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MTHFR promotes heterochromatin maintenance

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ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in the folate cycle, catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine. Methionine serves as the precursor of the active methyl donor *S*-adenosylmethionine, which provides methyl groups for many biological methylations. It has been reported that MTHFR is highly phosphorylated under unperturbed conditions and T34 is the priming phosphorylation site. In this report, we generated a phospho-specific antibody that recognized T34-phosphorylated form of MTHFR and revealed that MTHFR was phosphorylated at T34 in vivo and this phosphorylation peaked during mitosis. We further demonstrated that the cyclin-dependent kinase 1 (CDK1)/Cyclin B1 complex is the kinase that mediates MTHFR phosphorylation at T34 and the MTHFR immunocomplex purified from mitotic cells exhibited lower enzymatic activity. Inhibition of MTHFR expression resulted in a decrease of H3K9me3 levels, and an increase of transcription of the centromeric heterochromatin markers. Taken together, our results demonstrated that CDK1/Cyclin B1 phosphorylates MTHFR on T34 and MTHFR plays a role in the heterochromatin maintenance at the centromeric region.

1. Introduction

Methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), which is a ubiquitously expressed cytoplasmic flavoenzyme involved in the folate cycle, catalyzes the reduction of 5,10-methylenetetrahydrofolate (5,10-CH2-THF) to 5-methyltetrahydrofolate (5-CH3-THF). 5-CH3-THF, the most abundantly circulating form of folate, supplies methyl groups for methylation of homocysteine to methionine. Methionine is then converted into *S*-adenosylmethionine (SAM), which is the universal methyl donor for many biological methylation reactions, such as protein and nucleic acid methylation. 5,10-CH2-THF and its derivate, 10-formyl-THF, are indispensable substrates for thymidylates and purine nucleotides synthesis, respectively [1]. Thus, MTHFR couples folate cycle and methionine cycle to accomplish one-carbon metabolism in cells, regulating the balance between cellular methylation reactions and nucleic acid synthesis by channeling one-carbon unit distribution.

Genetic variation in MTHFR influences susceptibility to occlusive vascular disease [2–5], neural tube defects (NTD) [6], colon cancer [7–10] and acute leukemia [11,12]. The most extensively investigated polymorphism in MTHFR is C667T, in which its enzymatic activity decreases 30–40% in vitro [3,4]. Several retrospective clinical studies indicate that this polymorphic allele may have a protection role in colon cancer [7–9] and adult leukemia [11,12]. However, given that MTHFR deficiency both in human beings and mice leads to genome-wide DNA hypomethylation [13,14], which is a common feature of cancer and is associated with genomic and chromosomal instability, it warrants further investigation to clarify how MTHFR links to genome instability.

Heterochromatin is a highly packed and condensed DNA and protein complex. There are two types of heterochromatin, namely, facultative and constitutive heterochromatin. Facultative heterochromatin is the result of gene silencing, whereas constitutive heterochromatin usually occurs around the chromosomal centromeric and telomeric regions [15–19]. Heterochromatin, which often associates with di and tri-methylation of H3K9, mainly regulates gene expression and protects the chromosomal integrity [20–25].

It has been reported that MTHFR is phosphorylated on T34 and this phosphorylation inhibits its catalytic activity [26]. In this report, we generated a phospho-specific antibody recognizing the

Abbreviations: 5,10-CH2-THF, 5,10-methylenetetrahydrofolate; 5-CH3-THF, 5-methyltetrahydrofolate; *α-Sat*, *alpha satellite*; CDK1, cyclin-dependent kinase 1; H3K9me1, monomethylation of H3K9; H3K9me2, dimethylation of H3K9; H3K9me3, trimethylation of H3K9; *Maj-Sat*, *major satellite*; MTHFR, methylenetetrahydrofolate reductase; NTD, neural tube defect; SAM, *S*-adenosyltetrahydrofolate; *Sat2*, *satellite 2*.

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T34-phosphorylated form of MTHFR and demonstrated that MTHFR is phosphorylated on T34 in vivo by CDK1/Cyclin B1 under unperturbed conditions. We also establish a functional link between MTHFR and heterochromatin maintenance at the centromeric regions.

2. Materials and methods

2.1. Cell lines, plasmids, siRNA oligos and antibodies

HEK293T, HeLa, A549, HT29 and U2OS were purchased from ATCC. All the cell lines were grown in DMEM (Thermo) containing 10% fetal bovine serum (FBS, Hyclone) and 1% standard antibiotics (Hyclone), at 37 °C in a 5% CO2 incubator (Thermo).

The full-length cDNA of MTHFR (GeneBank accession number: NM_005957) was amplified by reverse-transcriptase polymerase chain reaction and subcloned into pcDNA-3HA vector, pcDNA-3FLAG vector and pTrc-HIS vector, resulting in pcDNA-3HA-MTHFR, pcDNA-3FLAG-MTHFR and pTrc-HIS-MTHFR. The point-mutant construct MTHFR(T34A) and MTHFR(T34D) were generated using the QuickChange Site Directed Mutagenesis kit (Stratagene).

All siRNA oligo duplexes (Ontarget plus option) were purchased from Dharmacon (Thermo). Control siRNA oligonucleotide duplex was siCTR: CGU ACG CGG AAU ACU UCGA. MTHFR siRNA oligonucleotide duplexes were si1-MTHFR, AGU GAG AGC UCC AAA GAUA; si2-MTHFR, GAC CAA AGA GUU ACA UCUA; si3-MTHFR, GAU CAU CAA GCC CAC CGUA; si4-MTHFR, AAA CCG GAA UGG UCA CAAA.

