairs, was utilized for PCMT1 and MAPK. Amplifying conditions were: 95 °C for 15 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Expression levels after treatment were evaluated by comparison of samples treated with siRNAs or shRNAs with control samples, transfected with scrambled sequence siRNA or shRNA. All qPCR results were obtained from the mean of triplicate measurements for the gene of interest in each sample, normalized for the mRNA levels of the housekeeping reference gene (GAPDH, also amplified in triplicate). Relative expression was calculated using the ΔCt method. A value of 2^ΔΔCt > 1 reflects increased expression of the relevant gene, whereas a value of 2^ΔΔCt < 1 points to a decrease in gene expression.

Cell Extracts—After transfection, cells were trypsinized, washed twice in PBS, and resuspended in a lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, and 1 X protease inhibitor mixture (Roche Diagnostics) for 30 min at 4 °C. Lysates were then centrifuged at 13,000 × g for 20 min at 4 °C and the supernatants were stored at −20 °C. Protein concentration was determined according to Bradford (31).
Targeting of Protein Repair Methyltransferase by RNAi

Western Blotting—A loading buffer of 2 M Tris-HCl, pH 6.8, 20% SDS, 5% β-mercaptoethanol, glycerol, and bromphenol blue was added to transfected HepG2 cell aliquots, containing 20 μg of total proteins and incubated for 5 min at 95 °C to allow protein denaturation. SDS-PAGE was performed according to Laemmli, utilizing 12% resolving gel and running buffer containing 192 mM glycine, 25 mM Tris, and 0.1% SDS. After electrophoresis, proteins were transferred to nitrocellulose membrane (Hybond-Extra, GE Healthcare), utilizing a TransBlot system apparatus (Bio-Rad) at 100 V for 1 h using as transfer buffer 20% methanol and 5% Tris glycine. Nitrocellulose membrane was then colored with Ponceau Red for 5 min, de-colored, washed with distilled water, and incubated overnight at 4 °C with TBS solution containing 0.1% Tween 20, 5% blotting grade blocker Non-fat Dry Milk (Bio-Rad). Membranes were then incubated with a primary monoclonal antibody against the protein of interest: anti-Caspase-3 (C9598 SIGMA), anti-PARP (AF600NA, R&D System), anti-BclxL (610211, BD Transduction Laboratories), anti-PCMT1 (610772, BD Transduction Laboratories), and anti-ACTIN as a loading control (sc-1616, Santa Cruz Biotechnology). The membrane was then washed with TBS solution and incubated with secondary antibody conjugated with horseradish peroxidase. Immunocomplex visualization was obtained through chemiluminescence, utilizing ECL Plus kit (GE Healthcare). Western blot images were acquired through Adobe Photoshop software.

MicroRNA Target Prediction—Bioinformatic prediction of microRNA targets was made by using the following algorithms: TargetScan that predicts biological microRNA:mRNA heteroduplex formation; searching for the presence of complete match with seed region (nucleotides 2–7 at the 5′ end) that match the seed region of each microRNA, PicTar that predicts microRNA targets by searching for conserved 8-mer and 7-mer sites with TBS solution and incubated with secondary antibody conjugated with horseradish peroxidase. Immunocomplex visualization was obtained through chemiluminescence, utilizing ECL Plus kit (GE Healthcare). Western blot images were acquired through Adobe Photoshop software.

Statistical Analysis—An unpaired Student's t test was performed to compare means from results of silencing experiments. All results are presented as the mean ± S.D. All experiments were done in triplicate.

