Role of the N-terminal Domain of the Human DMC1 Protein in Octamer Formation and DNA Binding*

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The DMC1 protein, a eukaryotic homologue of RecA that shares significant amino acid identity with RAD51, exhibits two oligomeric DNA binding forms, an octameric ring and a helical filament. In the crystal structure of the octameric ring form, the DMC1 N-terminal domain (1–81 amino acid residues) was highly flexible, with multiple conformations. On the other hand, the N-terminal domain of Rad51 makes specific interactions with the neighboring ATPase domain in the helical filament structure. To gain insights into the functional role of the N-terminal domain of DMC1, we prepared a deletion mutant, DMC1-(82–340), that lacks the N-terminal 81 amino acid residues from the human DMC1 protein. Analytical ultracentrifugation experiments revealed that, whereas full-length DMC1 forms a octamer, DMC1-(82–340) is a heptamer. Furthermore, DNA binding experiments showed that DMC1-(82–340) was completely defective in both single-stranded and double-stranded DNA binding activities. Therefore, the N-terminal domain of DMC1 is required for the formation of the octamer, which may support the proper DNA binding activity of the DMC1 protein.

Recombination is the exchange or transfer of information between DNA molecules, and it occurs in all organisms. During meiosis, homologous recombination takes place as part of the normal meiotic process in order to generate genetic diversity. Through homologous recombination in meiosis, the genes from each parent recombine at frequencies 100- to 1000-fold higher than those of vegetative cells, leading to different genomic signatures (1, 2). In meiotic homologous recombination, double-stranded breaks are introduced by the Spo11 protein, and 3′-single-stranded tails are produced at the double-stranded break sites (3–5). Then, the homologous pairing proteins, such as Rad51 and Dmc1, and their activators are recruited to the single-stranded tails. The Rad51 and Dmc1 proteins are eukaryotic homologues of the bacterial RecA protein that promotes homologous pairing between single-stranded DNA (ssDNA)1 and double-stranded DNA (dsDNA). Dmc1 is a meiosis-specific factor, whereas Rad51 is required for both meiotic and mitotic homologous recombination (6, 7).

DMC1 and RAD51 share ~54% amino acid sequence identity (8). Like RecA, Rad51 and Dmc1 promote homologous pairing between two DNA molecules in vitro (9–16). Despite these similarities, Rad51 and Dmc1 require different factors to promote homologous pairing efficiently. Factors such as Rad52, Rad54, and BRCA2 mediate the homologous pairing and strand exchange reaction promoted by Rad51 (17–19). On the other hand, the mouse TBPIP/Hop2 protein (16) and the yeast Hop2-Mnd1 complex (20) stimulate the Dmc1-dependent homologous pairing. Furthermore, a Dmc1-specific mediator, the Sae3-Mei5 complex, has been found in Saccharomyces cerevisiae and may promote the assembly of Dmc1 on the double-stranded break site (21, 22), although the Schizosaccharomyces pombe Swi5-Sfr1 complex, composed of homologues of the S. cerevisiae Sae3-Mei5 complex, functions with Rad51 (23).

The S. cerevisiae and human RAD51 proteins bind DNA as helical nucleoprotein filaments (24, 25). In contrast, the human DMC1 protein assumes an octameric ring structure, which forms a complex composed of stacked rings on DNA (26, 27). Recently, electron micrographs of the human DMC1 protein showed that DMC1 forms a helical filament on ssDNA (15). Therefore, unlike RAD51, DMC1 exhibits two oligomeric DNA binding forms: an octameric ring and a helical filament.

The crystal structures of the RecA superfamily members, such as bacterial RecA, archaeal RadA, and eukaryotic Rad51 and Dmc1, revealed that whereas their monomeric topologies are highly conserved, their oligomeric states differ from protein to protein. The Escherichia coli RecA (28), S. cerevisiae Rad51 (29), and Methanococcus voltae RadA (30) proteins all formed helical filaments with six monomers/turn in their crystal structures, but their helical pitches were different. The Pyrococcus furiosus Rad51 protein, which functions as a helical filament, formed a heptameric ring (31). The crystal structure of the DMC1 protein revealed a highly symmetrical octameric ring (14). These structures suggest a dynamic oligomeric property of the RecA superfamily in which these proteins interconvert between rings and helical filaments.

