The DNA-binding activity of mouse DNA methyltransferase 1 is regulated by phosphorylation with casein kinase 1δ/ε

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Dnmt1 (DNA methyltransferase 1) is an enzyme that recognizes and methylates hemimethylated DNA during DNA replication to maintain methylation patterns. The N-terminal region of Dnmt1 is known to form an independent domain structure that interacts with various regulatory proteins and DNA. In the present study, we investigated protein kinases in the mouse brain that could bind and phosphorylate the N-terminal regulatory domain of Dnmt1. A protein fraction containing protein kinase activity for phosphorylation of Dnmt1(1–290) was prepared using Dnmt1(1–290)-affinity, DNA–cellulose and gel-filtration columns. When the proteins in this fraction were analysed by LC-MS/MS (liquid chromatography tandem MS), CK1δ/ε (casein kinase 1δ/ε) was the only protein kinase identified. Recombinant CK1δ/ε was found to bind to the N-terminal domain of Dnmt1 and significantly phosphorylated this domain, especially in the presence of DNA. Phosphorylation analyses using various truncation and point mutants of Dnmt1 revealed that the major priming site phosphorylated by CK1δ/ε was Ser146, and that subsequent phosphorylation at other sites may occur after phosphorylation of the priming site. When the DNA-binding activity of phosphorylated Dnmt1 was compared with that of the non-phosphorylated form, phosphorylation of Dnmt1 was found to decrease the affinity for DNA. These results suggest that CK1δ/ε binds to and phosphorylates the N-terminal domain of Dnmt1 and regulates Dnmt1 function by reducing the DNA-binding activity.

Key words: casein kinase 1 (CK1), DNA-binding activity, DNA methylation, DNA methyltransferase, domain structure, protein phosphorylation.

INTRODUCTION

In mammals, the fifth position of cytosines in CpG sequences in genomic DNA is often methylated [1]. DNA methylation is essential for embryonic development and has been shown to be important for transcriptional repression of imprinted genes [2,3]. The methylation of DNA at CpG dinucleotides is catalysed by two classes of enzymes: the de novo type of DNA methyltransferase, Dnmt3a and Dnmt3b, and the maintenance type, Dnmt1. Dnmt1 has a strong preference for hemimethylated CpG sites, which are the products of DNA replication, and the enzyme plays an important role in maintaining the methylation pattern of DNA [4].

Dnmt1 is composed of a 180 kDa single polypeptide comprising the N-terminal regulatory domain, which covers two-thirds of the molecule, and the C-terminal catalytic domain, which contains essential motifs for the methyltransferase activity. The N-terminal region of the regulatory domain has a DNA-binding motif [5] and the binding sites for various protein factors. In particular, the N-terminal 36 kDa sequence is known to form an independent domain [6], which serves as a platform for the recruitment of regulatory proteins, such as PCNA (proliferating cell nuclear antigen) [7], the transcription factor DMAP1 [8], Dnmt3a and Dnmt3b [9], and MeCP2 (methylated-CpG-binding protein 2) [10]. Although the N-terminal region of 119–197 is known to interact preferentially with the minor groove of AT-rich sequences in DNA [6], the physiological significance of the DNA-binding activity of this region is still unclear.

Most cellular events are regulated by protein phosphorylation, and one-third of cellular proteins are believed to be phosphorylated. In previous studies, phosphorylation of Dnmt1 at multiple sites, including Ser146, was reported [11,12]. Although the regulation of gene expression through phosphorylation of Dnmt1 by protein kinases is a very important and attractive issue, there is little available information concerning the protein kinases involved in the phosphorylation and regulation of Dnmt1. In previous studies, we produced monoclonal antibodies, designated Multi-PK antibodies, that can detect a wide variety of protein kinases [13–17]. Using these antibodies, we detected a protein kinase of approx. 110 kDa in a mouse brain extract that interacted with Dnmt1, and identified it as CDKL5 (cyclin-dependent kinase-like 5) [18]. However, the phosphorylation of Dnmt1 by CDKL5 was very weak, and the role of this phosphorylation remained unclear. Therefore, we hypothesized that certain other protein kinases may phosphorylate Dnmt1.

In the present study, we used a GST (glutathione transferase)–Dnmt1(1–290) affinity column to isolate protein kinases that associate with and phosphorylate Dnmt1, since Dnmt1(1–290) contains the N-terminal regulatory domain for binding various protein factors. We monitored the DBK (Dnmt1(1–290)-binding kinase) activity by autoradiography, using Dnmt1(1–290) as a substrate, and Phos-tag SDS/PAGE, and identified it as CK1δ/ε.

Abbreviations used: CDKL5, cyclin-dependent kinase-like 5; CK1, casein kinase 1; DBK, Dnmt1(1–290)-binding kinase; DMEM, Dulbecco's modified Eagle's medium; Dnmt, DNA methyltransferase; FL, full-length; GST, glutathione transferase; KD, kinase-dead; LC-MS/MS, liquid chromatography tandem MS; PCNA, proliferating cell nuclear antigen.

