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The E3 ubiquitin ligase CHIP mediates ubiquitination and proteasomal degradation of PRMT5

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ABSTRACT

Protein arginine methyltransferase 5 (PRMT5) is an important member of the protein arginine methyltransferase family that regulates many cellular processes through epigenetic control of target gene expression. Because of its overexpression in a number of human cancers and its essential role in cell proliferation, transformation, and cell cycle progression, PRMT5 has been recently proposed to function as an oncoprotein in cancer cells. However, how its expression is regulated in cancer cells remains largely unknown. We have previously demonstrated that the transcription of PRMT5 can be negatively regulated by the PKC/c-Fos signaling pathway through modulating the transcription factor NF-Y in prostate cancer cells. In the present study, we demonstrated that PRMT5 undergoes polyubiquitination, possibly through multiple lysine residues. We also identified carboxyl terminus of heat shock cognate 70-interacting protein (CHIP), an important chaperone-dependent E3 ubiquitin ligase that couples protein folding/refolding to protein degradation, as an interacting protein of PRMT5 via mass spectrometry. Their interaction was further verified by co-immuoprecipitation, GST pull-down, and bimolecular fluorescence complementation (BiFC) assay. In addition, we provided evidence that the CHIP/chaperone system is essential for the negative regulation of PRMT5 expression via K48-linked ubiquitin-dependent proteasomal degradation. Given that down-regulation of CHIP and overexpression of PRMT5 have been observed in several human cancers, our finding suggests that down-regulation of CHIP may be one of the mechanisms underlying PRMT5 overexpression in these cancers.

1. Introduction

Protein arginine methyltransferase 5 (PRMT5) is a type II methyltransferase that can symmetrically methylate arginine residues of histones and non-histone substrates [1]. The symmetric methylation on histone H4 at arginine 3 (H4R3) and/or histone H3 at arginine 8 (H3R8) is generally thought to result in transcriptional repression of target genes such as suppressor of tumorigenicity 7 [1,2], nonmetastatic 23 [1], p53 [3], and RBs (RB1, RBL1, RBL2) [4]; whereas methylation of non-histone substrates including E2F1, p53, RelA/p65, epidermal growth factor receptor (EGFR), RAD9, and programmed cell death 4 generates more diverse cellular effects [5,6]. For example, the methylation of E2F1 at R111 and R113 by PRMT5 reduces its ability to suppress cell growth and to promote apoptosis, conferring a survival advantage to tumor cells [7]. Also, methylation of p65 at R30 activates NF-κB signaling pathway and facilitates the expression of its target genes including tumor necrosis factor (TNF), TNF receptor-associated factor 1, interleukin-8, and interleukin 1A [8]. It has been proposed that PRMT5 functions as an oncoprotein by either silencing the expression of tumor suppressors or activating the signaling molecules that are crucial for cancer cells [5]. In fact, recent studies have shown that up-regulation of PRMT5 expression correlates with the development and progression of several human cancers, such as breast cancer [9], gastric cancer [10], colorectal cancer [7], ovarian cancer [11], leukemia, and lymphoma [2]. However, how PRMT5 expression is regulated in cancer cells remains largely unknown.

We have previously demonstrated that in human prostate cancer cells, PRMT5 can be transcriptionally activated by nuclear factor Y (NF-Y), and that the protein kinase C (PKC)/c-Fos signaling pathway negatively regulates PRMT5 expression through transcriptional down-regulation of NF-Y [12]. Recent research has also found that MYC directly up-regulates the transcription of the core small nuclear ribonucleoprotein particle (snRNP) assembly genes, in which PRMT5 is the key

Abbreviations: PRMT5, protein arginine methyltransferase 5; EGFR, epidermal growth factor receptor; TNF, tumor necrosis factor; NF-Y, nuclear factor Y; PKC, protein kinase C; Hsp90, heat shock protein 90; UPS, ubiquitin–proteasome system; CHIP, carboxyl terminus of heat shock cognate 70-interacting protein; TPR, tetratricopeptide repeat; Hsp70, heat shock protein 70; HEK293T, human embryonic kidney 293 T; CHX, cycloheximide; PMA, phorbol-12-myristate-13-acetate; GA, geldanamycin; 17-AAG, 17-(Allylamino)-17-demethoxygeldanamycin; WT, wild-type; MS, mass spectrometry; GST, glutathione S-transferase; MEP50, methylosome protein 50; WCL, whole cell lysate.