Rad51 and Dmc1 are composed of two domains: the highly conserved ATPase domain, which makes up the core oligomeric structure, and the N-terminal domain, which is not conserved in the bacterial RecA protein. The ATPase domain is important for DNA binding and ATP hydrolysis and hence is also a core

1 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.
catalytic domain. The N-terminal domain of the human RAD51 protein physically contacts both ssDNA and dsDNA, as determined in previous NMR titration experiments and mutational analyses (32). Furthermore, the N-terminal domain of Rad51 specifically interacts with the neighboring ATPase domain in the helical filament structure (29, 30). By contrast, the N-terminal domain of DMC1 did not stably associate with the ATPase domain of the neighboring subunit in the octameric ring, and its functional role is unknown.

In the present study, a DMC1 mutant that lacks the N-terminal domain, DMC1-(82–340), was purified. Biochemical analyses revealed that the N-terminal domain of DMC1 is important for the DMC1 octamer formation and DNA binding. On the basis of these results, the functional role of the N-terminal domain of DMC1 is discussed.

MATERIALS AND METHODS

Protein Purification—The full-length DMC1 protein was purified as described previously (14). The DMC1-(82–340) gene was inserted into the pET-15b plasmid (Novagen), and the protein was overexpressed in the E. coli strain BL21(DE3) Codon Plus (Stratagene) as an N-terminal hexahistidine-tagged protein. DMC1-(82–340) was purified from a 10-liter Luria-Bertani culture incubated at 30 °C. When the A600 of the culture was 0.4–0.6, the protein expression was induced by adding isopropyl-1-thio-/beta-D-galactopyranoside to a final concentration of 1 mM. The cells were harvested after an overnight incubation and were lysed by sonication in buffer A (50 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, 10 mM 2-mercaptoethanol, 10% glycerol, and protease inhibitors (Complete EDTA-free; Roche Applied Science)) on ice. The cell lysate was centrifuged at 27,700 g for 20 min, and the supernatant was gently mixed by the batch method with 4 ml of nickel-nitrilotriacetic acid-agarose beads (Qiagen) for 1 h. The protein-bound beads were packed into an Econo-column (Bio-Rad) and were washed with 30 column volumes of buffer A containing 5 mM imidazole. The DMC1-(82–340) protein was eluted in a 20-column volume linear gradient of 5–300 mM imidazole in buffer A. The peak fractions, which predominantly contained DMC1-(82–340), were collected, and thrombin protease (5 units/mg of DMC1-(82–340); Amersham Biosciences) was added to cleave off the His tag. The thrombin-containing fractions were subjected to chromatography on a 4-ml Heparin-Sepharose (Amersham Biosciences) column. The column was washed with 20 column volumes of buffer B, and the protein was eluted with a 20-column volume linear gradient of 0.2-1.2 M KCl in buffer B. The DMC1-(82–340) protein was eluted in a sharp peak at ~0.5 M KCl. The protein concentration was determined with a Bio-Rad protein assay kit with bovine serum albumin (Pierce) as the standard.

Crystallization of DMC1-(82–340)—DMC1-(82–340) crystals were grown by the hanging drop method at 20 °C. The hanging drop was formed by adding 1 µl of DMC1-(82–340) (concentrated to 8 mg/ml) to 1 µl of the reservoir solution (1.4M sodium formate). Crystals typically appeared after 3 days and reached the maximum size (~0.15 x 0.15 x 0.15 mm) after 1 week. For data collection, the DMC1-(82–340) crystals were harvested in a reservoir solution containing 5 M sodium formate and were flash frozen in a stream of N2 gas (100 K). The data set of the crystal was collected at the SPring-8 BL44B2 beamline (Harima, Japan).

Assay for DNA Binding—All reaction mixtures contained final buffer concentrations of 20 mM Tris-HCl (pH 8.0), 1 mM MgCl2, 0.1 mM bovine serum albumin, 1 µM ATP, 2 mM creatine phosphate, and 75 µM

FIG. 2. Crystallization of DMC1-(82–340). A, SDS-PAGE of the purified full-length DMC1 and the DMC1-(82–340) mutant. B, DMC1-(82–340) crystals.

