Deficient in DNA Methylation 1 (DDM1) Defines a Novel Family of Chromatin-remodeling Factors*

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Deficient in DNA Methylation 1 (DDM1) protein is required to maintain the DNA methylation status of Arabidopsis thaliana. DDM1 is a member of the broad SWI2/SNF2 protein family. Because of its phylogenetic position, DDM1 has been speculated to act as a chromatin-remodeling factor. Here we used a purified recombinant DDM1 protein to investigate whether it can remodel chromatin in vitro. We show that DDM1 is an ATPase stimulated by both naked and nucleosomal DNA. DDM1 binds to the nucleosome and promotes chromatin remodeling in an ATP-dependent manner. Specifically, it induces nucleosome repositioning on a short DNA fragment. The enzymatic activity of DDM1 is not affected by DNA methylation. The relevance of these findings to the in vivo role of DDM1 is discussed.

The compaction of eukaryotic genomes into chromatin structures has profound implications for nuclear processes such as replication (1, 2), transcription (3, 4), DNA repair (5), and recombination (6, 7). Evolution has created a diverse repertoire of regulatory mechanisms affecting the dynamics of chromatin structure. One broad group of mechanisms involves a battery of the enzymes that covalently modify histones and DNA (8–10). Specific regulatory factors recognize the modification status of chromatin fibers and render the structure active or inactive (8). To ensure sufficient structural flexibility of the chromatin, the cell employs a specialized class of multiprotein complexes, which utilize the energy of ATP hydrolysis to change chromatin folding in the poorly understood process of “chromatin remodeling” (11, 12). Central to this activity are SWI2/SNF2-type ATPases that form the catalytic core of these remodeling complexes. The SWI2/SNF2 protein family falls within the large superfamily of DEXD/H-ATPases (13). The distinctive signature of the SWI2/SNF2 family is the SNF2_N domain, a variant of the typical DEXD/H domain that contains a well conserved C-terminal extension of −100 amino acids. Phylogenetic analysis divides the SWI2/SNF2 family into a number of subfamilies (13), which appear to be present in all major taxa. Outside the SNF2_N and HeliC domains, which form the catalytic module, these subfamilies are not well conserved. Members of three closely related subfamilies (ISWI, Mi2/CHD, and SWI2/SNF2) have been shown to serve as the catalytic subunits of chromatin-remodeling complexes (15–18). Recently, remodeling activity has also been demonstrated for complexes built around the more distantly related INOSO ATPase (19). All catalytic subunits of the chromatin-remodeling complexes that have been analyzed can act outside the complex context, although they require associated proteins to achieve their full activity (16–18, 20). CSB/ERCC6 defines a fifth group of remodelers, although it has been analyzed only as the isolated, recombinant protein (21). During recent years it has become clear that energy-dependent remodelers act in concert with histone modifying enzymes (22–27).

So far only very few family members have been analyzed in biochemical assays. At least one of them, MOT1, acts as a transcriptional repressor that regulates TATA binding protein binding to promoters (28–30). Therefore, it is likely that only a subset of SWI2/SNF2-like proteins serve as true chromatin-remodeling factors.

SWI2/SNF2-like proteins participate in various nuclear activities including transcriptional control (24, 31), DNA repair (Ref. 13 and references therein), chromosome segregation (32), and chromosome folding (32, 33). The members of two SWI2/SNF2-type subfamilies, ATRX and DDM1, are involved in the control of DNA methylation status (34, 35). In the plant Arabidopsis thaliana, the ddm1 genetic background results in a 70% decrease in the DNA methylation level (36). In consequence, this leads to deregulation of gene expression and the reactivation of silent transposons (37–42). DDM1 belongs to a small protein family conserved in plants (A. thaliana DDM1, NCBI accession number AAD28303; Zea mays, NCBI AAL73042), fungi (Saccharomyces cerevisiae Ypr038wp, NCBI NP_116696; Aspergillus fumigatus, NCBI CAD28443), and mammals (Mus musculus lymphocyte-specific helicase, NCBI NP_032260 and Homo sapiens proliferation-associated SNF2-like gene, NCBI AAAP2262). No genes encoding DDM1-like proteins have been found in the two fully sequenced invertebrate genomes. Evolutionary analysis places the DDM1 subfamily close to ISWI and SNF2/SWI2 groups (43). It is presently unclear how DDM1 acts to maintain DNA methylation status, although it has been speculated that it could remodel chromatin structure to facilitate the access of DNA methylases to the substrate (35). However, the remodeling activity of DDM1 has not been analyzed, and this hypothesis is based solely on the phylogenetic position of the DDM1 subfamily.

