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Specificity of Dnmt1 for Methylation of Hemimethylated CpG Sites Resides in Its Catalytic Domain

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SUMMARY

The maintenance methylation of hemimethylated CpG sites by the DNA methyltransferase Dnmt1 is the molecular basis of the inheritance of DNA methylation patterns. Based on structural data and kinetics obtained with a truncated form of Dnmt1, an autoinhibition model for the specificity of Dnmt1 was proposed in which unmethylated DNA binds to Dnmt1's CXXC domain, which prevents its methylation. We have prepared CXXC domain variants that lost DNA binding. Corresponding full-length Dnmt1 variants did not display a reduction in specificity, indicating that the autoinhibition model does not apply in full-length Dnmt1. Furthermore, we show that the Dnmt1 M1235S variant, which carries an exchange in the catalytic domain of the enzyme, has a marked reduction in specificity, indicating that the recognition of the hemimethylated state of target sites resides within the catalytic domain.

INTRODUCTION

The methylation of the 5-position of cytosine in DNA is an important epigenetic signal (Feng et al., 2010; Jurkowska et al., 2011; Klose and Bird, 2006; Law and Jacobsen, 2010). In mammals, it predominantly occurs within CpG dinucleotides (approximately 70% of which are modified in a cell-type-specific pattern in human cells). It is introduced by three different DNA methyltransferases (MTases) called Dnmt1, Dnmt3a, and Dnmt3b (Jurkowska et al., 2011). All these enzymes consist of a large multidomain N-terminal part and a smaller C-terminal catalytic domain. Starting from the N terminus, Dnmt1 comprises one N-terminal domain interacting with PCNA, a targeting domain that directs the enzyme to replication foci (RFTS domain), a CXXC domain, and two BAH domains that are linked to the catalytic domain via a GK linker. The C-terminal domains of Dnmt3a and Dnmt3b are active in an isolated form (Gowher and Jeltsch, 2002), but this is not the case for Dnmt1 (Fatemi et al., 2001). Dnmt1 shows a high preference for hemimethylated DNA over unmethylated substrates, and it is responsible for the maintenance of DNA methylation patterns through cell divisions by catalyzing the specific methylation of the hemimethylated CpG dinucleotides produced during DNA replication. Currently, DNA methylation is the only epigenetic process the heritability of which is understood mechanistically at molecular level.

The mechanistic basis of Dnmt1's specificity for hemimethylated DNA has been studied intensively during the past decade (Jeltsch, 2006). The enzyme contains several DNA binding sites in the N-terminal, CXXC, and catalytic domains (Araujo et al., 2001; Fatemi et al., 2001; Pradhan et al., 2008). Since 2004, CXXC domains were recognized as binding modules specific for unmethylated CpG sites (Allen et al., 2006; Ayton et al., 2004; Lee and Skalnik, 2005), and the CXXC domain of Dnmt1 was shown to bind specifically to unmethylated CpG sites as well (Pradhan et al., 2008). This was an unexpected result given the specificity of the enzyme for methylation of hemimethylated CpG sites. Recently, the structure of a truncated Dnmt1 containing the CXXC, BAH, and catalytic domains in complex with an unmethylated DNA was solved, which showed specific binding of the unmethylated CpG site to the CXXC domain (Song et al., 2011). Based on this observation, an autoinhibition model was proposed in which the binding of the unmethylated CpG sites to the CXXC domain prevents their methylation. In the same work, Song et al. (2011) presented kinetic data to support that model. They showed that the truncated Dnmt1, which contains the CXXC domain, has an about 17-fold preference for methylation of hemimethylated over unmethylated substrates. A shorter Dnmt1 not containing the CXXC domain only showed a 2.2-fold preference, suggesting that the CXXC domain provided a dominating contribution to its specificity. Similarly, a Dnmt1 version with a mutated CXXC domain (K686A/Q687A), in which two residues critical for the interaction with the CpG site were exchanged, also showed a reduced specificity, because the preference for hemimethylated substrates was only 3.8-fold (corresponding to a 4.5-fold reduction in specificity when compared to the corresponding wild-type truncated Dnmt1).

