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Suppression of PRMT6-mediated arginine methylation of p16 protein potentiates its ability to arrest A549 cell proliferation

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ABSTRACT

The tumor suppressor p16^{INK4A} (p16) blocks the cell cycle progression by inhibiting phosphorylation of the retinoblastoma protein. We describe here a novel aspect of the posttranslational control that has an important functional consequence on p16 protein. We first discovered that the p16 protein was methylated in various cell lineages. We then determined that the arginine 22, 131 and 138 of p16 were the main methylation sites. Western blotting and TUNEL analyses revealed that the p16 protein bearing these point mutations induced a higher apoptosis ratio than wild-type p16 in A549 cells. Furthermore, co-immunoprecipitation assays suggested that decrease of p16 arginine methylation level promoted the association of p16 with CDK4. Additionally, we determined that the protein arginine methyltransferase 6 (PRMT6) was responsible for the p16 arginine methylation. Results from flow cytometric analysis demonstrated that PRMT6 overexpression counteracted the cell cycle arrest at G1 phase induced by wild-type p16 in A549 cells. We also provided evidence that PRMT6 was able to interact with p16, and that the intensity of p16-CDK4 association was reduced upon PRMT6 overexpression. Together, data presented in this report establish that methylation at specific arginine residues of p16 protein by PRMT6 may be critical for the activity of p16.

1. Introduction

p16^{INK4a} (hereafter p16), as a cyclin-dependent kinase inhibitor, inhibits the phosphorylation of pRb by binding specifically to CDK4 and CDK6, thereby arrests cell progression in S phase (Nobori et al., 1994; Serrano et al., 1993). Abnormalities in p16 have been detected in about 70% of NSCLC (non-small cell lung cancer) cells, and loss of p16 protein has been reported in up to 50% of resected primary lung cancer tissue samples (Kratzke et al., 1996; Otterson et al., 1994). Exogenous expression of wild-type p16 in NSCLC A549 cells led to cell apoptosis by activating caspase-3, which in turn induced downregulation of Rb (Katsuda et al., 2002). Previous studies indicated that expression of p16 is regulated primarily at the transcriptional level, and a variety of transcription factors, such as Sp1 and Ets1, participate in the transcriptional regulation (Gizard et al., 2005; Ohtani et al., 2001). In our earlier studies, we found that the histone acetyltransferase p300 and the histone deacetylases HDAC3/4 were involved in p16 transcriptional regulation (Feng et al., 2009; Wang et al., 2008a,b). Moreover, there have been indications that phosphorylation of the Ser152 of p16 protein promotes its association with CDK4, suggesting that the posttranslational modification of p16 is important for the regulation of its function (Gump et al., 2003). Nevertheless, whether other posttranslational modifications, e.g., arginine methylation, participate in p16 functional regulation has not been elucidated.

The methylation of arginine residues of proteins has recently come into light as an important posttranslational modification (Bedford and Richard, 2005). Arginine residues are methylated by protein arginine methyltransferases (PRMTs). To date, four distinct PRMT activities have been identified in eukaryotic cells; types I and II are able to transfer methyl groups from S-adenosyl-L-methionine (AdoMet) to the guanidine group of arginine residues (Alessandra and Bedford, 2011). PRMT1, PRMT3, CARM1/PRMT4, PRMT6 and PRMT8 belong to type I PRMT enzymes that catalyze the formation of ω-monomethylarginine and asymmetric dimethylarginine, while type II enzymes, including PRMT5, PRMT7

Abbreviations: TUNEL, terminal deoxynucleotidyl transferase-mediated (dUTP) nick end-labeling; PRMT, protein arginine methyltransferase; FACS, flow cytometric analysis; CoIP, co-immunoprecipitation; FBS, fetal bovine serum; RNAi, RNA interference; siRNA, small interfering RNA; NSCLC, non-small cell lung cancer; AdoMet, S-adenosyl-L-methionine; GAR, glycine- and arginine-rich; ASYM, anti-dimethylarginine (asymmetric).

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and PRMT9, catalyze the formation of symmetric dimethylarginine residues (Alessandra and Bedford, 2011; Pahlich et al., 2006). Certain substrates are only monomethylated by PRMT7, which is referred to as type III enzymatic activity. Type IV enzyme activity catalyzes the monomethylation of the internal guanidine nitrogen atom and this type of activity has only been described in yeast (Alessandra and Bedford, 2011).

PRMT6 localizes exclusively in the nucleus, and can methylate arginine that resides within glycine- and arginine-rich (GAR) motifs (Frankel et al., 2002). However, PRMT6 can also methylate some specific cellular targets in non-GAR sequence, including HMGA1a/b (Sgarra et al., 2006), HIV TAT protein (Boulanger et al., 2005) and DNA polymerase β (El-Andaloussi et al., 2006). Moreover, PRMT6 methylates Histone 3 on arginine 2 (H3R2), as well as H2AR3 and H4R3 (Guccione et al., 2007; Hyllus et al., 2007; Iberg et al., 2008). Previous studies showed that the protein expression level of PRMT6 increased when the cell cycle proceeded from G0/G1 to S phase in HeLa cells (Kim et al., 2010), and reduced during the replicative senescence in WI-38 cells (Lim et al., 2008), suggesting that PRMT6 may be critical for cellular proliferation. We thus hypothesize that PRMT6 could abrogate the function of p16 in cancer cells that express p16, such as HeLa cells.

To the best of our knowledge, the present study is the first to investigate the novel form of p16 protein modification mediated by PRMT6, which brings about a profound functional consequence of the p16 activity. We discovered that hypomethylation of p16 protein exhibited a potentiated function in preventing cell proliferation. These results may provide the theoretical and experimental basis for new strategies in p16-related gene therapies.