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(casein kinase 1δ) by LC-MS/MS (liquid chromatography tandem MS) analysis. Recombinant CK1 isoforms were found to associate with Dnmt1 and efficiently phosphorylate it. Phosphorylation assays using various truncation and point mutants of Dnmt1 revealed that the major phosphorylation site in Dnmt1 for CK1δ was Ser146. In addition, the DNA-binding affinity of the N-terminal region of Dnmt1 was significantly reduced after phosphorylation by CK1δ.

MATERIALS AND METHODS

Materials

ATP, BSA, α-casein from bovine milk and mouse and rabbit anti-FLAG antibodies were purchased from Sigma Chemicals. An anti-His6 antibody was obtained from Roche Diagnostics. [γ-32P]ATP (111 TBq/mmol) was purchased from PerkinElmer. An anti-GST antibody, glutathione–Sepharose 4B and HiTrap Chelating HP columns were obtained from GE Healthcare Biosciences. The acrylamide-dependant Phos-tag ligand was obtained from the Phos-tag consortium (http://www.phos-tag.com). Dnmt1[FL] (full-length); 1–1620), GST–Dnmt1(1–290), GST–Dnmt1(1–197) GST–Dnmt1(1–146), GST–Dnmt1(1–118), GST–Dnmt1(197–290), and His6-tagged PCNA were prepared as described previously [6]. An anti-C-terminal Dnmt1 antibody was raised in rabbit and affinity purified with antigen-coupled Sepharose CL-4B as described previously [19]. Dnmt1(ΔN; 291–1620) was expressed and purified as described previously [20]. A monoclonal antibody against the mouse Dnmt1(72–86) peptide was produced as described previously [13].

Construction of plasmids

For mouse CK1δ, a 5′-upstream primer containing an NheI site (underlined) (5′-GGTACGGATCCGCTACGCGCC-3′) and a 3′-downstream primer containing an XhoI site (bold) (5′-GGTACGGATCCGCTACGCGCCGCGA-3′) were used for PCR with a mouse brain cDNA library as a template. Mouse CK1ε was generated by amplification of cDNA fragments using a 5′-upstream primer containing an Nhel site (underlined) (5′-GGTACGGATCCGCTACGCGCCGCGA-3′) and a 3′-downstream primer containing an XhoI site (bold) (5′-GGTACGGATCCGCTACGCGCCGCGA-3′). The PCR fragments were digested by NheI and XhoI, and ligated into pET-23a(+) (Novagen) to generate the plasmids pET-CK1δ and pET-CK1ε respectively. For FLAG−CK1 isoforms, the Nhel-EcoRI fragments from pET-CK1δ and pET-CK1ε were ligated into pcDNA3.1 (+) to generate pcDNA3.1(FLAG)-CK1δ and pcDNA3.1(FLAG)-CK1ε respectively.

Isolation and identification of protein kinases that bind to and phosphorylate Dnmt1(1–290)

Dnmt1(1–290)-binding proteins were prepared as described previously [18]. The Dnmt1(1–290)-binding proteins (12 ml) were loaded on to a DNA–cellulose (Deoxyribonucleic Acid–Cellulose Double-stranded from calf thymus DNA; Sigma) column prewashed with 1 mg/ml BSA and equilibrated with buffer B containing 0.15 M NaCl. After washing with 50 ml of buffer B containing 0.15 M NaCl, proteins including putative Dnmt1-binding kinases were sequentially eluted with buffer B containing 0.3 M NaCl and 1 M NaCl. The active fractions were pooled and loaded on to a HiLoad 16/60 Superdex 200 pg column (GE Healthcare Biosciences) prewashed with buffer B containing 0.15 M NaCl, 1 M EDTA and 0.5 M EGTA. Chromatography was performed with a flow rate of 1.25 ml/min, and 1.2 ml fractions (80 fractions in total) were collected after a 50 min run. The separation properties were determined in a first run with molecular-mass standard proteins (β-amylose: 200 kDa, transferrin: 81 kDa, ovalbumin: 43 kDa, and carbonic anhydrase: 29 kDa).

Fractions 37–46 (DBKs) were pooled, dialysed against buffer B (20 mM Tris/HCl, pH 7.5, containing 0.05% Tween 40 and 1 mM 2-mercaptoethanol) and stored in aliquots at −30°C until use.

GST-pulldown assay

Approx. 10 μg of CK1 or PCNA in 200 μl of buffer B containing 0.15 M NaCl was added to 20 μl of glutathione–Sepharose 4B.
prebound with 10 μg of GST–Dnmt1(1–290). After incubation with gentle rotation at 2°C for 2 h, the matrixes were washed five times with buffer B containing 0.15 M NaCl, and the proteins were eluted with SDS/PAGE sample buffer.