FIG. 1. A, the domain structures of full-length DMC1 and DMC1(82–340). B, the N-terminal domain is invisible in the crystal structure of full-length DMC1. The 2Fo–Fo electron density map, contoured at 1.0 σ, is shown in blue. The octameric ring structure of DMC1 is shown as a ribbon diagram. Each monomer is colored differently. The location of the N-terminal domain is shown in white squares. C, SDS-PAGE of the purified DMC1 and the DMC1 crystals.
creatinine phosphokinase. The indicated amounts of DMC1 were incubated with 1 μM SAT-1 ssDNA (5'-ATTTCTAGTCAGACGAAGATATCTGAGTAACTTTGCTGTGTA-3') for 6 min at 37 °C. The DMC1-ssDNA complexes were then fixed with 0.04% glutaraldehyde for 20 min. The complexes were resolved by 1% agarose gel electrophoresis in 0.5× TBE buffer at 3.3 V/cm for 2.5 h and were visualized by autoradiography of the dried gel. Products and reactants were visualized using a Fuji BAS2500 image analyzer. For the dsDNA binding assay, 10 μM X174 DNA, linearized with PstI, was incubated with the indicated amounts of DMC1 for 5 min at 37 °C. The complexes were resolved by 1% agarose gel electrophoresis in 0.5× TBE buffer at 3.3 V/cm for 2.5 h and were visualized by ethidium bromide staining. All DNA concentrations are expressed in moles of nucleotides.

**Assay for D-loop Formation**—The reactions were started by incubating the indicated amounts of DMC1 with 1 μM SAT-1 ssDNA for 5 min. Afterward, the supercoiled pGsat4 (3,218 bp) DNA (final concentration of 30 μM) was added along with MgCl₂ (final concentration of 10 mM). After the reaction mixtures were further incubated for 10 min, 1 μl of 5% SDS followed by 1 μl of 6 mg/ml proteinase K was added, and the reactions were incubated for 15 min. The products were resolved by 1% agarose gel electrophoresis in 0.5× TBE buffer at 3.3 V/cm for 2.5 h and were visualized by autoradiography of the dried gel.

**Analytical Ultracentrifugation**—Sedimentation equilibrium experi-

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**FIG. 3. Oligomerization state and thermal stability of DMC1-(82–340).** Sedimentation equilibrium analysis of full-length DMC1 (A) and DMC1-(82–340) (B). For the molecular mass analysis, the data were fit to an ideal, single component model. C, the CD effect measured at 222 nm, as a function of temperature, for the full-length DMC1 (blue) and DMC1-(82–340) (red).
Equilibrium distributions were analyzed after 18 h of centrifugation at extensively dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, and 10% glycerol. Equilibrium distributions were analyzed after 18 h of centrifugation at 9,000 rpm and 20 °C. For the molecular weight analysis, a partial specific volume of 0.675 cm³/g and a solution density of 1.05 g/cm³ were used.

**RESULTS**

**Flexible Nature of the DMC1 N-terminal Domain**—The DMC1 protein consists of two distinct domains, the N-terminal and ATPase domains (Fig. 1A). Fig. 1B shows the electron density map calculated in our previous x-ray structural analysis of the full-length DMC1 protein (14). As shown in this figure, the electron density of the ATPase domain of Dmc1 (amino acid residues 83–340) was clearly observed. In contrast, the electron density of the N-terminal domain (amino acid residues 1–82) was not detected. The absence of the electron density was not the result of protein degradation, as confirmed by the SDS-PAGE analysis of the dissolved DMC1 crystal (Fig. 1C).

Therefore, these observations suggest that the N-terminal domain of DMC1 exhibits high flexibility or multiple conformations.

**Construction and Purification of DMC1-(82–340)—**To gain insights into the functional role of the N-terminal domain of DMC1, we constructed a deletion mutant, DMC1-(82–340), which lacks the N-terminal 81 amino acid residues (Fig. 1A). The DMC1-(82–340) mutant was overexpressed in *E. coli* as an N-terminal hexahistidine-tagged protein and was purified by chromatography on a Ni-chelated-agarose column. The hexahistidine tag was removed with thrombin protease and was further purified by Heparin-Sepharose chromatography (Fig. 2A). Initial crystallization screens of DMC1-(82–340) yielded cubic-shaped microcrystals of ~20 μm in length (Fig. 2B). These DMC1-(82–340) crystals were single crystals, and their x-ray diffraction limit was ~8 Å at the SPring-8 synchrotron facility. Further attempts to extend the diffraction limit by improving the size and quality of the crystals and by testing different cryoprotectants were unsuccessful. However, the fact that DMC1-(82–340) crystallized suggests that DMC1-(82–340) could be structurally stable and uniform.