3′ Rapid Amplification of cDNA Ends (RACE)—3′ RACE was utilized to clone a cDNA molecule containing the PCMT1 seed sequence, and was carried out by the 3′ RACE System (Invitrogen) according to the manufacturer’s protocol. Briefly, first strand cDNA synthesis was initiated at the poly(A) tail of PCMT1 mRNA using an oligo(dT)-adapter primer and PCMT1 mRNA was converted into cDNA using reverse transcriptase. Specific cDNA was then amplified by PCR, using a PCMT1-specific primer and an adapter primer (AP) that targeted the poly(A) tail region. The AUAP is designed to synthesize first strand cDNA from all polyadenylated mRNAs. A certain sequence specificity in the amplification reaction was therefore provided solely from the specific primer. For this reason, a second PCMT1 nested primer was utilized, together with the adapter primer to further amplify the product of the first PCR, in a second amplification reaction, to yield a second highly specific amplification product of the 3′ RACE procedure. Primer sequences used were: adapter primer (AP), 5′-GGCCACGCGTCGACTAGTACCTCTTTC-; AUAP primer, 5′-GGCCACGCGTCGACTAGTACCTCTTTC-; GSP-PCMT: 5′-ACAGTGATTGCATCTCAG-3′; GSP-PCMT nested: 5′-TCAGTGATAGGATAAACACACACATTCAG-3′.

The final PCR product containing the PCMT1 seed sequence was then purified, using the GenElute Gel Extraction kit (Sigma) and sequenced at the S.U.N. DNA sequencing Facility (directed by Prof. Vincenzo Nigro), using an ABI PRISM DNA System (Applied Biosystems).

Dual Luciferase Reporter Assay—For luciferase reporter experiments, a PCMT1 3′ UTR segment of 480 bp was amplified by PCR 3′ RACE from human cDNA and inserted, between the Xbal/Sall sites (Takara Bio Inc., Otsu, Shiga, Japan), into a pGL3 control vector with a TK promoter (kindly provided by Dr. Giulio Piluso, Dept. of General Pathology and Oncology, S.U.N.), immediately downstream of the stop luciferase codon. Luciferase activity was measured 24 h after transfection, using a Dual Luciferase Reporter Assay System (Promega, Faggiano, Milan, Italy). Relative luciferase activity was calculated by normalizing the firefly luminescence to Renilla luminescence. The experiments were performed in triplicate.

Cells Silenced with siRNA—Silencing was quantitated by a luciferase activity assay described by Macfarlane (32).

In Vitro Enzymatic Methylation of Alkali-treated BclxL—Aliquots of alkali-treated BclxL were directly lyophilized and resuspended in the PCMT1 assay buffer and assayed for methylation by human recombinant PCMT1 (33). Sample aliquots to be subject to further SDS-PAGE and Western blot analysis were incubated in the presence of unlabeled S-adenosylmethionine as previously described (33) and analyzed by electrophoresis. Parallel sample aliquots were assayed for PCMT1, in the presence of S-adenosyl-[methyl-14C]methionine to evaluate the presence of the isoAsp residues, using human recombinant PCMT1 and the vapor diffusion radiochemical assay as previously described (33).
RESULTS

PCMT1 Is a Target for MicroRNA

MicroRNA are one of the most important post-transcriptional regulators of several genes involved in key cell functions including apoptosis. To investigate the potential regulatory properties of microRNAs on PCMT1 expression, we first checked whether PCMT1 could be predicted as a possible microRNA target, by using bioinformatic analysis. To this purpose we employed three of the most widely utilized algorithms (TargetScan, PicTar, and MiRanda), which are based on different prediction criteria and, therefore, used in combination, provide the highest probability of target identification. We identified a perfect complementarity (seed region) between microRNA 15a/16–1 cluster sequences and the 3’UTR of human PCMT1 (Fig. 1A).

This seed sequence, representing the potential recognition region for microRNA 15a/16–1 on PCMT1 3’UTR, is also highly conserved in other species (Fig. 1B).