Independently, two groups published the structure of Dnmt1 fragments including the RFTS domain but without DNA and

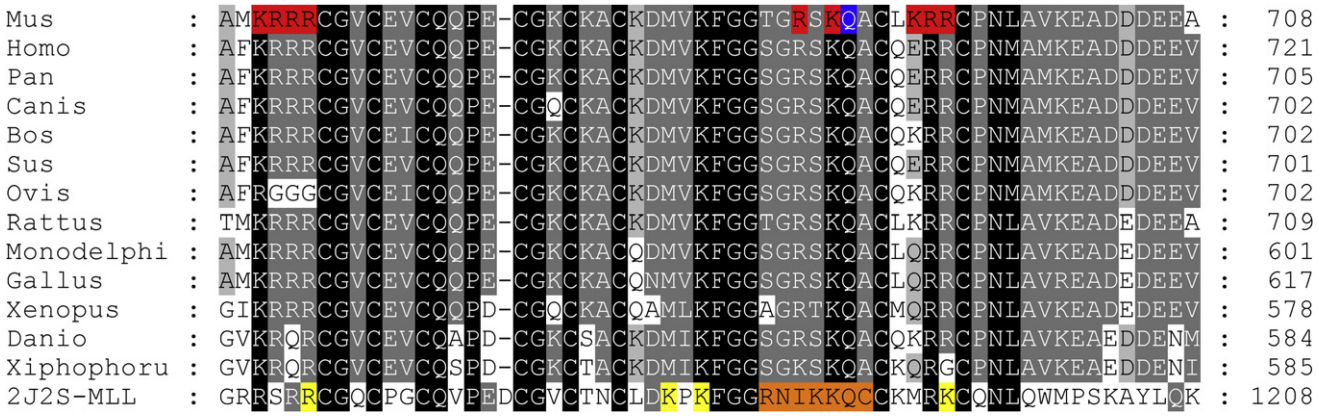

Figure 1. Multiple Sequence Alignment of Several Animal Dnmt1 CXXC Domains and the MLL CXXC Domain

The basic residues subjected to mutagenesis in this study are shaded red, and Q687 is shaded blue. The residues shown to be involved in DNA interaction in the MLL CXXC domain (R1154, K1176, K1178, and K1193) are shaded yellow, and the loop from R1182-C1188, which was shown to be involved in sequence-specific interactions, is shaded orange (Allen et al., 2006).

showed that the RFTS domain occupied the DNA binding pocket of the enzyme in the absence of DNA (Syeda et al., 2011; Takeshita et al., 2011). Syeda et al. (2011) supported that model with kinetic data, indicating that the RFTS domain strongly inhibits DNA binding and methylation. Takeshita et al. (2011) suggested that recognition of the methylcytosine of the hemimethylated CpG site resides in the catalytic domain. Considering an intrahelical or extrahelical position of this base, they mutated two tryptophane residues to alanine (W1500A and W1512A), which were candidates for involvement in the formation of hydrophobic pockets, and showed that both variants were catalytically inactive.

It was the aim of this work to investigate the role of the CXXC domain for the specificity of Dnmt1 in the full-length enzyme and study the potential recognition of hemimethylated DNA by the catalytic domain.

RESULTS

The specificity of Dnmt1 for methylation of hemimethylated CpG sites is a fundamental process required for the inheritance of site-specific DNA methylation patterns. Dnmt1's CXXC domain has been shown to bind to unmethylated CpG sites and to be essential for catalysis (Pradhan et al., 2008). It was our aim to disrupt the DNA binding of the CXXC domain and study the effect of this on the specificity of Dnmt1. In an initial step, we generated mutants in the isolated CXXC domain, purified the mutated domains from *Escherichia coli*, and studied their DNA binding. In a following step, mutations that disrupted or strongly reduced DNA binding were generated in the context of full-length Dnmt1. Afterward, the mutant Dnmt1 enzymes were purified from insect cells and their specificity for hemimethylated DNA studied in DNA methylation kinetics.

DNA Binding of CXXC Domain Variants

We were interested to investigate the role of the CXXC domain binding to unmethylated DNA in the function of Dnmt1. Since this project was started before the structure of this domain bound to DNA became available, we designed several variants with the aim to disrupt DNA binding. To identify target residues, we inspected the alignment of several animal Dnmt1 CXXC domains for conserved patches of basic residues, which are the best candidates to contact the DNA and contribute to an electrostatic interaction with the DNA (Figure 1). The first candidate regions were R652, K653, K654, K655 and K691, R692, R693. Another basic patch was identified at R684 and K686. At that time, the only structural information available was a nuclear magnetic resonance structure of the mixed-lineage leukemia (MLL) CXXC domain in which DNA interacting residues were identified by nuclear Overhauser enhancement shifts (Allen et al., 2006). A comparison of the Dnmt1 and MLL CXXC domains showed that K655 corresponds to R1154 in MLL and R692 to K1193 in MLL, which contribute to DNA binding of the MLL CXXC domain. Since these regions both lie at the edge of the CXXC domain, we did not expect these residues to mediate direct sequence-specific contacts but rather to contact the DNA backbone and/or contribute to electrostatic interaction with the DNA. Therefore, we mutated all residues in each region at once. To keep the hydrophilic nature of the regions, we mutated all residues to serine, giving rise to the 4S (R652S, K653S, K654S, K655S) and 3S (K691S, R692S, R693S) variants. Furthermore, the structural studies in MLL revealed a particular role of a loop comprising R1182 to C1188 for specific DNA binding, which corresponds to R684 and K686 of Dnmt1. Since R684 and K686 lie within the loop identified in MLL to mediate sequence contacts, we mutated both residues individually to serine. Later, when the Dnmt1 structure became available, which showed a direct contact of Q687 to the CpG sites (Song et al., 2011), we mutated Q687 to alanine as well.