**DMC1-(82–340) Forms a Heptamer in Solution**—To determine the oligomerization state of DMC1-(82–340) in solution, we performed an analytical ultracentrifugation experiment. As we previously reported, full-length DMC1 formed octamers (molecular mass of 304,411 daltons) in solution (14) (Fig. 3A). Interestingly, the molecular mass of DMC1-(82–340) was 199,678 daltons, which corresponds exactly to a heptamer and not an octamer (Fig. 3B). The *P. furiosus* Rad51 protein formed a heptameric ring structure in its crystal (31), and a heptameric ring structure of human DMC1 was observed by electron microscopy (27). Therefore, the heptamers of Dmc1-(82–340) to further characterize the heptameric oligomerization. The circular dichroism (CD) spectra of the mutant were monitored in the range of 20 to 295 °C. As shown in Fig. 3C, the ellipticities of DMC1-(82–340) and the full-length protein were unchanged up to 70 °C and increased thereafter. This result shows that the thermal stability of the DMC1-(82–340) heptamer is similar to that of the full-length DMC1 octamer.

**DMC1-(82–340) Is Defective in DNA Binding and Homologous Pairing**—We tested whether the N-terminal domain of DMC1 has a role in DNA binding, because the RAD51 N-terminal domain is known to directly bind to DNA (32). To do so, we performed a gel mobility shift assay with DMC1-(82–340). A 50-mer ssDNA and linearized ϕX174 phage dsDNA were used as substrates in the DNA binding assays. As shown in Fig. 4, A and B, full-length DMC1 efficiently bound to both ssDNA and dsDNA. However, DMC1-(82–340) was completely defective in both ssDNA and dsDNA binding (Fig. 4, A and B). The DMC1-(82–340) mutant was also defective in homologous pairing between a 50-mer ssDNA and a homologous superhelical dsDNA, probably because of its defective DNA binding (Fig. 4C).

Therefore, the N-terminal domain of DMC1 plays an essential role in DNA binding as well as in octamer formation.

**DISCUSSION**

Recent structural studies of Dmc1 and Rad51 have begun to uncover the dynamic oligomeric properties of these proteins. Rad51 binds to DNA as helical filaments. The crystal structures of the *S. cerevisiae* Rad51 protein and the *M. voltae* RadA protein, an archaeal Rad51, revealed that the N-terminal domain of Rad51 specifically interacts with the ATPase domain of the neighboring subunit in the helical filament (29, 30). These structural studies suggested that the N-terminal domain of Rad51 plays an important role in the filament formation. In contrast, the N-terminal domain of the DMC1 octameric ring was not visible in the crystal structure, suggesting that it is flexible or has multiple conformations in the octameric ring structure (14, this study). In the present study, however, we found that DMC1-(82–340) formed a heptamer, and not an octamer, indicating that the N-terminal domain is still essential for the DMC1 octameric ring formation, despite its flexible nature. Therefore, the N-terminal domain may have essential
roles in the quaternary structure formation by the Rad51 and DMC1 proteins.

Interestingly, it has been reported that DMC1 forms helical nucleoprotein filaments in addition to the stacked octameric rings on ssDNA (15, 26, 27). The amino acid sequences of the human RAD51 and DMC1 N-terminal domains are highly conserved. Therefore, an interaction similar to that observed between the N-terminal domain and the ATPase domain in the Rad51 filament may take place between the corresponding domains in the DMC1 helical filaments. We hypothesize that the N-terminal domain of DMC1 functions in the conversion from ring to filament and propose a model, as shown in Fig. 5. In the model, the conversion may be caused by ATP binding. In the DMC1 octameric ring structure, the ATP-binding site is located at the monomer-monomer interface, and the subsequent binding of ATP is likely to induce conformational changes at the interface. An ATP-driven change in the quaternary structure has been observed in several proteins, including RecA (33), T7 gene 4 protein (34), and SV40 T antigen (35). This change may facilitate the formation of a DMC1 helical filament. Consequently, the N-terminal domain of DMC1 may actively interact with the ATPase domain of another DMC1 molecule and stabilize the helical filament form. This conversion from ring to filament may take place on DNA, because both oligomeric states of DMC1 have been observed to bind DNA. If the DMC1 helical filament is an active form for its strand-exchange activity (15), then the DMC1 octameric ring may be a resting DNA binding form that is waiting for the initiation signal for homologous recombination on the double-stranded break site.

Interestingly, a comparison between the crystal structures of the human DMC1 protein and the S. cerevisiae Rad51 protein suggests that the Tyr-194 residue of DMC1 is located at the monomer-monomer interface in both the octameric ring form and the helical-filament model. In the crystal structure of the DMC1 octameric ring, the Tyr-194 residue in the ATPase domain forms a hydrogen bond with the Glu-258 residue in the ATPase domain. This interface is stabilized by tripartite hydrogen bonding between ATPase domains. When the octameric ring converts to the helical filament, the N-terminal domain interacts with the respective ATPase domain. This interconversion may be caused by ATP binding.
neighboring ATPase domain. On the other hand, in the crystal structure of the S. cerevisiae Rad51 filament, Tyr-253 (corresponding to the Tyr-194 residue of DMC1) in the ATPase domain hydrophobically interacts with the Tyr-112 residue in the neighboring N-terminal domain (29). Therefore, in the DMC1 helical filament, the Tyr-194 residue is a potential candidate that directly interacts with the neighboring N-terminal domain, like the Tyr-253 residue of the S. cerevisiae Rad51 filament.