To elucidate the role of microRNA 15a/16–1 cluster in regulating PCMT1 expression, parallel cell samples were transfected with pre-microRNA 15a/16–1 to verify their ability to influence PCMT1 expression. Treatment with pre-microRNA 15a (Fig. 2) and pre-microRNA 16–1 (Fig. 3) determined a significant decrease of PCMT1 expression, an effect starting as early as 48 h after transfection even at the lowest microRNA concentrations. Cell treatment with pre-microRNA 15a/16–1 at 50–100 nM pre-microRNA concentrations resulted in a significant decrease of PCMT1 expression at prolonged incubation times (Figs. 2 and 3). Transfection with anti-microRNA 15a and anti-microRNA 16–1 up-regulates PCMT1 expression, and their effects were detected as soon as 48 h after transfection (Figs. 4 and 5) even at the lowest anti-microRNA concentrations. Data in Figs. 2–5, as a whole, demonstrated that: (a) transfection with pre-microRNA 15a or pre-microRNA 16–1 were able to silence PCMT1 expression levels; (b) both pre-microRNA 15a and pre-microRNA 16–1 were almost equally effective, on a concentration basis, in inducing PCMT1 silencing; and (c) their relevant anti-microRNA 15a and anti-microRNA 16–1 both specifically prevented the effects of pre-miRNA 15a or pre-miRNA 16–1 on PCMT1 expression.

To demonstrate a specific interaction between the PCMT1 transcript and microRNA 15a/16–1 cluster, we used a luciferase reporter assay approach. According to this strategy we cloned the putative seed sequence, identified through bioinformatic analysis (Fig. 1A), immediately downstream of the stop codon of the luciferase reporter gene in a pGL3TK vector. HepG2 were co-transfected with this construct (pGL3TKpcmt) and pre-microRNA 15a or 16–1. In cells co-transfected with pGL3TKpcmt and microRNA 15a/16–1 the firefly luciferase activity was significantly reduced, whereas in both controls (cells co-transfected with pGL3TKCT and microRNA 15a/16–1) the luciferase activity was not significantly reduced.
microRNA scrambled) firefly luciferase activity was unchanged with respect to control (Fig. 6). Results showed the occurrence of specific, mutual interactions between microRNA 15a/16–1 and the PCMT1 putative seed sequence, which cause a reduction of firefly luciferase activity expressed by the pGL3TKpcmt recombinant plasmid.

We could conclude that transcripts encoding for PCMT1 are effectively targeted by the microRNA 15a/16–1 cluster. Therefore, the effects we observed in PCMT1 expression upon microRNA 15a/16–1 transfection of HepG2 occurred at the specific interaction site of such microRNAs with the PCMT1 transcript actually representing the seed sequence we previously identified in silico.

**PCMT1 Is Effectively Silenced by Various siRNA and shRNA Approaches**—Although the potential role of microRNA 15a/16–1 in PCMT1 expression was thus established, to study the biological meaning of PCMT1 silencing in our model, a different approach, based on a more selective way of switching off the expression of the gene, was undertaken. To assess the direct effects of PCMT1 silencing on apoptosis, we designed an approach possibly devoid of pleiotropic effects on other apoptosis mediators, as well as off-target effects on genes structurally unrelated to any PCMT1 substrates. Selective down-regulation of PCMT1 on HepG2 cells treated with cisplatinum, a well known apoptosis inducer, was performed by various siRNA and short hairpin RNA (shRNA).

Experimental conditions to trigger the very initial stages of apoptosis were explored, so that cells would not be irreversibly committed to programmed death, and allowing us to still detect and appreciate even small variations of cell response, possibly due to PCMT1 down-regulation. We then preliminarily tried various conditions including treatments with cisplatinum for various times (data not shown), so that we finally set treatment conditions of HepG2 with cisplatinum at 35 mM for 6 h (condition A), as shown in Fig. 7A. Under condition A, we could observe an initial activation of apoptosis cascade, in that PARP cleavage appeared to be just starting, compared with the pattern detected at the 18-h
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Effects of HepG2 transfection with anti-miRNA 15a on PCMT1 expression. The results of the treatment for 48 h with increasing concentrations of anti-miRNA 15a (as indicated at the top of each lane) are indicated. Western blot analysis for PCMT1 (upper panel) with the relevant densitometric scan (lower half) are reported. The first lane, marked with c, is relevant to a scrambled miRNA control. *, p < 0.05.