2. Materials and methods

2.1. Cell culture and transient transfection

The cell lines (HeLa, T47D, DU145, Jurkat, HCT116, LNcap, NCI-H460, 293 T and A549) were cultured in appropriate media supplemented with 10% FBS (fetal bovine serum), 100 U/ml penicillin and 100 μg/ml streptomycin, and kept in a humidified atmosphere containing 5% CO_2 at 37 °C. The transfection of HeLa and A549 cells were carried out using the Lipofectamine 2000 reagent (Invitrogen).

2.2. Plasmids

The p16-EGFP-N1 plasmid was provided by Dr. Jun Chen (New York Medical College, USA). Three specific site mutations of the arginine residues were introduced into the p16 cDNA region by using a two-step PCR procedure as described previously (Han et al., 2006). Briefly, two simultaneous PCR reactions using p16-EGFP-N1 as template were performed. Amplified fragments from each PCR reaction were purified, mixed, and subjected to a second round of PCR using two external primers. The amplified PCR products were inserted into the *Hind*III and *BamH*I sites of EGFP-N1 vector, and the correct insertion was verified by DNA sequencing. PRMT6 was kindly supplied by Dr. Stephane Richard (Lady Davis Institute, Montreal, Quebec, Canada) (Boulanger et al., 2005).

2.3. Western blot and co-immunoprecipitation (CoIP)

Endogenous expression of PRMTs and exogenous expression of p16 protein were detected by Western blotting. A549 cells were harvested 48 h after transfection with the wild-type p16-GFP or the mutation p16-GFP plasmids. 1×10^6 cells were digested and lysed in lysis buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 0.5% NP-40; 1 mM EDTA; protease inhibitors cocktail) for 30 min at 4 °C after they were washed twice with PBS buffer. Total cell extracts were separated in 12% SDS-polyacrylamide gel electrophoresis (PAGE), then transferred to a polyvinylidene fluoride membrane. The membrane was incubated with anti-PRMT6 (Sigma, p6495), anti-p16 (Sigma, SAB330036), anti-GFP (Abcam, ab1218), anti-cleaved caspase-3 (Cell Signaling Technology, Asp175), anti-phospho-Rb (ser 807/811) (Sigma, R3903) or anti-β-actin (Sigma, A1978) antibodies, and visualized by using the Chemiluminescent Substrate method with the SuperSignal West Pico kit provided by Pierce Co. β-actin was used as an internal control for normalizing the loading materials.

Co-precipitation of p16 with PRMT6, or p16 with CDK4 was performed in HeLa or A549 cells. Cells were lysed in lysis buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 0.5% NP-40; 1 mM EDTA; protease inhibitor cocktail). Total cell extracts were precleared with 40 μl Protein A-agarose at 4 °C for 1 h. The supernatant was incubated with the anti-GFP (or anti-p16), anti-CDK4 (Santa Cruz, sc-260) and anti-PRMT6 antibodies, with gentle shacking for 1 h at 4 °C, followed by the addition of 40 μl of Protein A-agarose and an incubation for another 3 h. The pellets were collected and washed twice with Buffer A (20 mM Tris–HCl, pH 8.0; 10 mM NaCl; 0.5% NP-40; 1 mM EDTA). The beads were resuspended in 50 μl of 5× loading buffer and boiled for 10 min. The proteins were separated in a 12% SDS-PAGE gel and then transferred to polyvinylidene fluoride membrane for immunoblotting detection with anti-p16, anti-PRMT6, anti-CDK4, anti-cyclin D (Santa Cruz, sc-753) or anti-dimethyl-arginine (asymmetric) (Millipore, 07-414, ASYM24) antibodies.

2.4. RNA interference (RNAi)

The p16- and PRMT6-targeting siRNAs were synthesized according to the published data. Specifically, the target sequence for PRMT6 was 5′-gcaagacacgcacgtttca-3′ (Boulanger et al., 2005), and the p16-targeting sequence was 5′-gaggaggtgcgggcgctgc-3′ (Voorhoeve and Agami, 2003). Oligonucleotides that represent small hairpin RNAs (shRNAs) targeting these sequences were designed and cloned into the pSliencer2.0-U6 vector (Ambion) between *Bam*HI and *Hind*III restriction sites according to the manufacturer's instructions, as described. Cells were seeded in 6-well plates, cultured for 18 h and then transfected with 5 μg of PRMT6siRNA, p16siRNA or control vectors. Cells were incubated for another 48 h and collected for immunoblotting analysis.

2.5. Immunofluorescence and TUNEL assay

Apoptosis of A549 cells was measured using terminal deoxynucleotidyl transferase-mediated (dUTP) nick end-labeling (TUNEL) stain (KeyGEN, Nanjing, China). Briefly, the treated A549 cells grown on coverslip were washed twice with PBS, fixed in 4% paraformaldehyde for 30 min, followed by blockage of endogenous peroxidase activity with 3% hydrogen peroxide diluted in methanol for 10 min at room temperature. Cells were then incubated in 0.1% Triton X-100 for 2 min on ice. After washing with PBS, cells were covered with 50 μl of TUNEL reaction mixture and incubated in this solution for 60 min at 37 °C in a humidified dark chamber. Finally, cells were stained with DAPI before they were visualized under an Olympus FV1000 (Olympus, Japan) confocal microscope.

2.6. Flow cytometric analysis

A549 cells were trypsinized and washed with cold PBS once and then fixed in 70% ethanol and stored at 4 °C for 30 min. Fixed cells were washed with PBS and suspended in 100 μl of PBS, added with 1 μl 10 mg/ml RNAaseA and 100 μl propidium iodide. Stained cells were incubated at room temperature for 30 min in the dark. The