**Cell culture, transfection, immunoprecipitation and co-precipitation**

Transfection of pcFLAG-CK1 isoforms into Neuro2a cells was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, Neuro2a cells (5 × 10⁵) were plated in 35 mm dishes in 2 ml of DMEM (Dulbecco’s modified Eagle’s medium; Sigma) containing 10% fetal calf serum at 23°C before transfection. Transfection was performed by incubation of the Neuro2a cells in 1 ml of DMEM containing 5% fetal calf serum, 6 μl of Lipofectamine™ 2000 and 2.5 μg of plasmid DNA for 24 h. For immunoprecipitation, Protein G-Sepharose 4 Fast Flow (GE Healthcare Biosciences) was mixed with a mouse anti-FLAG antibody in 200 μl of buffer C (20 mM Tris/HCl, pH 7.5, 1 mM EDTA and 0.5% Triton X-100) containing 300 mM NaCl at 4°C for 2 h, and washed three times with buffer C containing 300 mM NaCl. Cell extracts of Neuro2a cells expressing FLAG–CK1δ/ε were incubated with anti-FLAG antibody-bound Sepharose at 4°C for 2 h, and washed three times with buffer C containing 300 mM NaCl and buffer C containing 150 mM NaCl. Then, the mixtures were incubated with 4 μg of Dnmt1(FL) in 200 μl of buffer C containing 150 mM NaCl, and washed five times with buffer C containing 150 mM NaCl. The pulled-down proteins were subjected to Western blotting with an anti-C-terminal Dnmt1 antibody and a rabbit anti-FLAG antibody.

**Protein phosphorylation**

Phosphorylation of Dnmt1 and Dnmt1 mutants by CK1 was carried out essentially as described previously [18]. CK1 (10 ng) was incubated with 500 ng of Dnmt1(FL) and Dnmt1 mutants in the presence or absence of mouse genomic DNA (10 μg/ml). The reactions were carried out in 20 mM Hepes/NaOH buffer (pH 7.4) containing 10 mM MgCl₂, 1 mM dithiothreitol and 100 μM ATP or [γ-³²P]ATP at 30°C for 30 min. The reactions were stopped by adding an equal volume of 2 × SDS/PAGE sample buffer. The mixtures were electrophoresed on a SDS/polyacrylamide gel or an SDS/polyacrylamide gel containing Phos-tag [24], and the phosphorylated proteins were visualized by autoradiography or subjected to Western blotting.

The stoichiometry of phosphate incorporation into GST–Dnmt1(1–290) was determined as follows. GST–Dnmt1(1–290) was phosphorylated by CK1 in an aforementioned reaction mixture in a final volume of 70 μl. After incubation at 30°C for 30, 60, 90 and 120 min, a 10 μl aliquot of the mixture was withdrawn, spotted on to a 2 cm square of the Whatman 3MM chromatography paper (Whatman), and immediately placed in 75 mM phosphoric acid. The ³²P-phosphate incorporation into GST–Dnmt1(1–290) was measured essentially according to the method of Corbin and Reimann [25]. Phosphate incorporated into GST–Dnmt1(1–290) was calculated as mol phosphate/mol GST–Dnmt1(1–290) by subtracting the radioactivity into CK1 from that into CK1 plus GST–Dnmt1(1–290).

**DNA–cellulose binding assay**

Approx. 5 μg of GST–Dnmt1(1–290), GST–Dnmt1(1–290)(S146A) or Dnmt1(FL) was phosphorylated by CK1δ (1 μg) essentially as described above, except that mouse genomic DNA (1 μg) was removed from the reactions. Samples in 300 μl of buffer B containing 0.15 M NaCl were mixed with 20 μl of DNA–cellulose and rotated at 2°C for 2 h. After the incubation, the matrixes were washed three times with buffer B containing 0.15 M NaCl, and the proteins were eluted with SDS/PAGE sample buffer.

**DNA–cellulose dissociation assay**

Approx. 5 μg of GST–Dnmt1(1–290) or Dnmt1(FL) in 250 μl of buffer B containing 0.15 M NaCl was mixed with 20 μl of DNA–cellulose. The mixtures were rotated at 2°C for 30 min, and the matrixes were washed three times with buffer B containing 0.15 M NaCl. The matrixes were incubated with CK1δ (2 μg) in the presence or absence of 100 μM ATP at 30°C for 15, 30 or 45 min under genomic DNA-free conditions. After the incubations, the supernatants were added to an equal volume of 2 × SDS/PAGE sample buffer, and analysed by SDS/PAGE followed by Western blotting.

**Protein determination, SDS/PAGE, Phos-tag SDS/PAGE and Western blotting**

Protein concentrations were determined by the method of Bensadoun and Weinstein [26] using BSA as a standard. SDS/PAGE was performed essentially according to the method of Laemmli [27] on slab gels consisting of a 6% or 10% acrylamide separating gel and a 3% stacking gel. The resolved proteins were transferred electrophoretically to nitrocellulose membranes, and immunoreactive protein bands were detected essentially as described previously [13]. For Mn²⁺-Phos-tag SDS/PAGE, 20 μM acrylamide-pendant Phos-tag ligand and 40 μM MnCl₂ were added to a 6% separating gel before polymerization. The Phos-tag SDS/PAGE was performed essentially as described previously [24].