To address the above issue we have expressed recombinant DDM1 and characterized its biochemical activities. We find that DDM1 is an ATPase stimulated by both naked and nucleosomal DNA. It binds to nucleosomes and promotes nucleosome repositioning in an ATP-dependent manner. Our data indicate that DDM1 defines a novel class of chromatin-remod-
The DNA was labeled with $^{32}$P by PCR. After gel purification, the PCR nucleosomes were assembled on a 248-bp fragment of mouse rDNA (45). Micrococcal nuclease digestion. For nucleosome mobility assay, monochicken erythrocyte histones (44). Assembly quality was verified by and did not show any activity in either the enzymatic or band-shift using uninfected cells. This preparation was tested in all experiments and bands were visualized with the PhosphorImager.

**RESULTS**

**DDM1 Interacts with Nucleosomal DNA**—Sequence analysis of proteins of the DDM1 family did not reveal any known domain that might be implicated in DNA or nucleosome recognition. Therefore, it was necessary to determine whether DDM1 itself can bind to its potential substrates, naked DNA, or nucleosomes. To this end purified, recombinant DDM1 was used in band-shift assays with short fragments of DNA and with in vitro assembled mononucleosomes. To produce sufficient quantities of DDM1 protein, a recombinant baculovirus expressing *A. thaliana DDM1* was constructed and used to infect SF21 cells. The expressed DDM1 protein was isolated and found to be $\sim 90\%$ pure (Fig. 1A). A 248-bp fragment of mouse rDNA was amplified by PCR and used as free DNA or in a form of mononucleosomes assembled with purified chicken core histones. A band-shift assay with DDM1 and free DNA demonstrated the formation of nonspecific DNA-protein complexes (Fig. 1B). Increasing the protein concentration resulted in a smearing pattern, indicating the binding of DDM1 to multiple sites. In contrast, the interaction of DDM1 with the mononucleosomes resulted in the formation of one distinct shifted band (Fig. 1C), indicating the production of one bound species. Despite these differences in the nature of the complexes formed, band-shift assay showed that DDM1 binds to both targets with similar affinity.

**ATPase Activity of DDM1 Is Stimulated by Both Free and Nucleosomal DNA**—Although SWI2/SNF2-type chromatin-remodeling ATPases occur in vivo in multi-protein complexes, all of the enzymes analyzed thus far display ATPase activity in the absence of other subunits. To examine the activity of purified DDM1 protein, the ATPase assays were performed in the presence of core histones, plasmid DNA, or plasmid-assembled chromatin (Fig. 2A). Buffer was used as reference. To exclude any effect of potential contaminants, the mock preparation from uninfected cells was assayed in parallel reactions. To optimize the assay, DDM1 activity was analyzed under a broad spectrum of different conditions. The optimal reaction conditions were a buffer of pH 8.0 containing 50 mM NaCl, 100 mM bovine serum albumin, 1 mM MgCl$_2$ and a temperature of 25°C. In such a reaction ATPase activity of DDM1 was clearly stimulated by free DNA. Very little additional stimulation was seen in the presence of an equivalent amount of DNA assembled into chromatin. The addition of core histones had no effect on the DDM1 activity. These findings are consistent with the results of band-shift assays and imply that interaction with both DNA and nucleosomes stimulates the ATPase activity to similar levels.