All mutants were generated in the context of the isolated CXXC domain fused to glutathione S-transferase (GST), purified from *E. coli*, and their DNA binding was initially analyzed by gel shift assays using an unmethylated 30-mer oligonucleotide containing a single CpG site as substrate (Figure 2; Table S1 available online). The results showed that the 4S and 3S variants had lost or greatly reduced DNA binding and that the R684S and K686S variants showed reduced DNA binding, while DNA binding of the Q687A variant was not changed. To allow

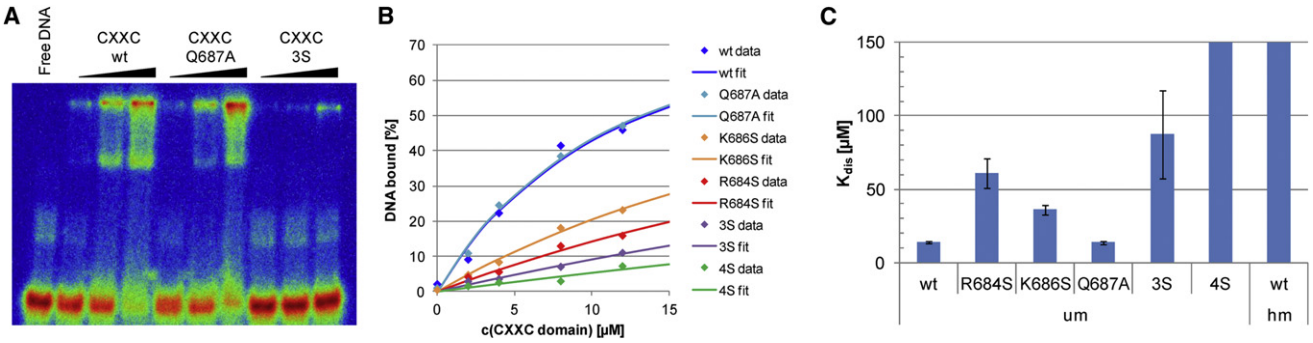

Figure 2. DNA Binding of the Isolated GST-CXXC Domain and Its Variants

(A) Example of the results obtained in the gel shift experiments. Protein concentrations were 3, 6, and 9 μM each.

(B) Examples of the results obtained in the nitrocellulose filter binding experiments.

(C) Binding constants derived from the quantitative analysis of the nitrocellulose filter binding experiments. Error bars indicate the SEM of the averages. See also Table S1.

a more quantitative analysis, the DNA binding of the mutants was also studied by nitrocellulose filter binding experiment, which in general confirmed the results of the gel shift studies (Figure 2; Table S1). Using a hemimethylated 30-mer as substrate, we also confirmed the specific binding of unmethylated DNA to the CXXC domain (Figure 2; Table S1). We concluded that the 4S, 3S, R684S, and K686S variants are valid model systems to study the role of DNA binding by the CXXC domain for the specificity of Dnmt1.

Specificity of Dnmt1 Containing CXXC Mutations Assayed Using a 30-mer Substrate

We generated the 4S, 3S, R684S, and K686S variants in the context of full-length Dnmt1. The mutated enzymes were expressed in insect cells using a baculovirus expression system and purified. Since the 3S and K686S variants could not be purified after overexpression, we focused our initial work on the 4S and R684S variants. We investigated the activity and specificity of both mutant enzymes using 30-mer oligonucleotide substrates containing one CpG site, which either was in hemimethylated or in unmethylated state, and compared the results with those of the wild-type enzyme purified in parallel (Figure 3; Table S1). We observed that wild-type Dnmt1 showed an approximately 10-fold preference for the methylation of hemimethylated over unmethylated CpG sites under our conditions. However, we did not detect a loss or reduction of specificity with any of the variants, although both had almost completely lost DNA binding to the CXXC domain. The 4S variant showed the same specificity as wild-type, and the specificity of the R684S variant was even increased. This result was unexpected given that Song et al. (2011) observed a marked reduction of specificity with CXXC domain variants in the context of the truncated Dnmt1 used as model system by them (Song et al., 2011). To compare our results with the data presented by Song et al. (2011) more directly, we wanted to investigate the K686A/Q687A double mutant used by them. We generated the double mutant in the Dnmt1 full-length context and purified the protein. However, like the R684S variant, the K686A/Q687A double mutant even showed an increased specificity (Figure 3; Table S1). We observed that the activities of two of our variants are increased (about 1.5- to twofold with 4S and about two- to threefold with the K686A/Q687A double mutant, which is comparable to the increase in activity observed by Song et al. [2011] with this mutant). This result may suggest that DNA binding to the CXXC domain has a repressive effect on Dnmt1's catalytic activity as proposed by Song et al. (2011). However, all three Dnmt1 variants, which had lost DNA binding to the CXXC domain, showed wild-type-like or even higher specificity, indicating that the DNA binding to the CXXC domain does not contribute to specificity under these conditions. We conclude that the results obtained by Song et al. (2011) with a truncated Dnmt1 could not be extrapolated to full-length Dnmt1.