Antibodies against MTHFR, pH3(S10) and HA were purchased from Bethyl Laboratories Inc. Antibodies against H3K9me1 and H3K9me3 were purchased from Abcam. Antibodies against FLAG and β-actin were purchased from Sigma. Antibodies against H3 and H3K9me2 were purchased from Cell Signaling Technologies.

2.2. Cell synchronization

HeLa cells were synchronized at G2/M border by thymidine–nocodazole arrest. Firstly, the cells were blocked with 2 mM thymidine in medium for 24 h, then the thymidine-arrested cells were released into fresh medium for 3 h and 340 nM nocodazole was added. 16 h Later, the nocodazole-arrested cells were released into fresh medium and collected at 0, 2, 4, 6, 8, 10, 12, 14, and 16-h time points.

2.3. CDK1/Cyclin B1 kinase assay

For in vitro kinase assays, bacterially produced GST, GST-MyPT1(297–600aa), HIS-MTHFR or HIS-MTHFR(T34A) recombinant proteins were incubated with active baculovirus-expressed human CDK1/Cyclin B1 complex (Millipore) in the kinase buffer. The kinase assay was carried out in a 30-μl reaction, containing 50 mM Tris–HCl, 10 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA, 0.01% Brij35 at pH 7.5, 50 mM cold ATP (or 5 μCi [γ-^{32}P]-ATP), 50 ng CDK1/Cyclin B1 complex and 1 μg purified recombinant substrates. The reactions were incubated at 30 °C for 30 min, quenched with SDS sample buffer and analyzed by SDS–PAGE followed by western blot or autoradiography.

2.4. NADPH-menadione oxidoductase assay

MTHFR enzyme activity was determined by using NADPH-menadione oxidoductase assay as described [27,28], except that the assay volume was reduced to 1 ml. The reaction was started by addition of menadione at 25 °C and absorbance changes were monitored at 340 nm.

2.5. Real-time PCR assay

Total RNA was extracted from HeLa cells with depletion of endogenous MTHFR by siRNA or mock treatment using TriZol (Invitrogen). To exclude potential contamination of DNA, extracted RNA was treated by DNase I (Takara) for 30 min at 37 °C and then reverse transcription PCR was performed by PrimeScript RT-PCR Kit (Takara). The relative expression of *α-Sat*, *Sat2*, or *Major-Sat* was measured by real-time PCR with SYBR-green dye (Bio-Rad) and CFX manager 3.0. Primers used were: *α-Sat*: CTGCACTACCTGAAGAGGAC (sense), GATGGTTCAACACTCTTACA (anti); *Sat2*: CATCGAATGGAAATGAA AGGAGTC (sense), ACCATTGGATGATTGCAGTCAA (anti); *Major-Sat*: GACGACTTGAAAAATGACGAAATC (sense), CATATTCCAGGTCCTTCA GTGTGC (anti); GAPDH: GAAGGTGAAGGTCGGAGTC (sense), GAA GATGGTGATGGGATTTC (anti) [29].

3. Results

3.1. T34 is the major phosphorylation site of MTHFR

It came to our attention that the anti-MTHFR antibody was reactive to two distinct bands, of which the slower migration form was dominant over the faster migration form, in both immunoblotting analysis and immunoprecipitation/immunoblotting analysis in a variety of human cancer cell lines, including cervical cancer line HeLa, lung cancer line A549, osteosarcoma line U2OS, and colon cancer line HT29 (Fig. 1A and data not shown). When the immunoprecipitated MTHFR was treated with the calf intestinal alkaline phosphatase, most, if not all of the slower migration form of MTHFR was compressed into the faster migration form (Fig. 1A). When HA-tagged MTHFR was expressed in HeLa cells, it behaved

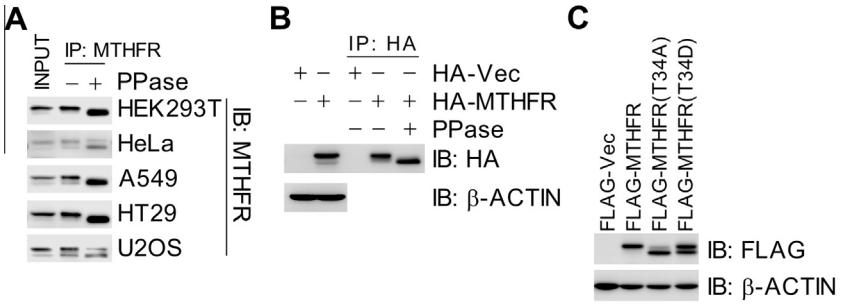

Fig. 1. The T34 residue drives MTHFR phosphorylation in vivo. (A) MTHFR is a phosphoprotein. Endogenous MTHFR was immunoprecipitated from different cancer cell lines as indicated. The MTHFR immunoprecipitates were divided into two parts for mock treatment and phosphatase (PPase) treatment respectively. The treated MTHFR immunoprecipitates were blotted with an anti-MTHFR antibody. (B) Ectopically expressed HA-MTHFR is a phosphoprotein. HA-MTHFR was transiently expressed in HeLa cells, and the HA immunoprecipitates were treated as described in (A). (C) The T34 residue drives MTHFR phosphorylation. FLAG-tagged MTHFR and its mutants were expressed in HeLa cells. Total lysates were extracted for immunoblotting with antibodies as indicated.