FIGURE 4.

Effects of HepG2 transfection with anti-miRNA 16–1 on PCMT1 expression. Results of the treatment for 48 h with increasing concentrations of anti-miRNA 16 (as indicated at the top of each lane) are indicated. Western blot analysis for PCMT1 (upper panel) with the relevant densitometric scan (lower half) are reported. The first lane, marked with c, is relevant to a scrambled miRNA control. *, p < 0.05.

FIGURE 5.

The effects of PCMT1 enzyme activity in cells upon its gene silencing using a radiochemical methyltransferase assay (32) (Fig. 8). All siRNAs (Fig. 8A) and shRNAs (Fig. 8B) employed were able to cause a significant reduction of PCMT1 activity, in HepG2 cells transfected with the constructs, by at least 80% (p < 0.05).

Characterization of Isomerized and Methylated Bcl<sub>©</sub>. Products with Respect to Their Electrofroject Behavior—Several reports from the literature described the Bcl<sub>©</sub> shift on SDS-PAGE as the result of “deamination” of this protein (21, 25, 26). However, it should be pointed out that, taking into account the mechanism for asparaginyl deamination (1, 2), its most abundant product, in most proteins, is in fact the isoaspartyl-containing derivative. Unfortunately this cannot be characterized on the simple basis of band shift, neither previous work done by others has accomplished a thorough characterization particularly regarding the possible isoaspartyl nature of the “deamidated” residue (21, 25, 26, 42). To confirm the identity of deamidated/isoaspartyl “n” form in the alkaline buffer and its product called the “isomerized” by immunoblotting. Results were displayed slowly on SDS-PAGE, (Fig. 9, panel A). This retarded band was described by previous authors as a shift due to mass spectral analysis of the Bcl<sub>©</sub> i form and to the consistent deamidation/isoaspartyl residues at positions 52 and 66 of Bcl<sub>©</sub> (Fig. 9, inset). The relevant mass fingerprint, obtained from MALDI-TOF mass spectral analysis of the Bcl<sub>©</sub> i form is shown in Table 1. All experimental mass values are consistent with the masses of expected peptides from Bcl<sub>©</sub>, as theoretically predicted by calculation (Table 1).

Moreover all the mass values of peptides containing Asp<sup>52</sup> or Asp<sup>66</sup> were 1 mass unit higher than expected, confirming deamination of the corresponding Asn precursors. In addition, the pattern of peptide fragments in the alkali-treated sample for 18 h is highly comparable with that of the sample deamidated for 6 h, thus allowing us to conclude that all susceptible Asn residues had been extensively deamidated and therefore the Bcl<sub>©</sub> i form is stable (Fig. 9, panel B). The relevant mass fingerprint, obtained from MALDI-TOF mass spectral analysis of the Bcl<sub>©</sub> n form showed a signal profile consistent deamidation/isomerization (Fig. 9, inset). The relevant mass fingerprint, obtained from MALDI-TOF mass spectral analysis of the Bcl<sub>©</sub> i form was analyzed by immunoblotting. Results were displayed slowly on SDS-PAGE, (Fig. 9, panel A). This retarded band was described by previous authors as a shift due to mass spectral analysis of the Bcl<sub>©</sub> i form and to the consistent deamidation/isoaspartyl residues at positions 52 and 66 of Bcl<sub>©</sub> (Fig. 9, inset). The relevant mass fingerprint, obtained from MALDI-TOF mass spectral analysis of the Bcl<sub>©</sub> i form is shown in Table 1. All experimental mass values are consistent with the masses of expected peptides from Bcl<sub>©</sub>, as theoretically predicted by calculation (Table 1).