Specificity of Dnmt1 Containing CXXC Mutations Assayed Using a 40-mer Substrate with Two Target Sites

To further confirm these findings, we have developed an additional assay, which is based on restriction protection and allows the direct comparison of the methylation of hemi- and unmethylated target sites on one 40-mer DNA substrate that contains one hemimethylated and one unmethylated CpG site. Methylation of both sites can be measured by digestion of the DNA with restriction enzymes that cleave recognition sequences that overlap with the CpG sites. Two enzymes were used for cleavage: either Sau3AI, which is inhibited by methylation of the hemimethylated site (Hermann et al., 2004), or HpaII, which is inhibited by methylation of the unmethylated site (Figure 4). After methylation for 30 min, we observed almost complete methylation of the hemimethylated CpG site with wild-type Dnmt1. The 4S and R686S variants showed a similar time course of methylation of the hemimethylated site. However, no methylation of the unmodified site was detectable, neither by wild-type Dnmt1 nor by any of the variants even after incubation for 3 hr. If one assumes that 10% of methylation should have been detectable, this corresponds to an at least 60-fold preference of methylation for hemimethylated over unmethylated sites when both sites are presented on one substrate molecule. We conclude that none of the variants displayed any apparent reduction in specificity, which confirms the results obtained with the 30-mer substrate.

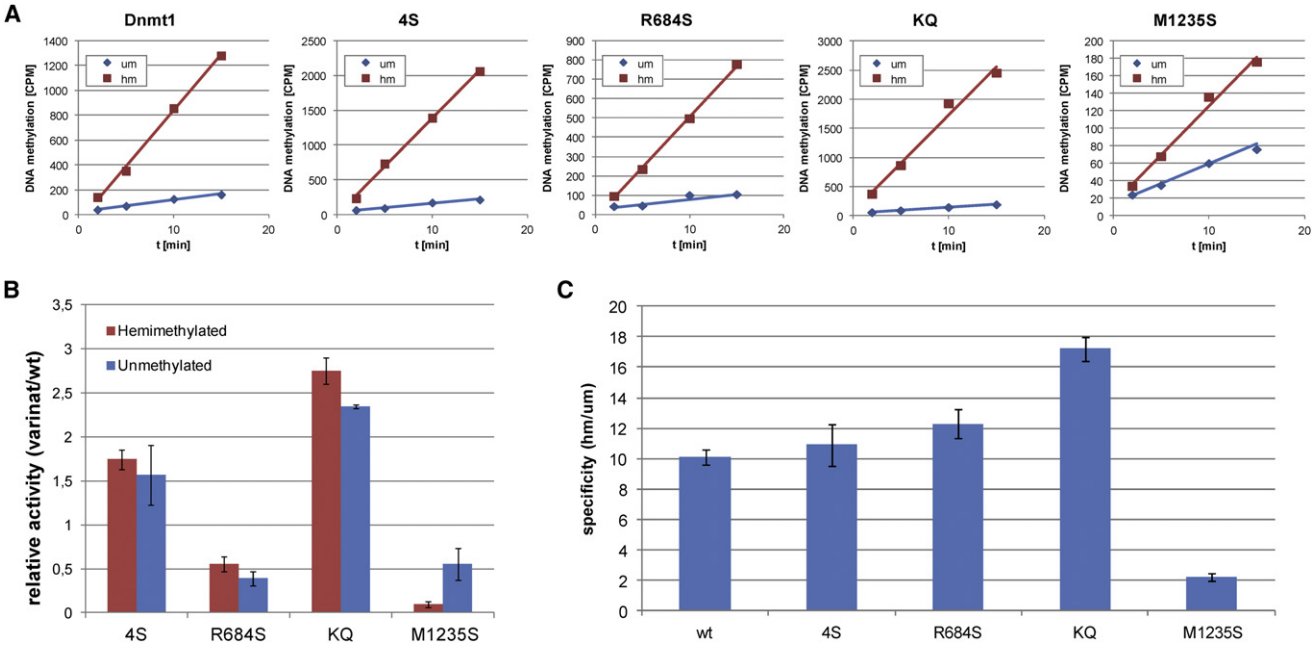

Figure 3. Kinetics of Methylation of the Hemimethylated and Unmethylated 30-mer Oligonucleotide Substrates

(A) Example kinetics with the different Dnmt1 variants. The reactions were carried out using 0.4 μM enzyme, except with M1235S where 0.7 μM was used.