**DNA methylation assay**

DNA methylation activities were determined essentially as described previously [28]. Briefly, the methylation reaction mixture contained 80 ng of Dnmt1(FL), 30 nM hemimethylated oligonucleotide substrate (42-mer, 12CpG) and 5.3 μM S-[methyl-³H]adenosyl-L-methionine (15 Ci/mmol; Amersham Biosciences) in 25 μl of reaction buffer comprising 5% glycerol, 0.5 mM EDTA, 0.2 mM dithiothreitol, 0.1 mg/ml BSA and 20 mM Tris/HCl (pH 7.4). After 1 h of incubation at 37°C, the radioactivity was determined using a scintillation counter.

**RESULTS**

**Identification of protein kinases that bind to and phosphorylate Dnmt1(1–290)**

The N-terminal domain of Dnmt1 interacts with various proteins, indicating that it functions as a platform for the recruitment of regulatory factors. However, there have been no reports regarding protein kinases that interact with the N-terminal regulatory domain of Dnmt1, except our previous report [18]. In our previous study, we investigated a Dnmt1-binding protein kinase in a mouse brain extract by Western blotting with Multi-PK antibodies, and identified it as CDKL5 [18]. Although CDKL5 was found to bind to and phosphorylate Dnmt1, the phosphorylation of Dnmt1 by CDKL5 was not sufficiently strong enough to explain the phosphorylation of Dnmt1 by a partially purified kinase preparation. Therefore, we assumed that there may be other protein kinases responsible for more significant phosphorylation of Dnmt1.

To identify the protein kinase that phosphorylates Dnmt1, we prepared a protein fraction that contained the protein kinase responsible for phosphorylation of Dnmt1(1–290) from the
mouse brain. First, the mouse brain extract was incubated with GST–Dnmt1(1–290) bound to glutathione–Sepharose 4B, washed with an equilibration buffer and then eluted with a buffer containing 0.3 M NaCl. The Dnmt1(1–290)-bound fractions were loaded on to a DNA–cellulose column, washed with a buffer containing 0.15 M NaCl, and then eluted with a buffer containing 0.3 M or 1 M NaCl. Protein kinase activity toward Dnmt1(1–290) was measured by incubating each fraction with GST–Dnmt1(1–290) in the presence of [γ-32P]ATP and analysed by SDS/PAGE followed by autoradiography. Protein kinase activities that phosphorylated Dnmt1(1–290) were detected in both the flow-through fraction and the 0.3 M NaCl fractions (Figure 1A, upper panel).

The above fractions were also analysed by Phos-tag SDS/PAGE [24], which detects differently phosphorylated proteins as differently upward-shifted protein bands. When GST–Dnmt1(1–290) was incubated with the brain extract (input) fraction and analysed by Phos-tag SDS/PAGE followed by Western blotting, two differently shifted bands, a significantly shifted band (designated a super-shifted band) and a slightly shifted band, were observed (Figure 1A, upper panel). The slightly shifted band was observed in the pass, wash and eluted fractions, whereas the super-shifted band was only detected in the 0.3 M NaCl fractions (Figure 1A, lower panel). In the present study, we attempted to identify the protein kinase that caused this super-shift of GST–Dnmt1(1–290) upon phosphorylation.

For further purification of the protein kinase responsible for causing the super-shifted product, a 0.3 M NaCl fraction was loaded on to a Superdex 200 column and eluted fractions of 1.2 ml were collected. Each fraction was incubated with GST–Dnmt1(1–290) in the presence of [γ-32P]ATP and analysed by Phos-tag SDS/PAGE followed by autoradiography. Two peaks of Dnmt1(1–290)-phosphorylating activities were detected, comprising a weak phosphorylation activity at approx. 150 kDa (fractions 13–19) and a significant phosphorylation activity corresponding to approx. 38 kDa (fractions 37–46) that produced a super-shifted band of GST–Dnmt1(1–290) (Figure 1B). These results suggested that fractions 37–46 contained the protein kinase activity responsible for the significant phosphorylation of Dnmt1(1–290), and we therefore designated this fraction the DBK fraction.

To identify the protein kinase that phosphorylates Dnmt1, the DBK fraction was subjected to SDS/PAGE and 14 gel slices in the region between 20 and 80 kDa were subjected to LC-MS/MS analysis (Figure 1C). Among the gel slices evaluated, the CK1 isoforms CK1δ and CK1ε were identified when the gel slice containing the 45-kDa protein band was analysed (Figure 1C and Table 1). Molecular masses of CK1δ and CK1ε were calculated to be 46789 and 47122 respectively (Table 1). Since no other protein kinases were detected in the other slices, we concluded that the protein kinase in the DBK fraction responsible for the significant phosphorylation of Dnmt1(1–290) was CK1δ/ε.