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PCMT1 assay showed a significant substrate activity for the Bcl<sub>©</sub> i form, which was quantitatively enzymatically transformed in its product: Bcl<sub>©</sub> n form (Fig. 9, panel B). This result allowed us to correctly identify the Bcl<sub>©</sub> i form as “isoaspartyl-containing” Bcl<sub>©</sub>. Therefore a more appropriate denomination for this Bcl<sub>©</sub> i form is “isomerized” rather than simply deamidated.

The effects of PCMT-dependent methylation on Bcl<sub>©</sub> were monitored by band shift. Isomerized Bcl<sub>©</sub> (Bcl<sub>©</sub> i form) was incubated in vitro with human recombinant PCMT1, under conditions in which extensive methylesterification of the isoaspartyl sites gives way to the repair reaction sequence (1, 2). The possibility of any off-target effects occurring under such experimental conditions.

Therefore parallel samples of HepG2 cells were treated with cisplatinum, under condition A, and then transfected with either siRNA designed against 3’ and 5’ UTRs of the PCMT1 transcript or with four different pGFP-V-RS vectors carrying shRNA constructs against the 5’ UTR and ORF of PCMT1. Quantitation of PCMT1 transcription levels after transfection was estimated by real time PCR, showing that both siRNAs (siRNApcmt3 and siRNApcmt5; Fig. 7B) and all the shRNAs (shRNAsv5'UTRpcmt(a), shRNAsv5'UTRpcmt(b), shRNAsvORFpcmt(a), and shRNAsvORFpcmt(b)) (Fig. 7C), were almost equally highly effective in switching the PCMT1 gene off within 96 h of treatment (p < 0.01). The high consistency of the results with various RNAi approaches allowed us to rule out the
Targeting of Protein Repair Methyltransferase by RNAi

In the present paper we show that PCMT1 is effectively silenced, in HepG2 hepatoma cells, by microRNA 15a/16–1, a cluster involved in down-regulation of various antiapoptosis mediators (i.e. Bcl2 and MCL1) (28, 29). Down-regulation of PCMT1 in HepG2 depends on the specific interaction of microRNA 15a/16–1 with a seed sequence located at the 3′ UTR of the PCMT1 transcripts. In addition, we showed that PCMT1 is effectively silenced by various specific siRNAs and shRNAs. The increased susceptibility to apoptosis induced by PCMT1 silencing was monitored by Caspase 3 activation and PARP cleavage markers. Isomerized BclxL was also involved in this process, possibly involving identification of human recombinant PCMT1 activity in the presence of oxidative stress conditions (e.g. induced by oxidative treatment) in this model (19). Therefore no specific information on increased susceptibility to apoptosis induced by PCMT1 knock-out mice. These previous reports in the literature point...

product was then analyzed by SDS-PAGE, in parallel with aliquots of the BclxL n and BclxL i forms, for comparison of the relevant band positions (Fig. 9, panel C).

Results once again confirmed migration delay, on SDS-PAGE for the BclxL i form, whereas the BclxL m form displayed a pattern of migration absolutely comparable with that of the BclxL n form, thus allowing us to conclude that the BclxL n and m forms are structurally similar at least for what is related to their electrophoresis behavior.

PCMT1 Silencing Make HepG2 Cells More Susceptible to Apoptosis through BclxL Isomerization—The biological effects of PCMT1 silencing on both BclxL isomerization and apoptosis induction were investigated. HepG2 were co-transfected with this recombinant plasmid and either pre-miR 15a or 16 –1. Levels of luciferase activity were normalized with cell samples transfected with random siRNA (Fig. 10 panel A) or shRNA (Fig. 10, panel B). The migration pattern is comparable with what was observed in PCMT1 assay. This correct assignment of this retarded band as isomerized. This migration behavior is comparable with what previously reported for “native” and “deamidated” BclxL (21, 25, 26, 38, 42); and 3) apoptosis markers (Caspase 3 and PARP) showed a significant increase upon cisplatinum apoptotic stimulus, in cells where the enzyme was silenced by siRNA (Fig. 10A) or shRNAs (Fig. 10B).