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enzymatic component [13]. In addition to the transcriptional regulation of PRMT5 expression, PRMT5 is also regulated by miR-92b/96 in mantle cell lymphoma [2]. Research from the same group also demonstrates that down-regulation of another three miRNAs (miR-19a, miR-25, and miR-32) in several lymphoid cancer cell lines leads to an increase of PRMT5 protein expression [4]. Recently, it has been observed that treatment of three different human cancer cell lines (ovarian, colon, and melanoma) with the heat shock protein 90 (Hsp90) inhibitor 17-AAG reproducibly down-regulates the expression of PRMT5 at the protein level [14]. Given the role of Hsp90 in the regulation of protein folding and degradation, it is reasonable to postulate that PRMT5 may be a putative client protein for Hsp90 [14].

Ubiquitination is one of the most important post-translational modifications that regulate diverse cellular signaling [15]. To execute the ubiquitination process, the consecutive action of three enzymes, including the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligases, is required for the attachment of ubiquitin to a substrate [16,17]. The ubiquitin–proteasome system (UPS) is often utilized to fine-tune the expression of target proteins that are associated with cancer development and progression. As a mechanism of quality control for protein folding, ubiquitin-dependent proteasomal degradation is often coupled with the molecular chaperone system to remove misfolded proteins [16,18,19]. In this system, E3 ubiquitin ligases appear to be the key regulators that function together with the chaperone system to regulate protein degradation. Carboxyl terminus of heat shock cognate 70-interacting protein (CHIP), also known as STUB1/STIP1 homology and U-Box containing protein 1, is a chaperone-dependent E3 ubiquitin ligase [20,21]. CHIP contains three tandem tetratricopeptide repeat (TPR) motifs, through which it interacts with the chaperones including heat shock protein 70 (Hsp70) and Hsp90, and a U-box domain, which is responsible for ubiquitination of the chaperone-bound substrates. Recently, CHIP has been proposed as a tumor suppressor since lower expression of CHIP promotes cell proliferation and/or inhibits apoptosis in breast cancer [22,23], gastric cancer [24], pancreatic cancer [25], and colorectal cancer [26]. Specifically, the role of CHIP in these cancers is to control the expression of several crucial proteins, such as ErbB2 [22], hypoxia-inducible factor-1a [27], c-Myc [28], p65 [26], and EGFR [25].

In the present study, we demonstrated that PRMT5 can undergo polyubiquitination both *in vivo* and *in vitro*. We also provided evidence that the E3 ubiquitin ligase CHIP couples to the molecular chaperone system (Hsp70/Hsp90) and mediates ubiquitin-dependent proteasomal degradation of PRMT5. Our work provides a new mechanism underlying PRMT5 overexpression in cancer cells.

2. Materials and methods

2.1. Cell culture and reagents

Prostate cancer cell line LNCaP, human embryonic kidney 293 T (HEK293T), and COS-1 cells were purchased from American Type Culture Collection (ATCC) and were maintained in RPMI 1640 or DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS) supplemented with penicillin/streptomycin, sodium pyruvate, and L-glutamine. All cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. Cycloheximide (CHX) and MG132 were purchased from Sigma. GA and 17-AAG were purchased from Tocris Bioscience.