(B) Catalytic activities of the variants for methylation of the unmethylated and hemimethylated substrates in relation to the activity of the wild-type enzyme (variant/wt).

(C) Specificity of the variants expressed as the ratio of the rate of methylation of the hemimethylated substrate divided by the rate of methylation of unmethylated substrate (hm/um). Activities and specificities were averaged over three to six independent experiments. Error bar indicates the SEM.

See also Table S1.

Specificity of the M1235S Variant Which Carries a Mutation in the Catalytic Domain

In light of these results, we wanted to investigate if the specificity of Dnmt1 could reside in its catalytic domain. We superimposed the structures of Dnmt1 and M.HhaI (Klimasauskas et al., 1994) and considered two models for the recognition of the 5-methylcytosine of a hemimethylated CpG site. One possibility would be that, in addition to the flipping of the target cytosine, the 5-methylcytosine in the opposite strand could also be flipped out of the double helix and bound into a hydrophobic pocket. Such "double flipping" appeared feasible, since it had been observed with other DNA MTases (Horton et al., 2006). Furthermore, the SRA domain of UHRF1 also recognizes a hemimethylated CpG site by flipping of the methylated base (Jeltsch, 2008). However, such mechanism would require a large conformational change of the enzyme and the DNA such that we could not predict any amino acid residue as a strong candidate to contact the methyl group on the basis of our static modeling. Therefore, we focused on the potential recognition of the 5-methyl group of the hemimethylated CpG site in an intrahelical conformation. We identified one methionine (M1235) that might be able to move into the major groove of the DNA and contribute to a hydrophobic pocket for the methyl group. Since recognition of the 5-methylgroup of thymidine in DNA by methionine residues is not uncommon in DNA binding proteins, we decided to mutate this methionine to serine in order to change the character of the residue from hydrophobic to hydrophilic. The mutant was generated in full-length Dnmt1, and the mutated protein was expressed and purified. The M1235S variant displayed a strongly reduced activity (about 10-fold reduction) of the hemimethylated 30-mer substrate (Figure 3). However, the methylation of the unmodified 30-mer substrate was only twofold reduced such that the overall specificity dropped to 2.2-fold. Due to its weaker activity, methylation of the 40-mer substrate by this mutant could not be detected.

DISCUSSION

Understanding the specificity of Dnmt1 for hemimethylated target sites is scientifically challenging, because the enzyme responds to the absence or presence of a single methyl group in a macromolecular substrate with a pronounced change of activity and this process provides the basis for the epigenetic function of DNA methylation. Recently, Song et al. (2011) solved the structure of a Dnmt1 fragment in complex with unmethylated DNA and observed binding of the DNA to the CXXC domain instead of binding to the catalytic domain (Song et al., 2011). Based on this, they proposed an autoinhibition model in which binding of unmethylated DNA to the CXXC domain prevents its methylation and provided kinetic evidence to support their model. However, their experiments were conducted using a Dnmt1 fragment starting with the CXXC domain. We have shown here in the context of full-length Dnmt1 that mutations within the CXXC domain, which strongly impair DNA binding of the isolated CXXC domain, did not reduce the specificity of the enzyme. In addition, the K686A/Q687A double mutant variant, which showed strong loss of specificity in the context of the