When the fractions from the Superdex 200 column were analysed by Western blotting using antibodies against CK1δ/ε, the immunoreactive band was detected in fractions 37–46 (results not shown). Although the elution position of CK1 from the gel filtration column was somewhat slower than that of the predicted molecular size, it was confirmed with three independent experiments. On the other hand, CDKL5 was detected in fractions 13–19 by Western blotting with an anti-CDKL5 antibody (results not shown). These results suggest that Dnmt1(1–290) was phosphorylated by both CK1δ/ε and CDKL5, but more significantly phosphorylated by CK1δ/ε.
was incubated with 4 μM Immunoprecipitation of CK1 isoforms with Dnmt1(FL). Cell extracts of Neuro2a cells expressing FLAG–CK1 transfected with or without FLAG–CK1 to Dnmt1(FL). Cell extracts were prepared from Neuro2a cells to Dnmt1(1–290) was specific. (Figure 2A, middle panel), suggesting that the binding of CK1 when GST alone was used instead of GST–Dnmt1(1–290) (Figure 2A, top panel). In contrast, no CK1 was coprecipitated GST-pulldown assays using GST–Dnmt1(1–290) (Figure 2A). Next, we examined whether or not CK1 isoforms could bind to Dnmt1(FL). Cell extracts were prepared from Neuro2a cells transfected with or without FLAG–CK1 ε. When Dnmt1(FL) was incubated with the expressed CK1 which had been bound to anti-FLAG antibody, Dnmt1(FL) was coprecipitated with CK1 isoforms (Figure 2B). These results suggest that CK1 isoforms are able to bind not only to Dnmt1(1–290) but also to Dnmt1(FL).

Phosphorylation of Dnmt1(1–290) by CK1 δ/ε

Next, we examined the phosphorylation of Dnmt1(1–290) by CK1 δ/ε. When GST–Dnmt1(1–290) was incubated with CK1 δ, CK1 ε or DBK in the presence of [γ-32P]ATP, Dnmt1(1–290) was significantly phosphorylated. GST-Dnmt1(1–290) served as an effective substrate for CK1 δ/ε, similar to the case for casein, which is known to be the most efficient substrate for CK1 (Figure 3A). Furthermore, phosphorylation of Dnmt1(1–290) by the CK1 isoforms was stimulated by the addition of DNA (Figure 3B). When phosphorylated GST–Dnmt1(1–290) was separated by Phos-tag SDS/PAGE, the phosphorylated bands showed essentially the same shift patterns when CK1 isoforms and DBK were used for phosphorylation of Dnmt1(1–290) (Figure 3C). When the phosphate incorporation into Dnmt1(1–290) was examined, at least two moles of phosphate were incorporated into one mole of GST–Dnmt1(1–290). Furthermore, the phosphate incorporation into Dnmt1 increased as the amount of the CK1 isoforms in the reaction mixture increased (results not shown). These results suggest that multiple phosphorylation sites for CK1 δ/ε are present in the N-terminal region of Dnmt1.

Table 1 List of identified proteins from the gel containing the 45-kDa band that phosphorylated Dnmt1(1–290)

<table>
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<th>Protein name</th>
<th>NCBI ID</th>
<th>Mass (Da)</th>
<th>Mascot score</th>
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</tr>
<tr>
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<td>126741</td>
<td>198860</td>
</tr>
</tbody>
</table>

Binding of CK1 δ/ε to Dnmt1

In the present study, the protein kinase responsible for phosphorylation of Dnmt1(1–290) was identified as CK1 δ/ε. Therefore we produced CK1 δ and CK1 ε using an E. coli expression system. The recombinant CK1 isoforms were expressed as soluble forms in E. coli, and purified using HiTrap Chelating columns.

Binding of CK1 δ and CK1 ε to Dnmt1 was examined by GST-pulldown assays using GST–Dnmt1(1–290) (Figure 2A). The CK1 isoforms were able to bind to GST–Dnmt1(1–290) (Figure 2A, top panel). In contrast, no CK1 was coprecipitated when GST alone was used instead of GST–Dnmt1(1–290) (Figure 2A, middle panel), suggesting that the binding of CK1 δ/ε to Dnmt1(1–290) was specific.

Next, we examined whether or not CK1 isoforms could bind to Dnmt1(FL). Cell extracts were prepared from Neuro2a cells transfected with or without FLAG–CK1. When Dnmt1(FL) was incubated with the expressed CK1 which had been bound to anti-FLAG antibody, Dnmt1(FL) was coprecipitated with CK1 isoforms (Figure 2B). These results suggest that CK1 isoforms are able to bind not only to Dnmt1(1–290) but also to Dnmt1(FL).