These results, as a whole, allow us to conclude that PCMT1 expression can be effectively down-regulated by the microRNA 15a/16–1 cluster in HepG2, and that PCMT1 silencing, carried out by an RNAi approach, which allowed us to rule out any pleitropic or off-target effects, makes the cells more susceptible to apoptosis induced by cisplatinum.

DISCUSSION

In the present paper we show that PCMT1 is effectively silenced, in HepG2 hepatoma cells, by microRNA 15a/16–1, a cluster involved in down-regulation of various antiapoptosis mediators (i.e. Bcl2 and MCL1) (28, 29). Down-regulation of PCMT1 in HepG2 depends on the specific interaction of PCMT1 with its target mRNA, which is complementary to the seed sequence of the microRNA 15a/16–1.
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Figure 7. Effects of siRNA and shRNA treatment on PCMT1 transcript levels. Panel A, set up of apoptosis condition. HepG2 were treated with cisplatinum for 6 h (defined as condition A in the text). Panel B, PCMT1 silencing estimated by real time PCR in HepG2 transfected with siRNA and then subject to condition A. C siRNA rd, negative control transfected with scrambled siRNA; siRNA MAPK, positive control (see "Experimental Procedures"); siRNA pcmt3, cell sample transfected with siRNA against PCMT1 3' UTR; siRNA pcmt5, cell samples transfected with siRNA against PCMT1 5' UTR. *, p < 0.01. Panel C, PCMT1 silencing estimated by real time PCR in HepG2 transfected with shRNA and then subject to condition A. C shRNA RD, negative control transfected with scrambled shRNA; shRNAAsS'UTRpcmt(a), shRNA directed against the 5' UTR of PCMT1 transcript; shRNAAsS'UTRpcmt5(b), shRNA directed against a different 5' UTR of the PCMT1 transcript; shRNAAsS'UTRfpcmt(a), shRNA directed against the PCMT1 transcript ORF; shRNAAsS'UTRfpcmt5(b), shRNA directed against a different sequence in the PCMT1 ORF. **, p < 0.01

to the interpretation that, even in the presence of apparently normal PCMT1 activity, various physiological or pathological conditions, mostly associated with human diseases or aging, may overcome the ability of PCMT1 to adequately prevent isoAsp accumulation. In addition, there are situations, such as cancer, in which resistance to apoptosis is a key feature of the pathophysiological mechanism (41). Therefore our findings suggest that the microRNA-dependent regulation mechanism may contribute to “tuning down” PCMT1 activity, under conditions in which cell commitment to apoptosis needs to be reinforced.

A possible question regards the specificity of the microRNA 15a/16–1 cluster activity on PCMT1 and its possible reflections on apoptosis regulation. These microRNAs down-regulate a number of other genes encoding for antiapoptotic mediators like Bcl2 (28). MicroRNA 15a/16–1 deregulation, through deletion or mutation, affects tumor resistance to apoptosis, by causing Bcl2 overexpression (37). It has been reported that the locus corresponding to microRNA 15a and microRNA 16–1 at 13q14 is deleted in more than half of B–cell chronic lymphocytic leukemias (34, 35). The undeleted microRNA 15a, miRNA 16–1 allele, in chronic lymphocytic leukemia patients, may also contain inactivating point mutations (36, 37). These observations led to the proposal that microRNA 15a and microRNA 16–1 may behave as tumor suppressors (37). Therefore, whereas the effects of the microRNA 15a/16–1 cluster on PCMT1 silencing were straightforward, any effect on apoptosis would lack specificity, due to wide pleiotropic effects of microRNAs on various other antiapoptotic mediators.