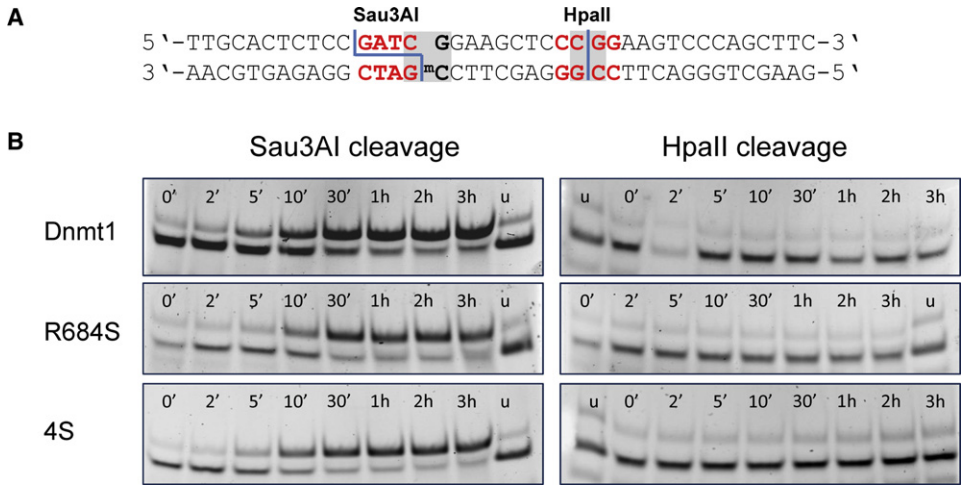

Figure 4. Specificity of Dnmt1 Analyzed by Methylation of a 40-mer Substrate Containing Two CpG Sites

(A) Design of the experiment. The sequence of the 40-mer is given. The two CpG sites are shaded gray, and the Sau3AI and HpaII recognition sites are in red. Methylation of the upper strand of the hemimethylated CpG site will protect the Sau3AI site from digestion. Methylation of any strand of the unmethylated CpG site will prevent digestion by HpaII.

(B) Examples of the kinetics observed with the different variants. Methylation times are indicated above the bands; u, unmethylated. The hemimethylated site is protected with similar kinetics by all variants, no protection was observed at the unmethylated CpG site.

See also Table S1.

truncated Dnmt1 studied by Song et al. (2011), did not show a reduction in specificity in the context of full-length Dnmt1. These data indicate that the autoinhibition model of Dnmt1 cannot be valid for the full-length enzyme. However, our data agree with the results reported by Song et al. (2011) in that two of the Dnmt1 variants that had lost DNA binding to the CXXC domain showed an increased catalytic activity, suggesting that binding of unmethylated DNA to the CXXC domain reduces activity. This effect may help to prevent Dnmt1 from methylation of an unmethylated CpG island where several unmethylated CpG sites occur next to another.

This conclusion that the autoinhibition model is not valid in the context of full-length Dnmt1 is in agreement with cellular data published recently by Frauer et al. (2011), who reported results of experiments in which Dnmt1 wild-type and mutants were reintroduced into dnmt1$^{-/-}$ embryonic stem cells (Frauer et al., 2011). They observed that neither wild-type Dnmt1 nor a variant lacking the CXXC domain showed detectable de novo DNA methylation activity at the H19a promoter and concluded that this argues against a role of the CXXC domain in restraining Dnmt1 methyltransferase activity on unmethylated CpG sites.

Furthermore, additional domains are involved in the regulation of the activity of Dnmt1. Recently, Takeshita et al. (2011) and Syeda et al. (2011) showed that, in the absence of DNA, the RFTS domain blocks the DNA binding site of the catalytic domain of Dnmt1 (Syeda et al., 2011; Takeshita et al., 2011). Thereby, it prevents access of the DNA and leads to a relocation of the CXXC domain. Syeda et al. (2011) observed a strong inhibition of Dnmt1 activity by the RFTS domain after comparing the activities of Dnmt1 351-1616 (which includes the RFTS domain) and Dnmt1 621-1616 (which excludes RFTS but still contains the CXXC domain; Syeda et al., 2011). In vivo, the arrangement of different domains like the CXXC domain and the RFTS domain might be regulated by the interaction with other proteins or post-translational modifications.

Comparison with Other Proofreading Enzymes with Nucleic Acid Substrates

The autoinhibition model for Dnmt1 specificity proposes that DNA bound to Dnmt1 partitions between the DNA binding sites present in the CXXC domain and in the catalytic domain, which implies that double-stranded DNA has to shuttle between these two independent binding sites rapidly. Other enzymes that interact with nucleic acids in a highly specific mode also use proofreading mechanisms that are based on the competition of two active sites for the bound substrate, which can be compared with the model proposed for Dnmt1. Many aminoacyl-tRNA-synthetases contain an esterase proofreading catalytic site in addition to the aminoacylation site, which is used for a double sieve proofreading mechanism. It is based on the flexible movement of the tRNA CCA end between the aminoacylation and esterase active sites. Similarly, most DNA polymerases possess a nuclease activity that can remove the last introduced base. Here, specificity depends on the partitioning of the 3′-end of the daughter strand between the polymerase and nuclease catalytic pockets and the activity of the polymerase with different substrates. Evidently, in both systems only small movements of the single-stranded 3′ terminus of the bound nucleic acids are required to switch from catalytic into proofreading mode back and forth. During this change, the main part of the nucleic acid remains bound by the enzyme, such that no substrate dissociation is necessary. In contrast, the autoinhibition model of Dnmt1 requires a large conformation change of the Dnmt1-DNA complex including the movement of the DNA out of one DNA binding site and into the other. Such mechanism is unprecedented in other enzymes acting on nucleic acids, which raises

some doubts on its general feasibility. Possibly, the truncation of Dnmt1 right in front of the CXXC domain used by Song et al. (2011) had put the CXXC domain in a more mobile situation, which may have increased its contribution to specificity readout in the truncated Dnmt1.