2.2. Plasmid construction

The pCMV-Myc-PRMT5 expression plasmid was previously constructed [12] and was used as a template to generate methyltransferase activity-deficient mutant pCMV-Myc-PRMT5-R368A [29], and a series of truncated fragments covering the residues 229–637, 284–637, 352–637, and 451–637. For mutagenesis, nucleotide substitutions (from lysine/K to arginine/R) were introduced into PRMT5 using ligation PCR as described previously [12,30]. pCMV-FLAG-PRMT5 was generated by subcloning PRMT5 into pCMV-FLAG expression vector (Sigma). Various truncated mutants and single-point mutations of PRMT5 were generated using PCR or ligation PCR, and then subcloned into pCMV-FLAG or pCMV-HA (Clontech). The chaperone-interaction-deficient K30A mutant (Lysine/K to alanine/A at position 30) and E3 ubiquitin ligase activity-deficient H260Q mutant (histidine/H to glutamine/Q at position 260) for CHIP were generated using the same methods. Two truncated fragments of CHIP were amplified by PCR using primers specific for ΔU-box (forward primer: 5′-ccggaattcggatgaagggcaaggagg-3′ and reverse primer: 5′-cggggtaccgaggtaggagtgcagctc-3′) and ΔTPR (forward primer: 5′-ccggaattcggatcgcgaagaagaagcg-3′ and reverse primer: 5′-cggggtaccgtagtcctccacccagcc-3′), and then were subcloned into pCMV-FLAG. To express CHIP as a fusion with GST, the cDNA encoding CHIP was subcloned into pGEX-4 T2 vector. For BiFC plasmid construction, pCMV-Myc and pCMV-HA were used to generate pBiFC-VN155(I152L)-N and pBiFC-VC155-N vectors, followed by the subcloning of the cDNAs encoding PRMT5 and CHIP into either of these two BiFC cloning vectors. cDNAs encoding wild-type (WT) ubiquitin, ubiquitin-K48R, and ubiquitin-K63R were kind gifts from Dr. Chittaranjan Das lab (Purdue University) and were then subcloned into pCMV-HA vector. All plasmid constructs were verified by enzymatic digestion or DNA sequencing.

2.3. In vivo ubiquitination assay

Cells were co-transfected with the plasmid encoding HA-Ubiquitin and Myc-PRMT5 or its various mutants, along with plasmids encoding FLAG-CHIP or CHIP mutants for the indicated time, followed by the treatment with MG132 (10 μM) for another 6 h. Whole cell lysate (WCL) was prepared, and 500 μg of the WCL was used for immunoprecipitation (IP) using the antibodies against PRMT5, HA, and Myc, followed by the detection of respective proteins by immunoblotting (IB). For the detection of protein ubiquitination, a final concentration of 10 mM NEM (Sigma, E3876-5G) was added to the IP buffer in order to inhibit protein deubiquitination.

2.4. Co-immunoprecipitation and immunoblotting

Cells were harvested and washed twice with cold phosphate buffered saline (PBS) and then lysed by sonication in lysis buffer (10 mM Tris–HCl pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl) containing 1 mM phenylmethlysulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), protease cocktail, 25 mM okadaic acid, and 1% Triton X-100 as described previously [31]. For the preparation of soluble and insoluble samples, supernatant was collected and saved as soluble fraction, and pellets were resuspended in the same volume of lysis buffer and sonicated on ice, and the boiled pellets were saved as insoluble fraction. For co-immunoprecipitation (Co-IP), cells were treated with or without 17-AAG for 24 h, and the cell lysate was prepared by sonication in IP buffer (50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1.5 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM beta-glycerolphosphate, 1 mM PMSF, and protease cocktail), and IP was performed following the same procedure as described previously [12,32]. The antibodies used for IB analysis were anti-β-actin (Cell Signaling Technology, 8H10D10), anti-PRMT5 (Millipore, 07–405), anti-CHIP (Santa Cruz, G-2 sc-133,066), anti-FLAG M2 (Cell Signaling Technology, 9A3), anti-HA (Cell Signaling Technology, 6E2), anti-GST (BD Biosciences), and anti-Myc (GenScript, A00704-100). Secondary HRP-conjugated antibodies were purchased from Amersham Biosciences.