To establish the potential biological role of microRNA 15a/16–1 on PCMT1 on apoptosis, we employed small noncoding RNAs, devoid of off-target or pleiotropic effects and specifically targeted on PCMT1 at different sites in cell cultures treated with cisplatinum. To this purpose we employed and showed that selective PCMT1 silencing in cells stressed with cisplatinum makes cells more sensitive to apoptosis, by inducing an increase of Bcl1, deamidation/isomerization, which is devoid of antiapoptotic activity, as previously reported on various models (21, 25, 26, 38, 42).

Our results in fact are in line with the reports by previous authors, except that, based on our characterization of deamidated Bcl1, we propose to more adequately denominate this protein subform as isomerized, also because, as previously shown, the simple replacement of Asn with normal Asp does not cause, per se, any functional loss (21, 43). Takara and Takashashi (26) showed decreased deamidation of Bcl1 in human hepatocellular carcinomas, on the basis of its band shift on SDS-PAGE. Therefore these authors hypothesized an association with increased protection against apoptosis in these tumors (26). Later, Zhao and colleagues (38) showed that inhibition of the Bcl1, deamidation pathway, detected merely on the basis of band shift, contributed to the development of myeloproliferative disorders. Deamidation of Bcl1 has been related to a mechanism involving up-regulation, under proapoptotic conditions, of a proton pump, which in turn causes alkalinization of the intracellular medium, thus creating conditions for protein deamidation to occur, with special regard to Bcl1 band, which in fact was generally referred as deamidated, without mentioning the possibility of isoaspartyl formation. On the
other hand isoAsp is the major product of protein deamidation under alkaline conditions in various proteins (5–9). In addition, it has been shown that isoaspartyl containing calmodulin displays an altered molecular radius, which is responsible for its aberrant electrophoretic migration (14).

More recently the isoaspartyl-containing derivative of deamidated BclxL has been characterized by proteome analysis, from cells treated with antisense PCMT1 plus oxidative stress. This was accomplished by using a purification protocol that employed a resin linked to human recombinant PCMT1, which can only recognize isomerized proteins but not the one carrying normal aspartyl (27). These latter experiments provided the first evidence that BclxL undergoes deamidation under cell stress conditions, yielding its isomerized derivative, which is devoid of antiapoptotic function.

In the present work we characterized a BclxL slower-migrating band on SDS-PAGE showing that under alkaline conditions,

FIGURE 8. Methyltransferase activity in HepG2 upon transfection with siRNAs and shRNAs against PCMT1. Cells were transfected with various siRNAs and shRNAs against PCMT1 and then treated with cisplatinum under condition A. Methyltransferase-specific activity was measured in cell extracts. A, C siRNA rd, negative control transfected with scrambled siRNA; siRNA pcmt3, cell sample transfected with siRNA against PCMT1 3’ UTR; siRNA pcmt5, cell sample transfected with siRNA against PCMT1 5’ UTR. *, p < 0.05. B, C shRNA RD, negative control transfected with scrambled shRNA; shRNA3’ UTRpcmt(a), shRNA directed against the 3’ UTR of PCMT1 transcript; shRNA5’ UTRpcmt(b), shRNA directed against a different 3’ UTR of PCMT1 transcript; shRNAORFpcmt(a), shRNA directed against the PCMT1 transcript ORF; shRNAORFpcmt(b), shRNA directed against a different sequence in the PCMT1 ORF. *, p < 0.05.