Phosphorylation of Dnmt1(1–290) by CK1 δ/ε

Next, we examined the phosphorylation of Dnmt1(1–290) by CK1 δ/ε. When GST–Dnmt1(1–290) was incubated with CK1 δ, CK1 ε or DBK in the presence of [γ-32P]ATP, Dnmt1(1–290) was significantly phosphorylated. GST-Dnmt1(1–290) served as an effective substrate for CK1 δ/ε, similar to the case for casein, which is known to be the most efficient substrate for CK1 (Figure 3A). Furthermore, phosphorylation of Dnmt1(1–290) by the CK1 isoforms was stimulated by the addition of DNA (Figure 3B). When phosphorylated GST–Dnmt1(1–290) was separated by Phos-tag SDS/PAGE, the phosphorylated bands showed essentially the same shift patterns when CK1 isoforms and DBK were used for phosphorylation of Dnmt1(1–290) (Figure 3C). When the phosphate incorporation into Dnmt1(1–290) was examined, at least two moles of phosphate were incorporated into one mole of GST–Dnmt1(1–290). Furthermore, the phosphate incorporation into Dnmt1 increased as the amount of the CK1 isoforms in the reaction mixture increased (results not shown). These results suggest that multiple phosphorylation sites for CK1 δ/ε are present in the N-terminal region of Dnmt1.

Determination of the phosphorylation sites of Dnmt1 by CK1 δ/ε

Next, the phosphorylation sites in Dnmt1 for CK1 δ/ε were investigated. For this experiment, we prepared purified preparations of Dnmt1(FL) and various deletion mutants of Dnmt1 (Figure 4A and B). Dnmt1(FL) and Dnmt1(ΔN; 291–1620), an N-terminal truncation mutant of Dnmt1, were incubated with CK1 δ and CK1 ε in the presence of [γ-32P]ATP and analysed by SDS/PAGE followed by autoradiography. Dnmt1(FL) and Dnmt1(1–290) were significantly phosphorylated by the CK1 isoforms, whereas Dnmt1(ΔN) was not (Figure 4C), suggesting that the phosphorylation sites are located in the N-terminal region. Therefore various truncation mutants of the N-terminal region of Dnmt1, such as GST–Dnmt1(1–197), GST–Dnmt1(1–146) and GST–Dnmt1(1–119), were prepared and compared with GST–Dnmt1(1–290). Among these mutants, Dnmt1(1–290) and Dnmt1(1–197) were efficiently phosphorylated by CK1. Although Dnmt1(1–146) was also significantly phosphorylated by CK1, the phosphate incorporation into Dnmt1(1–146) was reduced to 63% of that into Dnmt1(1–290) (Figure 4D). In contrast, Dnmt1(1–118) was not phosphorylated at all. These results suggest that the major phosphorylation sites in Dnmt1 are located in the N-terminal region between amino acids 119 and 146. To determine further the phosphorylation sites in this region, three mutants of Dnmt1(119–197) whose serine/threonine clusters were replaced by alanine residues were produced as follows: mutant-1 (S146A, T149A, S150A, S152A and S153A); mutant-2 (T156A, T159A and T160A); and mutant-3 (T163A, T164A and T166A). Mutant-2 and mutant-3 were phosphorylated by the CK1 isoforms to essentially the same extent as wild-type Dnmt1(119–197), whereas the

![Figure 2](image-url)
phosphorylated GST–Dnmt1(1–290) respectively.

The asterisks indicate autophosphorylation of CK1. (Phosphorylation of the proteins was analysed by SDS/PAGE followed by autoradiography. The DBK fraction (1 μl) or CK1 isoforms (10 ng) were incubated with 500 ng of GST–Dnmt1(1–290) or casein in the presence of 100 μM [γ-32P]ATP. Phosphorylation of the proteins was analysed by SDS/PAGE followed by autoradiography. The DBK/CK1 isoforms (10 ng) were incubated with 500 ng of GST–Dnmt1(1–290) in the presence or absence of mouse genomic DNA (10 μg/ml). Phosphorylation of the proteins was analysed by SDS/PAGE followed by autoradiography. The asterisks indicate autophosphorylation of CK1. (B) Effects of genomic DNA on the phosphorylation of Dnmt1(1–290). Approx. 10 ng of CK1 isoforms was incubated with 500 ng of GST–Dnmt1(1–290) in the presence or absence of mouse genomic DNA (10 μg/ml). Phosphorylation of the proteins was analysed by SDS/PAGE followed by autoradiography. The asterisks indicate autophosphorylation of CK1. (C) Phos-tag SDS/PAGE showing the shifted bands of phosphorylated Dnmt1(1–290). The phosphorylation of GST–Dnmt1(1–290) (500 ng) by DBK (1 μl) or CK1 isoforms (10 ng) was analysed by Phos-tag SDS/PAGE followed by autoradiography. The arrowhead and arrow indicate the super-shifted and shifted bands of phosphorylated GST–Dnmt1(1–290) respectively.