Recognition of the Hemimethylated State of the Substrate by the Catalytic Domain

Based on modeling, we have identified M1235 as one candidate for being involved in the recognition of the 5-methylcytosine in hemimethylated CpG sites if the methylated base would remain inside the DNA helix. We observed that methylation of the hemimethylated substrate, but not so much methylation of the unmodified substrate, was impaired, indicating that the mutant has a reduced specificity but not a general loss of activity. To our knowledge, this is the first example, of full length Dnmt1 variant with reduced specificity that has been described. Takeshita et al. (2011) also considered the recognition of intrahelical or extrahelical 5-methylcytosine by the catalytic domain, and they mutated two tryptophane residues (W1500A and W1512A) to alanine, which were candidates for intrahelical or extrahelical recognition (Takeshita et al., 2011). Unfortunately, both variants were catalytically inactive. Hence, no shift in specificity could be determined, and it could not be ruled out that the exchange from tryptophan to alanine affected the structure of the enzyme, and this led to loss of activity. We conclude that our results obtained with the M1235S variant strongly suggest that the mutation interferes with the recognition of the methyl group in the hemimethylated substrate. Consequently, the recognition of hemimethylated CpG sites resides at least in part in the catalytic domain of Dnmt1 where it recognizes the methylcytosine of the hemimethylated CpG site in an intrahelical conformation.

Conclusions

In this study, we investigated the validity of the CXXC-domain-based autoinhibition model of Dnmt1 specificity that has been put forward for a truncated Dnmt1 (Song et al., 2011). Our results suggest that this model cannot be extrapolated to full-length Dnmt1. Our conclusion is in line with the results from a recent study that did not show any change of Dnmt1's specificity in DNA methylation in cells after removing its CXXC domain (Frauer et al., 2011). Although it is possible in principle that binding to other factors might change the conformation and flexibility of Dnmt1 in the cell, we conclude that current in vitro and in vivo data with full-length Dnmt1 do not support a major role of the CXXC domain in the specificity of full-length Dnmt1. The inhibition of Dnmt1 after binding of unmethylated DNA to the CXXC domain may help to prevent methylation of unmethylated CpG islands. We observed a loss of specificity for hemimethylated substrates with a M1235S variant that carries a mutation in the catalytic domain but no changes in the CXXC and RFTS domains, suggesting that the recognition of hemimethylated CpG sites occurs at least in part in the catalytic domain of Dnmt1.

SIGNIFICANCE

The specific methylation of hemimethylated CpG sites by Dnmt1 is the molecular basis for the inheritance of DNA methylation patterns, which currently represents the only epigenetic process for which a mechanistic basis is understood at molecular level. Still, the mechanism of Dnmt1 to achieve that function is not known. Recently, the structure of a truncated Dnmt1 in complex with unmethylated DNA was reported. In that work, binding of the DNA to the CXXC domain of Dnmt1 was observed, which led the authors to propose an autoinhibition model for the mechanism of Dnmt1's specificity. Using the same truncated Dnmt1, they also provided kinetic data that supported their model. Using the same mutations and additional one, we now show that in full-length Dnmt1 the autoinhibition model does not apply. Based on the generation of a Dnmt1 variant with a mutation in the catalytic domain of the enzyme that displayed a reduced specificity, we propose that the recognition of the hemimethylated state of target sites resides within the catalytic domain. Thereby, our data shed new light on the mechanism of recognition of hemimethylated DNA by Dnmt1.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