In the present study, we also found that DMC1-(82–340) is completely defective in both ssDNA and dsDNA binding activities. A possible explanation for these observations is that the N-terminal domain plays a direct role in DNA binding, like RAD51 (32). Another possibility is that the heptameric form caused by the truncation of the N-terminal domain of DMC1 is inactive in DNA binding. In the octameric ring, the amino acid residues essential for DNA binding were located near the central channel, which is large enough to accommodate a duplex DNA (Fig. 6A). By contrast, the heptamer model (Fig. 6B) suggests that the central channel is too narrow to accommodate a duplex DNA.

DMc1 and Rad51 are known to co-exist in meiotic cells, and the disruption of either gene leads to defects in meiotic recombination (6, 7). Hence, Dmc1 and Rad51 are likely to have the disruption of either gene leads to defects in meiotic recombination (6, 7). Hence, Dmc1 and Rad51 are likely to have.

Function of the N-terminal Domain of Human DMC1

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Interaction of the mammalian endosomal sorting complex required for transport (ESCRT) III protein hSnf7-1 with itself, membranes, and the AAA⁺ ATPase SKD1.
Yuan Lin, Lisa A. Kimpler, Teresa V. Naismith, Joshua M. Lauer, and Phyllis I. Hanson

The plasmid used to express FLAG-hSnf7N (residues 1–116) in this paper had an unintended missense mutation changing serine at residue 2 to cysteine. We found that this cysteine was palmitoylated. Changing it back to serine decreased the amount of hSnf7N associated with membranes from all for the mutant fragment containing cysteine to approximately half for the wild-type fragment containing serine. The images of FLAG-hSnf7N in Figs. 7B and 8 represent average cells expressing mutant (Cys-2) hSnf7N, whereas for wild-type (Ser-2) hSnf7N, these images correspond to cells expressing high levels of protein. All other plasmids are as indicated, and the conclusions of the paper remain unchanged.

Role of the N-terminal domain of the human DMC1 protein in octamer formation and DNA binding.
Takashi Kinebuchi, Wataru Kagawa, Hitoshi Kurumizaka, and Shigeyuki Yokoyama

Due to an inadvertent error, the wrong image was presented in Fig. 4C. Fig. 4C should appear as shown below. The figure legend and text remain unchanged.

Dynamic changes in histone H3 lysine 9 methylations. IDENTIFICATION OF A MITOSIS-SPECIFIC FUNCTION FOR DYNAMIC METHYLATION IN CHROMOSOME CONGRESSION AND SEGREGATION.
Kirk J. McManus, Vincent L. Biron, Ryan Heit, D. Alan Underhill, and Michael J. Hendzel

The concentration of a drug that was employed, adenosine dialdehyde, was erroneously reported as 25 mM. The concentration that was employed was actually 250 μM.

Conditional deletion of hypothalamic Y2 receptors reverts gonadectomy-induced bone loss in adult mice.
Susan J. Allison, Paul Baldock, Amanda Sainsbury, Ronaldo Enriquez, Nicola J. Lee, En-Ju Deborah Lin, Matthias Klugmann, Matthew During, John A. Eisman, Mei Li, Lydia C. Pan, Herbert Herzog, and Edith M. Gardiner

Dr. Klugmann’s name was misspelled in the author line. The correct spelling is shown above.

The first structure from the SOUL/HBP family of heme-binding proteins, murine P22HBP.
Jorge S. Dias, Anjos L. Macedo, Gloria C. Ferreira, Francis C. Peterson, Brian F. Volkman, and Brian J. Goodfellow

Column 2, first line: The concentrations should read “(4.0 μM) or hemin (3.5 μM)…”
In Fig. 5, panel D was inadvertently omitted and is shown below. The figure legend is correct as it appears.
Correction of pulmonary abnormalities in Sftpd−/− mice requires the collagenous domain of surfactant protein D.

Paul S. Kingma, Liqian Zhang, Machiko Ikegami, Kevan Hartshorn, Francis X. McCormack, and Jeffrey A. Whitsett

PAGE 24501:

Fig. 5: An incorrect image was used for the panel A inset. The correct image is shown below.