Regulation of the DNA-binding activity of Dnm1 by phosphorylation with CK1δ/

phosphorylation of mutant-1 was almost completely abolished (Figure 4E). To identify further the phosphorylation sites in this sequence, five alanine residue substitutions of Dnmt1(119–197), namely S146A, T149A, S150A, S152A and S153A, were constructed and analysed for their phosphorylation by the CK1 isoforms. The phosphorylation of Dnmt1(119–197)(S146A) was almost completely abolished compared with that of wild-type Dnmt1(119–197) and the other mutants (Figure 4F). These results suggest that Ser46 of Dnmt1 is the major phosphorylation site for CK1δ/ε, and that phosphorylation at this site may play a potential role as the priming site for phosphorylation.

Regulation of the DNA-binding activity of Dnm1 by phosphorylation with CK1δ

The N-terminal region of Dnm1 has been reported to recognize and bind to AT-rich sequences in DNA [6]. To investigate the effect of Dnm1 phosphorylation by CK1δ on its DNA-binding activity, Dnm1(1–290) and Dnm1(1–290)(S146A) were incubated with CK1δ in the presence or absence of ATP and their DNA-binding activities were compared. Briefly, these proteins were incubated with DNA–cellulose, and their DNA-binding abilities were quantified by Western blotting. The DNA-binding activity of Dnm1(1–290) was decreased after incubation under phosphorylating conditions in the presence of ATP, whereas that of the nonphosphorylatable mutant Dnm1(1–290)(S146A) was not (Figure 5A, left panel). The DNA-binding activity of Dnm1 was also examined using Dnm1(FL) instead of Dnm1(1–290). In this experiment, we found that the DNA-binding activity of Dnm1(FL) was also decreased when it was phosphorylated by CK1δ (Figure 5A, right panel).

In the next experiment, we examined whether Dnm1(1–290) and Dnm1(FL) were dissociated from DNA–cellulose upon phosphorylation by CK1δ. When GST–Dnm1(1–290) or Dnm1(FL) bound to DNA–cellulose was incubated with CK1δ in the presence or absence of ATP, Dnm1(1–290) and Dnm1(FL) were gradually dissociated from DNA–cellulose when incubated under phosphorylating conditions (Figure 5B). These results suggest that the DNA-binding activity of the N-terminal domain of Dnm1 is decreased by phosphorylation with CK1δ and that the phosphorylation at the N-terminal domain of Dnm1 induces dissociation of Dnm1 from DNA.

Effects of phosphorylation by CK1δ/ε on the function of Dnm1

In the present study, we have demonstrated that CK1δ/ε-mediated phosphorylation of the N-terminal domain of Dnm1 reduces the affinity of Dnm1 for DNA. The next question to be resolved was the effect of phosphorylation on the function of Dnm1. To investigate the effects of phosphorylation on the DNA methylation activity of Dnm1, Dnm1 was incubated with wild-type or KD forms of CK1δ and CK1ε under phosphorylating conditions and the DNA methylation activities were measured. The DNA methylation activity of phosphorylated Dnm1 did not differ from that of non-phosphorylated Dnm1 (Figure 6A), indicating that the catalytic activity of Dnm1 remained unchanged upon phosphorylation by CK1δ/ε under the conditions used.

The N-terminal domain of Dnm1 functions as a platform for the binding of various modulator proteins. PCNA is known to bind to this region and Dnm1–PCNA complexes are recruited to replication forks at S-phase during the cell cycle [6,29]. Furthermore, since the DNA-binding sequence in this region partly overlaps with the PCNA-binding motif [6], we examined the effects of phosphorylation by CK1δ/ε on the PCNA-binding activity of Dnm1. GST–Dnm1(1–290) or GST was incubated with CK1δ in the presence or absence of ATP and then coupled to glutathione–Sepharose 4B. After washing of CK1δ with 0.3 M NaCl, the GST–Dnm1(1–290)-bound glutathione–Sepharose 4B preparations were mixed with PCNA and pulled down by centrifugation. PCNA was able to bind to GST–Dnm1(1–290), but not to GST alone, indicating that PCNA specifically associated with the N-terminal domain of Dnm1. However, the PCNA-binding ability to Dnm1 remained unchanged under both phosphorylating and non-phosphorylating conditions (Figure 6B), suggesting that the association of PCNA with Dnm1 is not regulated by phosphorylation with CK1δ/ε.