**To understand how DNA and nucleosomes stimulate the**
DDM1 is a Chromatin-remodeling Factor—The interaction with mononucleosomes and the stimulation of ATPase activity by nucleosomal DNA suggested that DDM1 could be a chromatin-remodeling factor. To address this issue directly a well established sliding assay, which measures the movement of a histone octamer along a short DNA fragment, was used (45). The histone octamers were deposited on a 248-bp rDNA fragment. Gel electrophoresis of the assembled products revealed two discrete species (Fig. 3A) that correspond to nucleosomes positioned at the center or at the end of the DNA fragment (45). Positioned nucleosomes were gel-purified and incubated with increasing amounts of DDM1 protein. DDM1 was able to induce, in an ATP-dependent manner, the redistribution of the histone octamer (Fig. 3B). DDM1 was found to move nucleosomes from the end to the center of the DNA fragment with much greater efficiency than in the opposite direction.

**DDM1 ATPase Activity Is Not Stimulated by DNA Methylation**—DDM1 is involved in the maintenance of CpG DNA methylation in vivo (36). Therefore, we wished to see whether DNA methylation would affect the activity of recombinant DDM1 in *in vitro* assays. To this end DDM1 activity was compared in the ATPase assay performed with nucleosomes assembled on methylated and on non-methylated DNA. We decided to use an ATPase assay since it gives the most quantitative and directly comparable results. A fragment of *A. thaliana* FWA gene promoter was used because it contains a high number of CpG sequences (18 methylcytosines in a 210-bp-long DNA fragment) and has been shown to undergo demethylation in *ddm1* mutant plants (40). The DNA was amplified by PCR and methylated with *SspI* methylase. The efficiency of DNA methylation was checked by cleavage with the restriction enzyme *Fnu4HI*, which is sensitive to methylation of its recognition sequence (Fig. 4A). Methylated and non-methylated FWA fragments were then used in ATPase assays as both free and nucleosomal DNA. This experiment showed that there was no influence of DNA methylation on the ATPase activity of DDM1 (Fig. 4B).

**DISCUSSION**

The SWI2/SNF2 protein family falls within the DEXD/H superfamily of ATPases. It is defined by the SNF2_N domain, a variant of DEXD/H-ATPase domain that contains an additional conserved region of about 100 amino acids extending C-terminally to the classical DEXD/H domain (15). Based on phylogenetic analysis the SWI2/SNF2 family can be divided into several subfamilies that share the SNF2_N and HelicC domains but are not well conserved outside these regions (13). Chromatin-remodeling activity has been demonstrated for proteins of the closely related SWI2/SNF2, ISWI, and Mi2 subfamilies (15–17, 18). Recently the proteins of two other more distant subfamilies, INO80 and ERCC6/CSB, have been shown to act in a similar manner (19, 21). Here we present experimental evidences of chromatin-remodeling activity by a member of the DDM1 subfamily.

**DDM1 Interacts with the Nucleosome**—Our results show that DDM1 binds to both free DNA and nucleosomes with similar affinity (Fig. 1). However, the binding to the nucleosomal target resulted in a different pattern of gel-resolved complexes compared with the binding to free DNA. The diffused pattern formed by free DNA-DDM1 complexes indicated nonspecific interactions, whereas binding to the nucleosome resulted in the formation of a single distinct complex. These results indicate that in contrast to free DNA, the nucleosomal structure has a preferred binding site for DDM1. We did not observe any stable interaction of DDM1 with core histones in a pull-down assay (data not shown). These data suggest that although DNA is a major target for DDM1 binding, it is the nucleosomal structure
above kinetic data indicate that both free DNA and nucleosomes stimulate the ATPase of DDM1 mainly by increasing its turnover number. Similar dependence was found earlier for ISWI (15) and dMi2 (16). Most likely, these observations reflect mechanistic differences between the remodelers. We reported that the ISWI containing complex ACF and purified dMi2 behave in exactly the opposite way (20, 16). Most likely, the same group has reported that the ISWI containing complex ACF and purified dMi2 behave in exactly the opposite way (20, 16). Most likely, these observations reflect mechanistic differences between the remodelers. We found that DDM1, like ACF and dMi2, was considerably more efficient at mobilizing nucleosomes occupying the fragment termini than located at the center of the 248-bp rDNA fragment but not those located at the end. Interestingly, the same group has reported that the ISWI containing complex ACF and purified dMi2 behave in exactly the opposite way (20, 16). Most likely, these observations reflect mechanistic differences between the remodelers. We found that DDM1, like ACF and dMi2, was considerably more efficient at mobilizing nucleosomes occupying the fragment termini than located at the center of the 248-bp rDNA. The mechanistic reasons for this directionality of nucleosome sliding are not clear.