FIGURE 9. Characterization of various molecular forms of BclxL. A, immunoblot analysis of the BclxL n (native-unmodified) and i (isomerized) forms. The latter was generated through alkali treatment (see "Experimental Procedures"). B, evaluation of PCMT1 substrate capability of BclxL i form. IsoAsp residues in the BclxL i form were identified by human recombinant PCMT1 assay in the presence of methyl labeled S-adenosylmethionine (see "Experimental Procedures"). The result is expressed as percentage of the fraction of methylesterified BclxL molecules over total. Inset, MALDI/TOF spectrum of the BclxL i form. Arrows mark the peptides containing deamidated Asn52 and Asn66 residues. C, electrophoretic behavior of the BclxL m form compared with the n and i forms. BclxL m form was obtained through enzymatic methylation of the i form (see "Experimental Procedures") by human recombinant PCMT1 in the presence of unlabeled S-adenosylmethionine.
closely resembling those occurring in apoptotic cells (25), BclxL isomerizes, because it is methylated in vitro by human recombinant PCMT1. This isomerized BclxL migrates slowly on SDS-PAGE, with a behavior totally comparable with what was observed by previous authors (21, 25, 26, 38, 42). In addition, enzymatic methylation of isomerized BclxL, by human recombinant PCMT1, reverts its aberrant pattern of migration so that methylated BclxL is no longer distinguishable from native BclxL by electrophoresis. This result is consistent with the interpretation of the repair role of PCMT1 in agreement as described for many other proteins (1–5). We then monitored the increase of isomerized BclxL under conditions in which PCMT1 had been silenced and apoptosis induced by cisplatinum (see Fig. 10).

Therefore, based on our present result, we can then hypothesize that miRNA-dependent down-regulation of PCMT1 can be envisioned as a way to increase susceptibility to apoptosis, and PCMT1 may represent an additional late checkpoint of apoptosis regulation. We may infer a possible role of PCMT1 up-regulation, induced by the microRNA 15a/16–1 defect, as a late mechanism for triggering apoptosis resistance in tumors. In this respect it may be interesting to look at PCMT1 activity in cancer, where microRNA 15a and 16–1 are suppressed and/or BclxL repaired by methylation.

The overall scenario may be even more complex, because of the pleiotropic activity of this microRNA cluster on a number of other antiapoptotic genes (23, 28–30, 35–37, 44), not just PCMT1 and also because other PCMT1 substrates, such as Hsp70, Hsp90, are involved in apoptosis (27), and both of these aspects may further complicate this model and certainly deserve further investigations. These results, as a whole, support a novel interpretation that PCMT1 should not be simply interpreted as a repair methyltransferase of aged/damaged proteins, but it may act as an effective modulator of apoptotic cell death, whose derangement may play a role in proliferative or degenerative disorders.

| Deamination time | Asp/isoAsp (sequence position) | BclxL peptides (sequence position) | m/z values of deamidated BclxL peptides, (theoretical) | m/z values of native BclxL peptides, (theoretical) | Error ppm |
|------------------|--------------------------------|-----------------------------------|------------------------------------------------------|------------------------------------------------------|----------|
| 6 h              | 66                             | 61–78                             | 175,699                                              | 175,582                                              | 108      |
| 6                | 52                             | 29–60                             | 349,885                                              | 349,753                                              | 94       |
| 6                | 52                             | 21–60                             | 444,156                                              | 444,091                                              | 64       |
| 6                | 52                             | 17–60                             | 489,754                                              | 489,720                                              | 31       |
| 18               | 66                             | 61–78                             | 175,672                                              | 175,680                                              | 45       |
| 18               | 52                             | 29–60                             | 349,825                                              | 349,852                                              | 77       |
| 18               | 52                             | 21–60                             | 444,056                                              | 444,091                                              | 78       |

* Sequence positions of Asp residues formed by deamination of Asn52 and Asn66 (each can be Asp or isoAsp, same theoretical m/z).

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REFERENCES
1. Galletti, P., Ingrosso, D., Manna, C., Clemente, G., and Zappia, V. (1995) Biochem. j. 306, 313–325
2. Clarke, S. (2003) Ageing Res. Rev. 2, 263–285
3. Robinson, N. E., and Robinson, A. B. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 944–949
4. Aswad, D. W., Paranandi, M. V., and Schurter, B. T. (2000) J. Pharm.