DISCUSSION

Dnm1 was reported to be phosphorylated in vivo and its function may be regulated through protein phosphorylation [11,12]. However, the protein kinases responsible for the phosphorylation of Dnm1 and the physiological significance of its phosphorylation remain to be elucidated. It is known that the N-terminal region of Dnm1 forms a structurally independent domain from the other parts of the enzyme, including the catalytic domain [6] and that the N-terminal domain serves as a platform for the recruitment of various regulatory proteins [7–10]. In our previous study, we identified CDKL5 as a protein kinase that
binds to the N-terminal region of Dnmt1 [18]. CDKL5 was found to bind to and phosphorylate Dnmt1 at its N-terminal domain, although the physiological significance of the phosphorylation by CDKL5 is still unclear. In the present study, we demonstrated that CK1δ/ε also binds to the N-terminal regulatory domain of Dnmt1 and phosphorylates Dnmt1 much more significantly than CDKL5. Furthermore, we identified the major phosphorylation site in the N-terminal region of Dnmt1 as Ser146. We also demonstrated that the DNA-binding affinity of the N-terminal region of Dnmt1 was significantly reduced by phosphorylation with CK1δ. These results indicate that CK1δ/ε binds to and phosphorylates the N-terminal region of Dnmt1, thereby regulating the functions of Dnmt1 through a reduction in its DNA-binding activity.

The final test would be to demonstrate that the CK1 phosphorylation site Ser146, in the N-terminal domain of Dnmt1, is phosphorylated in vivo. All our attempts, however, to detect phosphorylation at this site by MS analysis were unsuccessful (results not shown). Difficulty in detecting phosphorylation at Ser146 of Dnmt1 in vivo might be due to the occurrence of multiple phosphorylation sites in the serine/threonine cluster region adjacent to Ser146. It is known that the consensus sequences of CK1 are E/D-X₁₋₂-S/T and S/T(P)-X₁₋₂-S/T (where
X is any amino acid) [30,31]. Therefore a substrate including a serine/threonine cluster is often phosphorylated at multiple residues after initiation from a priming phosphorylation site. The N-terminal domain of Dnmt1 has a serine/threonine cluster, including the consensus sequences for CK1, and this domain was actually phosphorylated at multiple sites by CK1δ/ε. In addition, Dnmt1(119–197)(S146A) was not phosphorylated by CK1δ/ε at all. These results suggest that the multiple sites in the N-terminal region of Dnmt1 may be sequentially phosphorylated by CK1δ/ε after phosphorylation of Ser146 as the priming site.

Although the N-terminal region of Dnmt1 is known to interact with AT-rich sequences in DNA [6], the physiological meaning of the DNA-binding activity of this domain in Dnmt1 is unknown. In the present study, we found that the DNA-binding activity of the N-terminal region of Dnmt1 was decreased by phosphorylation with CK1δ. However, the DNA methylation activity of Dnmt1 was not changed by phosphorylation with CK1δ/ε under the present conditions. These results agree with previous reports that the N-terminal domain of Dnmt1 is not required for the DNA methylation activity [19,32]. Therefore the physiological role of the DNA-binding activity of the N-terminal domain of Dnmt1 is the next issue to be studied.

The N-terminal region of Dnmt1 is also known to act as a platform for interactions with various proteins. The N-terminal sequence of amino acids 119–197 is necessary for the DNA-binding activity, and this region also contains a PCNA-binding motif at amino acids 160–172 [6]. Therefore, we examined the effects of phosphorylation by CK1δ/ε on the PCNA-binding activity of Dnmt1. However, the PCNA-binding activity of Dnmt1 remained unchanged after phosphorylation with CK1δ/ε. The N-terminal region of Dnmt1(1–290) has an isoelectric point of 8.55, and this isoelectric point of the N-terminal region would be significantly changed by multiple phosphorylations in this region. Therefore we need to investigate the effects of phosphorylation by CK1δ/ε on the binding activities of the various proteins that associate with the N-terminal regulatory domain.

It is known that CK1 is mainly localized in the cytoplasm and that the activity of cytoplasmic CK1 is repressed by autophosphorylation at the C-terminal autoinhibitory domain [33]. The kinase activity of CK1δ is important for its localization and the KD form of CK1δ is localized in the nucleus. In addition, CK1δ has putative nuclear localization sequences and a significant proportion of this protein is often localized in the nucleus, including the central body and spindle poles during mitosis [34]. On the other hand, Dnmt1 is known to be a typical nuclear protein [4], although the majority of Dnmt1 in neurons is localized in the cytoplasm [35]. These findings suggest that both Dnmt1 and CK1δ may co-localize and interact with each other during the cell cycle or in cells such as neurons. Further investigations concerning the relationship between Dnmt1 and CK1δ in vivo are necessary to elucidate the mechanisms of the regulation of gene expression through DNA methylation.

**AUTHOR CONTRIBUTION**

Yasunori Sugiyama performed most experiments, summarized data and contributed to manuscript preparation. Naoya Hatano performed the LC-MS/MS analysis. Isao Suetake and Shoji Tajima contributed to studies involving the DNA methylation assay. Eiji Kinozita, Emiko Kinoshita-Kikuta and Tohru Koike contributed to studies involving Phos-tag SDS/PAGE. Noriyuki Sueyoshi and Isamu Kameshita designed experiments, analysed data and were involving in manuscript preparation.
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