The nucleosome sliding activity of DDM1 appears to be rel-

| Factor | Cofactor | $K_m$ (μM) | $V_{max}$ (no. ATP/min/factor) | References |
|--------|----------|-------------|--------------------------------|------------|
| DDM1   | DNA      | 55          | 138                            | This work  |
|        | Chromatin| 66          | 400                            | This work  |
|        | DNA      | 30          | 434                            | This work  |
|        | Chromatin| 38          | 37                             | 17         |
| Mi2α   | DNA      | 94          | 1111                           | 17         |
| NuRD   | DNA      | 112         | 4180                           | 17         |
| ySWI/SNF| DNA     | 100 ± 50    | 1000 ± 200                     | 14         |
|        | Chromatin| 100 ± 50    | 1000 ± 200                     | 14         |
| yRSC   | DNA      | 100 ± 50    | 1000 ± 200                     | 14         |
|        | Chromatin| 100 ± 50    | 1000 ± 200                     | 14         |

*—, no cofactor present.

**Fig. 3.** Recombinant DDM1 is capable of remodeling nucleosomes in vitro. A, mononucleosomes were assembled on a 248-bp rDNA fragment and resolved by native polyacrylamide gel electrophoresis to reveal two positioned nucleosomal species. B, upper panel, nucleosomes positioned at the center of an rDNA fragment (60 fmol) were gel-purified and incubated with recombinant DDM1 (lane 1, 1 fmol; lane 2, 5 fmol; lane 3, 50 fmol) in the presence of ATP. As a negative control DDM1 (50 fmol) was used in the absence of ATP (lane 4, no ATP). Lower panel, 60 fmol of isolated end-positioned nucleosomes were incubated with recombinant DDM1 (lane 1, 1 fmol; lane 2, 5 fmol; lane 3, 50 fmol) in the presence or absence (no ATP) of ATP. All samples were analyzed by electrophoresis in a native polyacrylamide gel. The positions of free DNA (double line) and two nucleosomal species (gray ovals) are indicated to the left of the figure.

**Fig. 4.** DNA methylation status does not influence the ATPase activity of DDM1. A, in vitro methylation of the FWA 210-bp fragment with SssI methylase demonstrated by Fnu4Hi digestion and gel electrophoresis (lane 1, nonmethylated DNA; lane 2, methylated DNA incubated with Fnu4Hi; lane 3, nonmethylated DNA incubated with Fnu4Hi). B, recombinant DDM1 (10 fmol) was used in an ATPase assay in the absence (buffer) or presence of 100 ng of nonmethylated 210-bp FWA naked DNA (DNA, gray bar) or 100 ng of methylated DNA (Met-DNA, black bar) or 100 ng of mononucleosomes assembled on nonmethylated (Chromatin, gray bar) or methylated (Chromatin, black bar) DNA.

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3 G. Langst, personal communication.
Chromatin Remodeling by DDM1 Protein

as an example of the genomic distribution of Lys-9-methylated histone H3 in ddm1 mutants. Therefore, we speculate that DDM1 could be involved in histone H3 Lys-9 methylation. According to this scenario the effect on DNA methylation, which is seen in ddm1 plants, would be indirect. We are actively investigating this possibility.

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