Dnmt1 and Dnmt1 mutants were cloned with N-terminal His_6 and YFP tag in pFastbac and confirmed by DNA sequencing. Baculovirus encoding for the mutated Dnmt1 was produced following the instructions of the supplier (Bactobac manual, Invitrogen). Wild-type and mutant Dnmt1 were expressed in Sf21 insect cells and purified basically as described (Fatemi et al., 2001; Goyal et al., 2006). Briefly, Sf21 cells were harvested 96 hr after infection and kept at −20°C. Cells were lysed on ice in 40 ml sonication buffer (20 mM HEPES, pH 7.2, 500 mM KCl, 20 mM imidazole, 10% glycerol, 0.5 mM dithiothreitol [DTT]) supplemented with 100 μl protease inhibitor cocktail (Sigma) and 1 mM phenylmethylsulfonyl fluoride. The lysate was sonicated 10 times for 15 s (4 cycles, 10% output and 30% power) with 3–5 min intervals between and centrifuged at 20,000 rpm for 30 min at 4°C. The supernatant was added to 1.5 ml Ni-NTA beads pre-equilibrated in sonication buffer and incubated overnight on a rotator at 4°C for protein binding. The beads were loaded on to a column and washed with 60 ml sonication buffer and then with 60 ml of washing buffer (20 mM HEPES, pH 7.2, 250 mM KCl, 20 mM imidazole, 10% glycerol, 0.5 mM DTT). After washing, the proteins were eluted with elution buffer (20 mM HEPES, pH 7.2, 250 mM KCl, 250 mM imidazole, 10% glycerol, 0.5 mM DTT) and dialyzed 2.5 hr against dialysis I buffer (20 mM HEPES, pH 7.2, 250 mM KCl, 10% glycerol, 0.5 mM DTT), followed by dialyses overnight against dialysis II buffer (20 mM HEPES, pH 7.2, 250 mM KCl, 70% glycerol, 0.5 mM DTT) and kept at −20°C. The Dnmt1 CXXC domain (amino acids 647-700 of murine Dnmt1) was cloned as GST fusion and expressed in BL21 cells. Purification was conducted following a general protocol as described elsewhere (Rathert et al., 2008).

DNA Binding of the CXXC Domain

For gel shift experiments, increasing concentrations of CXXC domain (3 to 9 μM) were incubated with 1 pmol of radioactively labeled unmethylated or hemimethylated 30-mer substrate in a total volume of 10 μl of reaction buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 25 μg/ml bovine serum albumin [BSA], 5% glycerol, and 100 μM ZnSO4 for CXXC) for 15 min at ambient temperature. Afterward, 1.5 μl of 86% glycerol was added, and 6 μl of the mixture was loaded and ran on 8% polyacrylamide gel in 1× Tris-phosphate-EDTA (0.5× Tris-borate-EDTA for CXXC) buffer. The resulting gel was dried and analyzed using a Phosphorimager (Fuji). Nitrocellulose filter binding experiments were conducted in 20 mM HEPES, pH 7.5, 50 mM KCl, 25 μg/ml BSA, 5% glycerol, and 100 μM $ZnSO_4$ using 1 nM of radioactively labeled 30-mer. After incubation with different amounts of protein, the samples were loaded on equilibrated nitrocellulose membrane using DotBlot apparatus (BioRad). The membrane was washed twice with 100 μl of wash buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 0.2 mM DTT) and dried, and the radioactivity was analyzed using a Phosphoimager.

Kinetic Assays

Methylation of the biotinylated 30-mer substrates was carried out basically as described elsewhere (Fatemi et al., 2001) using 2 μM DNA, 0.4–1 μM Dnmt1, 1.125 μM [methyl-^{3}H]AdoMet (GE Healthcare) in methylation buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM KCl) at 37°C. The sequence of the 30-mer substrate was as follows: TTG CAC TCT CCT CCC GGA AGT CCC AGC TTC. The central CpG site (underlined) was either unmethylated or hemimethylated with the methyl group in the lower strand. The upper strand was biotinylated at its 5′ end.

For additional assays, a 40-mer substrate containing two CpG sites, one of them hemimethylated and the other unmethylated, was used. The sequence of the 40-mer is given in Figure 4. Methylation was conducted in buffer (100 mM HEPES, pH 7.2, 1 mM EDTA, 0.5 mM DTT, 0.1 mg/ml BSA, 7% glycerol) at 37°C using 1 μM DNA and Dnmt1 and 0.5 mM AdoMet (Sigma). During the methylation reaction, samples were taken and frozen in liquid nitrogen. Afterward, the samples were split in two aliquots each containing 10 pmol of DNA, which were digested with HpaII (10 U/μl, Biolabs) or Sau3AI (4 U/μl, Biolabs) for 2 hr at 37°C. The digestion pattern was analyzed by gel electrophoresis followed by ethidium bromide staining and image acquisition with a Biodoc gel documentation system (Biometra). Quantitative analysis of band intensities was performed using ImageJ.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found with this article online at doi:10.1016/j.chembiol.2012.03.010.

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Note Added in Proof

After finishing the first round of reviewing of this article, a new structure of a truncated Dnmt1 comprising the BAH domains and the catalytic domain was reported (Song et al., 2012). In this structure, a hemimethylated DNA substrate was bound to the catalytic domain. Close contacts to the hemimethylated target site are indicative of a direct readout of the methylation state by the catalytic domain, as concluded in our work. In agreement with our modeling and biochemical data, the M1235 residue is located in the major grove of the DNA, where it directly contacts the hemimethylated CpG site.

